Safety Assessment of Ethyl Tafluprostamide and Isopropyl Cloprostenate as Used in Cosmetics

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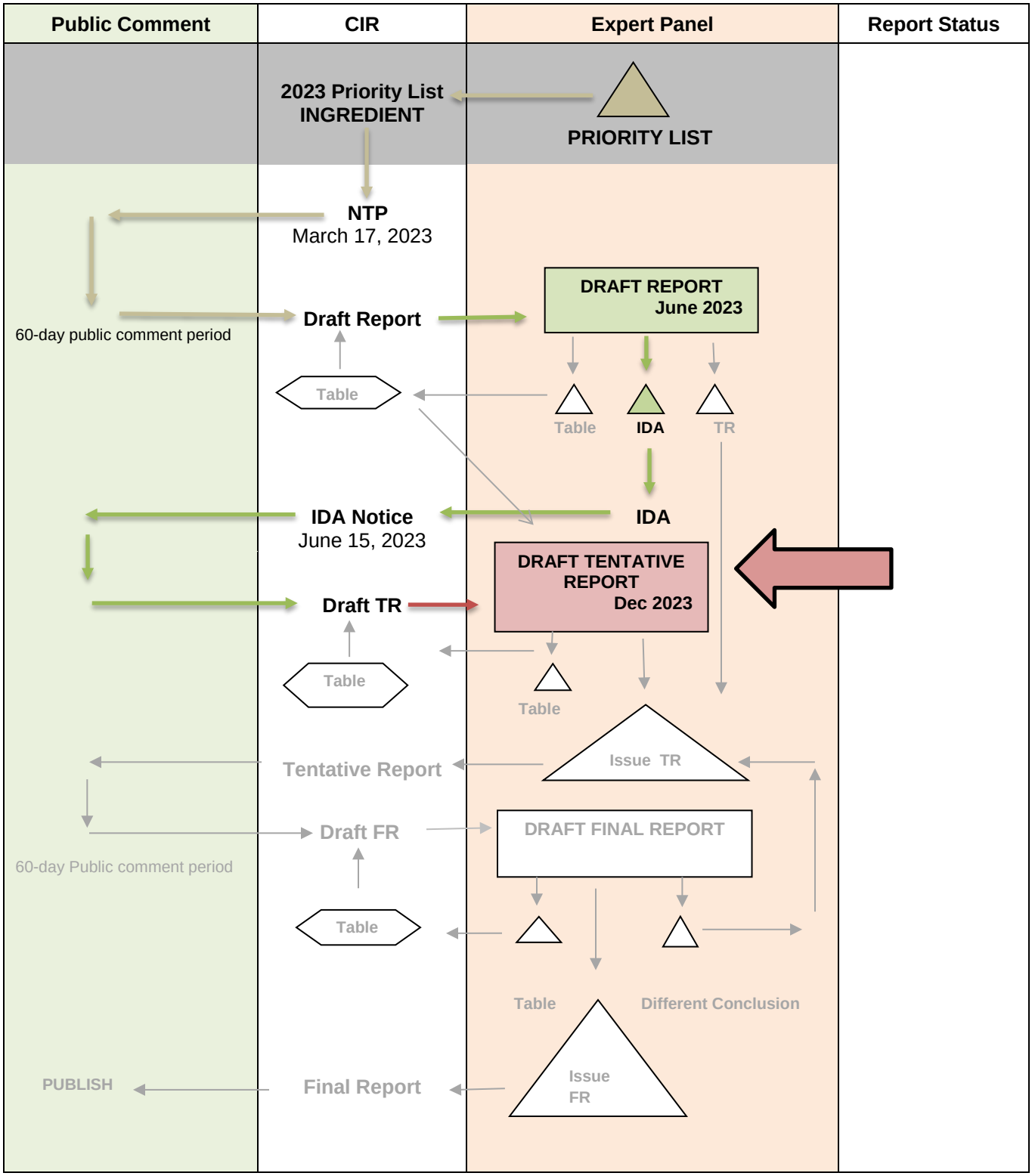
The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Thomas J. Slaga, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D., and the Senior Director is Monice Fiume. This safety assessment was prepared by Priya Cherian, M.S., Senior Scientific Analyst/Writer, CIR.

1620 L Street, NW, Suite 1200 ♢ Washington, DC 20036-4702 ♢ ph 202.331.0651
cirinfo@cir-safety.org


Public Comment
CIR
Expert Panel
Report Status
2023 Priority List
INGREDIENT
PRIORITY LIST
NTP
March 17, 2023
60-day public comment period
Draft Report
DRAFT REPORT
June 2023
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Memorandum

To: Expert Panel for Cosmetic Ingredient Safety Members and Liaisons
From: Priya Cherian, M.S., Senior Scientific Analyst/Writer, CIR
Date: November 9, 2023
Subject: Safety Assessment of Ethyl Tafluprostamide and Isopropyl Cloprostenate as Used in Cosmetics

Enclosed is the Draft Tentative Report on the Safety of Ethyl Tafluprostamide and Isopropyl Cloprostenate (identified in the pdf as *report_ProstaglandinAnalogues_122023*). At the June 2023 meeting, the Panel issued an Insufficient Data Announcement (IDA) for these ingredients, and requested the following data:

- concentration of use
- information on packaging of products and directions for consumer use
- 28-day dermal toxicity data; if absorbed, further systemic toxicological data may be needed
- dermal sensitization and irritation data at maximum concentrations of use (if maximum concentrations of use are higher than the concentrations used in dermal irritation/sensitization studies already present in report)
- intraocular pressure data on eyelash preparation containing Isopropyl Cloprostenate
- potency/inhibition constant (Ki) binding affinity data on Ethyl Tafluprostamide and Isopropyl Cloprostenate as compared to bimatoprost (FDA-approved prostaglandin analogue used for ocular hypertension/glaucoma treatment; also used as eyelash lengthener)

Since the issuing of the IDA, new data have been received on both Ethyl Tafluprostamide and Isopropyl Cloprostenate. These submissions are described below to help the Panel navigate what was received.

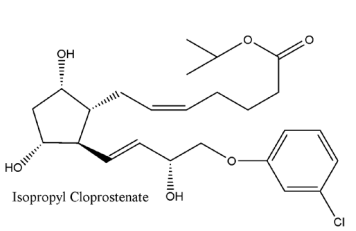
A data supplement containing information on various endpoints and summaries of toxicity data on Ethyl Tafluprostamide was provided (*data1_ProstaglandinAnalogues_122023*). In addition to the summary document, full-length versions of many of these studies were also provided by the submitter and have been included herein. A notation of the type of information that was provided, along with the file names where the full studies are found, is provided in the table below. Please note, a summary table of all of the studies provided below is found in *data1_ProstaglandinAnalogues_122023*.

Data on Ethyl Tafluprostamide	
Endpoint	**Data Source**
physical and chemical properties	*data1_ProstaglandinAnalogues_122023* *data2_ProstaglandinAnalogues_122023* *data3_ProstaglandinAnalogues_122023* *data4_ProstaglandinAnalogues_122023*
function and uses	*data1_ProstaglandinAnalogues_122023*
use instructions	*data1_ProstaglandinAnalogues_122023*
product amount per application	*data1_ProstaglandinAnalogues_122023*
in vitro percutaneous absorption	*data5_ProstaglandinAnalogues_122023*
in vitro percutaneous metabolism	*data6_ProstaglandinAnalogues_122023*
estimated oral absorption potential	*data1_ProstaglandinAnalogues_122023*
in vitro genotoxicity – Ames assay	*data7_ProstaglandinAnalogues_122023*
in vitro genotoxicity – micronucleus assay	*data8_ProstaglandinAnalogues_122023*

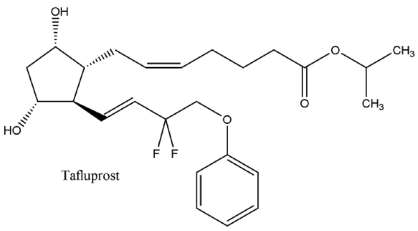
QSAR predictions for carcinogenicity	*data1_ProstaglandinAnalogues_122023*
intraocular pressure	*data9_ProstaglandinAnalogues_122023*
QSAR predictions for endocrine disruption	*data1_ProstaglandinAnalogues_122023*
in vitro dermal irritation	*data10_ProstaglandinAnalogues_122023* *data11_ProstaglandinAnalogues_122023*
in vitro/in chemico skin sensitization	*data12_ProstaglandinAnalogues_122023* *data13_ProstaglandinAnalogues_122023* *data14_ProstaglandinAnalogues_122023* *data15_ProstaglandinAnalogues_122023*
HRIPT	*data16_ProstaglandinAnalogues_122023* *data17_ProstaglandinAnalogues_122023*
estimated phototoxicity	*data4_ProstaglandinAnalogues_122023*
in vitro ocular irritation	*data18_ProstaglandinAnalogues_122023* *data19_ProstaglandinAnalogues_122023*
human eye irritation	*data9_ProstaglandinAnalogues_122023*
MOS calculation *it should be noted that this calculation was based on a PoD derived from a systemic toxicity assay performed using Tafluprost	*data1_ProstaglandinAnalogues_122023*

This data supplement (*data1_ProstaglandinAnalogues_122023*) also included information on a potentially related chemical, Tafluprost, along with the submitter's rationale for read-across justification. The numerous studies on Tafluprost are not summarized in the report at this time, awaiting input from the Panel as to whether data on Tafluprost is an appropriate read-across source to target either ingredient in this report. These data are summarized in an appendix that can be found in this packet as *appendix_ProstaglandinAnalogues_122023*.

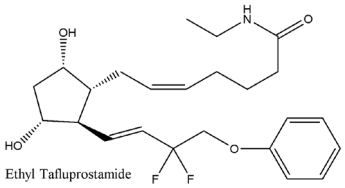
Furthermore, it should be noted that Tafluprost is a cosmetic ingredient listed in the *Dictionary*; however, no current uses are reported according to 2023 FDA VCRP data. **The Panel should review the data on Tafluprost and determine whether these data are appropriate for addition in the current prostaglandin analogues report.** If the Panel deems these data appropriate for addition, t**he Panel should determine whether this data should be added only as a read-across source, or, should Tafluprost be added to the report as an ingredient itself.** (The safety assessment would then include Ethyl Tafluprostamide, Isopropyl Cloprostenate, and Tafluprost.) The majority of the systemic toxicity studies performed on Tafluprost used methods of administration that are not relevant to cosmetic exposure (e.g., intravenous injection). The Panel should take this into account when deciding if these data are appropriate for addition.



Isopropyl Cloprostenate



Tafluprost



Ethyl Tafluprostamide

In addition to the data on Ethyl Tafluprostamide and Tafluprost, data on Isopropyl Cloprostenate were also submitted (*data20_ProstaglandinAnalogues_122023* and *data 21_ProstaglandinAnalogues_122023*). These data include a summary of a use assay (subjects used eyelash serum containing 0.0044% Isopropyl Cloprostenate for 8 mo (evaluated irritation, pigmentation, periorbital volume loss)), a 28-day intraocular pressure assay, a safety assessment of Isopropyl Cloprostenate in eyelash serums, concentration of use data, and packaging/directions for consumer use.

It should be noted that the safety assessment of Isopropyl Cloprostenate provided in the submission (i.e., *data20_ProstaglandinAnalogues_122023*) includes systemic toxicity data on cloprostenol and travoprost. (These are not cosmetic ingredients, according to the *Dictionary*.) The data on cloprostenol and travoprost have not been incorporated into the report as the use of cloprostenol as a read-across source was previously rejected by the Panel. If these data are deemed appropriate by the Panel for inclusion in this report, they will be added prior to the next iteration.

All new data on Ethyl Tafluprostamide and Isopropyl Cloprostenate have been incorporated into the report and are indicated by highlighted text.

No concentrations of use for Ethyl Tafluprostamide or Isopropyl Cloprostenate were submitted in response to the Council use survey performed in 2022. However, recently submitted data report that Isopropyl Cloprostenate is used in eyelash serums at up to 0.0075% (*data22_ProstaglandinAnalogues*; **please note, these new data indicate a higher use concentration than what was previously reported**), and Ethyl Tafluprostamide is used in products intended for eyelashes, eyebrows, or scalp hair at concentrations up to 0.02% (these data were included in the previous version of this report).

The following documents are also included in this packet:

- transcripts (*transcripts_ProstaglandinAnalogues_122023*)
- report history (*history_ProstaglandinAnalogues_122023*)
- data profile (*dataprofile_ProstaglandinAnalogues_122023*)
- search strategy (*search_ProstaglandinAnalogues_122023*)
- flow chart (*flow_ProstaglandinAnalogues_122023*)

A draft Abstract and Discussion have been included in this report version. The Panel should carefully consider and discuss the data (or lack thereof), and issue a Tentative Report with a safe, safe with qualifications, insufficient data, unsafe, or split conclusion, and identify any additional items for inclusion in the Discussion.

Prostaglandin Analogues – History

March 2023

NTP issued

April 2023

Concentration of use survey received – no reported uses for Ethyl Tafluprostamide or Isopropyl Cloprostenate

May 2023

Data received on Isopropyl Cloprostenate – concentration, ocular irritation, and dermal sensitization data

Data received on Ethyl Tafluprostamide (several endpoints)

June 2023

Panel reviews Draft Report

Panel issues IDA – needs include: concentration of use, application and packaging, instructions to consumers to prevent skin exposures, 28-d dermal toxicity (other endpoints, if absorbed), sensitization and irritation data, potency data (Ki values of Ethyl Tafluprostamide and Isopropyl Cloprostenate in comparison to bimatoprost)

October 2023

Data received on Ethyl Tafluprostamide (all systemic data in this packet is on tafluprost; Panel will review at December meeting if this data is appropriate for addition)

Data received on Isopropyl Cloprostenate (data in this packet also includes cloprostenol and travoprost data)

November 2023

Instructions for use of a product containing Isopropyl Cloprostenate received

Concentration of use received from Isopropyl Cloprostenate

December 2023

Panel reviews Draft Tentative Report

Prostaglandin Analogues Data Profile –December 2023 – Writer, Priya Cherian																																	
				Toxicokinetics			Acute Tox				Repeated Dose Tox				DART				Geno-tox		Carci			Dermal Irritation			Dermal Sensitization				Ocular Irritation		Clinical Studies	
	Reported Use	Method of Mfg	Impurities	log P/log K_{ow}	Dermal Absorption	ADME	Dermal	Oral	Inhalation	Parenteral	Dermal	Oral	Inhalation	Parenteral	Dermal	Oral	Parenteral	In Silico	In Vitro	In Vivo	Dermal	Oral	In Silico	In Vitro	Animal	Human	In Vitro	Animal	Human	Phototoxicity	In Vitro	Animal	Retrospective/ Multicenter	Case Reports
Ethyl Tafluprostamide			X	X	X	X												X	X				X	X				X			X		
Isopropyl Cloprostenate	X		X	X	X					X				X			X	X					X			X				X	X	X	X

* “X” indicates that data were available in a category for the ingredient

Prostaglandin analogues

Ingredient	CAS #	PubMed	FDA	HPVIS	NIOSH	NTIS	NTP	FEMA	EU	ECHA	ECETOC	SIDS	SCCS	AICIS	FAO	WHO	Web
Isopropyl Cloprostenate	157283-66-4	x											x				x
Ethyl Tafluprostamide	1185851-52-8												x				

Search Strategy

Search terms below searched in all listed links

Typical Search Terms (this is informational – not for inclusion for search strategy that goes to the Panel)

- INCI names
- CAS numbers
- chemical/technical names

LINKS

Search Engines

- Pubmed - http://www.ncbi.nlm.nih.gov/pubmed
  - appropriate qualifiers are used as necessary
  - search results are reviewed to identify relevant documents
- Connected Papers - https://www.connectedpapers.com/

Pertinent Websites

- wINCI - https://incipedia.personalcarecouncil.org/winci/ingredient-custom-search/
- FDA databases http://www.ecfr.gov/cgi-bin/ECFR?page=browse
- FDA search databases: http://www.fda.gov/ForIndustry/FDABasicsforIndustry/ucm234631.htm;,
- Substances Added to Food (formerly, EAFUS): https://www.fda.gov/food/food-additives-petitions/substances-added-food-formerly-eafus
- GRAS listing: http://www.fda.gov/food/ingredientspackaginglabeling/gras/default.htm
- SCOGS database: http://www.fda.gov/food/ingredientspackaginglabeling/gras/scogs/ucm2006852.htm
- Indirect Food Additives: http://www.accessdata.fda.gov/scripts/fdcc/?set=IndirectAdditives
- Drug Approvals and Database: http://www.fda.gov/Drugs/InformationOnDrugs/default.htm
- FDA Orange Book: https://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm
- (inactive ingredients approved for drugs: http://www.accessdata.fda.gov/scripts/cder/iig/
- HPVIS (EPA High-Production Volume Info Systems) - https://iaspub.epa.gov/oppthpv/public_search.html_page
- NIOSH (National Institute for Occupational Safety and Health) - http://www.cdc.gov/niosh/
- NTIS (National Technical Information Service) - http://www.ntis.gov/
  - technical reports search page: https://ntrl.ntis.gov/NTRL/
- NTP (National Toxicology Program) - http://ntp.niehs.nih.gov/
- Office of Dietary Supplements https://ods.od.nih.gov/

- FEMA (Flavor & Extract Manufacturers Association) GRAS: https://www.femaflavor.org/fema-gras
- EU CosIng database: http://ec.europa.eu/growth/tools-databases/cosing/
- ECHA (European Chemicals Agency – REACH dossiers) – http://echa.europa.eu/information-on-chemicals;jsessionid=A978100B4E4CC39C78C93A851EB3E3C7.live1
- ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) - http://www.ecetoc.org
- European Medicines Agency (EMA) - http://www.ema.europa.eu/ema/
- OECD SIDS (Organisation for Economic Co-operation and Development Screening Info Data Sets)- http://webnet.oecd.org/hpv/ui/Search.aspx
- SCCS (Scientific Committee for Consumer Safety) opinions: http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm
- AICIS (Australian Industrial Chemicals Introduction Scheme)- https://www.industrialchemicals.gov.au/
- International Programme on Chemical Safety http://www.inchem.org/
- FAO (Food and Agriculture Organization of the United Nations) - http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/
- WHO (World Health Organization) technical reports - http://www.who.int/biologicals/technical_report_series/en/
- www.google.com - a general Google search should be performed for additional background information, to identify references that are available, and for other general information

Botanical Websites, if applicable

- Dr. Duke's - https://phytochem.nal.usda.gov/phytochem/search
- Taxonomy database - http://www.ncbi.nlm.nih.gov/taxonomy
- GRIN (U.S. National Plant Germplasm System) - https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysimple.aspx
- Sigma Aldrich plant profiler- http://www.sigmaaldrich.com/life-science/nutrition-research/learning-center/plant-profiler.html
- American Herbal Products Association Botanical Safety Handbook (database) - http://www.ahpa.org/Resources/BotanicalSafetyHandbook.aspx
- National Agricultural Library NAL Catalog (AGRICOLA) https://agricola.nal.usda.gov/
- The Seasoning and Spice Association List of Culinary Herbs and Spices
- http://www.seasoningandspice.org.uk/ssa/background_culinary-herbs-spices.aspx

Fragrance Websites, if applicable

- IFRA (International Fragrance Association) – https://ifrafragrance.org/
- Research Institute for Fragrance Materials (RIFM) - https://www.rifm.org/#gsc.tab=0 http://fragrancematerialsafetyresource.elsevier.com/

JUNE 2022 PANEL MEETING – PRIORITY LIST DISCUSSION

Belsito Team – June 16, 2022

Dr. Belsito - So I had brought this up because of a colleague of mine who sits on the SCCS as chair, had asked me whether we were looking at this. And then at the last meeting.

We decided that this would probably be more in the purview of the FDA, but then we got, a report back from the FDA indicating that they've looked at the marketing claims and there was nothing that made it look like an OTC drug. So it was back in our court. I do feel like, I guess they're what, three products that have been voluntarily reported? I recall this correctly and yeah, frequency of use in the VCRP. And but, I still think we should reopen this for cause, even if there are three products out there. I mean, there's a prostaglandins and potential side effects or are considerable, depending upon how they're being used, which you haven't looked at, you know. One issue that I have is Bart added a long list of other potential prostaglandin analogues and I'm not sure how to handle that if we do reopen it. It's not what the SCCS looked at. So with that as background, I'll just open it up for everyone's comments.

Dr. Snyder - Well, my comment is I agree that we I can't agree with reopening, but I think we would have to consider all of them, wouldn't we?

Dr. Liebler - So can we only review things that are in the dictionary? Isn't that right?

Dr. Belsito - Yeah, yeah.

Dr. Liebler - We only can review things that are in the dictionaries as I understand it.

Dr. Eisenmann (PCPC) - You can easily review things that are in the VCRP but not the dictionary, but you don't review things that they're that are in neither.

Dr. Rettie - So am I hearing if it's in the dictionary or in the VCRP, then we review it, OK.

Ms. Kowcz (PCPC) - Correct.

Ms. Fiume (CIR) - Yes.

Dr. Liebler - Really. OK. I have no objection. I mean, this is obviously very downstream of my tenure, but I don't object to having any review of any of these that are in the dictionary.

If they're in the VCRP fine, there are a couple of structures that are not in the dictionary, but they on PDF page 4. If that's correct. Those are in the are those VCRP reported?

Dr. Belsito - Page what Dan?

Dr. Liebler - Page 4 of the PDF. But cloprostenate and travopost not in the dictionary, but they're in the VCRP?

Dr. Belsito - Yeah, I think those are actually the ones that are being used.

Dr. Liebler - Yeah. As I last conditioning agents.

Dr. Belsito - That's how they're marketed. But the prescription product, the drug product, but bimatoprost is called Latisse and is marketed as a prescription drug to actually grow the length of the eyelash.

Dr. Liebler - Uh-huh.

Dr. Belsito - A side effect is that if it gets in the eye, it can actually change the color of the eye from blue to brown.

Dr. Liebler - Oh.

Dr. Belsito - Is probably the most disturbing side effect that people do experience. But I don't do any cosmetics or it's not a product that I use at all.

Dr. Liebler - You know. So the question before us. This will work for to add these to the priority list or to proceed to a review of these?

Dr. Belsito - Well, to add them to the 2023 priority list for review, at some point, yes.

Dr. Liebler - Yeah. I mean, I think that's appropriate.

Dr. Belsito - Well, I do too. And then the question is do we add in all of these, I mean that that I couldn't answer.

Dr. Liebler - It seems to me any that are either in the dictionary or in the VCRP.

Dr. Belsito - OK, Carol.

Dr. Eisenmann (PCPC) - But there's a few eyelash conditioning agents that are not prostaglandins that I don't think should be belonged that belong in the report.

Dr. Liebler - Agreed.

Dr. Belsito - Which ones are those, Carol?

Dr. Eisenmann (PCPC) - Towards the end, black Widow Spider Peptide One SP Sr polypeptide. Well, all the ones at the end that that are proteins are mixtures.

Dr. Belsito - Like *(inaudible) adipose stromal cell conditioning media.

Dr. Eisenmann (PCPC) - Correct.

Dr. Belsito - So I guess it would start with glycerin Eitherconic acid peg, four Pinter erythritol crosspolymer, it starts there?

Dr. Eisenmann (PCPC) - I think so.

Dr. Liebler - Yeah. And then the one above it, the isopropyl dimethyl norocarp carbon phosphate. That would seem to potentially belong in the review, and then the one above it at the top of Table 3, the furanyl methylthio methyl sulfinyl triazole.

Dr. Belsito - No, OK. And the one any before that, Dan, that we should delete?

Dr. Liebler - I don't think so.

Dr. Rettie - But. What about the two unavailable on the first page, which certainly one of them sounds like prostaglandin for sure without the structures. Nor Alfa, Prosto and travoprost. They're both prostaglandins, OK.

Dr. Liebler - You know. Yeah, the structures unavailable, I guess, but they're.

Dr. Rettie - Yeah.

Dr. Liebler – Appropriate to include, so all these prostanoid structures, yeah.

Dr. Rettie - That would be 7. Of these.

Dr. Belsito - Yeah, I mean we I think we can use the data for the meta process to read across because that's been extensively studied for drug use. But it wouldn't be something that we would include in the report because it is a drug, not our cosmetic. But I think that data from that can be very helpful. So we would start with Cyclops purple, the bimatoprost. Processed in all travoprost, Roxy *(inaudible) Tanner Prostanoids or and. Nor be. Nor be not appraised, nor to floor Prost. Trifluoromethyl dehydro latanoprost. Method Burnett apros. Neural for procedural travoprostamide. And then we're deleting the fiorinal. We're including the isopropyl dimethyl neuroprosthetic and then from glycerin, itaconic acid peg, four entaerythritol crosspolymer down were eliminating. There was 1,2,3,4,5, 6-7 at the end of the list, so we're limiting those seven plus the. Be there and also that's eight and we're not going to include the metapress because that's a drug, but we'll use data on that to read across.

Dr. Liebler -Yeah. And I would just add that with prostanoids of relatively subtle appearing differences in structures can have dramatic difference in pharmacologic activity. So read across here is going to be a yeah, it's going to be a challenge.

Dr. Belsito - Carol, you still have your hand up. You're muted.

Dr. Eisenmann (PCPC) - No, I don't have any additional comments right now.

Dr. Belsito - OK.

Dr. Rettie - Yeah. So that's something more like 15 structures. I was missing a page when I was counting 7. So yeah, yeah, bigger load.

Dr. Belsito - So. Yes. So Monice were clear on this? We're adding it to the 2023 priority list and the ingredients that we're adding

Ms. Fiume (CIR) - Yes, and I'm assuming it'll be gone over again tomorrow so that Bart can definitely hear all of the names and the rationale behind it.

Dr. Belsito - OK

Dr. Snyder - You're presenting this one, Don.

Dr. Belsito - Okie doke.

Dr. Klaassen - It's going to be a huge task.

Dr. Belsito - Yeah.

Dr. Klaassen - I mean. I think we almost have to start off with the concept that you can't read across. Maybe you can for a few, but I think in general we need to be super, super confident about read across with these chemicals.

Dr. Liebler - Right. I think it'll depend on the endpoint of as usual, but it's going to be a delicate a delicate task.

Dr. Klaassen - Yeah. The good word a delicate task.

Dr. Belsito - OK. I mean, we're not going to know until we dive into it right Dan?

Dr. Liebler - Right.

Ms. Fiume (CIR) - And I do want to point out I'm just seeing it now. For the two that are not in the dictionary, it does say frequency of use not reported. I'm guessing there's suspected use, but I will let Bart speak to that because he is the one that prepared the submission.

Dr. Liebler - OK.

Dr. Belsito - Which two of those Monice that you're talking about are the?

Ms. Fiume (CIR) - Close prostanozol in the travoprost

Dr. Belsito -Yeah.

Ms. Fiume (CIR) - Yeah. So in that first column, he does indicate whether or not this frequency of use or not,

Dr. Belsito - Yeah. Were those in the EU document?

Ms. Fiume (CIR) - I do not know.

Dr. Belsito - Let me just scan that.

Ms. Fiume (CIR) - Yeah, there, there are a couple that are not in the dictionary that says frequency of use not reported.

Dr. Belsito - Yeah. So they actually were looking at there they looked at them. I don't know. They just looked at the whole class. They're not reporting that I can see their use and then they're just. I mean, it's a very helpful report and then and I think it's sort of shows that areas where you may be able to do some read across on PDF page 22 of the SCCS report.

Dr. Liebler - Got it. Thank you.

Dr. Belsito - And they also didn't have the formulas for those two. Yeah, they looked at them. OK. And then use. I like the idea of changing our use table? I don't know what other people thought?

Dr. Snyder - I think it's. I think it's improvement also.

Dr. Liebler - I like. Me too.

Dr. Belsito – Curt?

Dr. Klaassen - Sure.

Dr. Belsito - OK. Okie doke.

Cohen Team– June 16, 2022

Prostaglandins not discussed in this team.

Full Panel – June 17, 2022

Dr. Belsito - Uh. OK. Prostaglandins. Yeah, I still feel we need to open it. You know, we decided previously not to reopen it because we thought it was going to be in FDA issue. Then FDA got back to us and said, well, now that we looked at the marketing, they seemed to be marketing as a cosmetic, not as a drug. I think we need to look at it, you

know, again, VCRP is telling us there are only three products out there. I suspect they're much more. If we do reopen.

Dr. Shank - It's not a reopen, is it?

Dr. Bergfeld - It's a move it up on the priority list.

Dr. Shank - It's to add to the priority list.

Dr. Belsito - Put it on the property list. I'm sorry to put it back on.

Dr. Belsito - May 23 if we do that, there was a whole list of other products planned analog, some of which we did not feel should be in included. We could go through those if we decide to put it on the priority.

Dr. Bergfeld - Bart. Can you make comment and then tell you if the opinion just general open opinion is to put it back on the priority list or reinforce it on the priority list if we need?

Dr. Heldreth (CIR) - Yeah, as Doctor Belsito mentioned, we had brought the first four ingredients listed in this document as a draft priority back in March. And at the time it was unclear if this was within the purview of the panel or under the regulatory authority of FDA drugs. FDA cosmetics got back to us via email. Mentioning that at least some of these prostaglandin derivatives are being used in products that do not appear to make drug claims and therefore could be considered cosmetics. Specifically, they looked at one particular product that contained a ethyl tafluprostamide and the literature that surrounded it did not make any drug claims in particular and therefore it would not be under FDA drugs purview to regulate that product and then falls to this panel to evaluate the safety. So I also included table two as other structurally related prostaglandin derivatives and then only for the sake of being completely inclusive I included table three of other ingredients that are eyelash conditioning agents but are structurally diverse. Was not proposing that we add those. I just wanted to paint the entire picture for the panel.

Dr. Bergfeld - Ok. *(inaudiable)

Dr. Cohen - Yeah, I lost you. Well, I couldn't hear you. It might have been on my side. I.

Dr. Belsito - Yeah, I couldn't hear either.

Dr. Bergfeld - I said, let me see. I'm on. Can you hear me now?

Dr. Belsito - Yeah.

Dr. Heldreth (CIR) - Yes.

Dr. Cohen - Yes, yes.

Dr. Bergfeld - I got my microphone in my hand. My assumption is this was on the priority list. It was questioned. It's now been confirmed that is at cosmetic ingredient at this point in time we do not have to vote it. It's on the priority list. Is that correct?

Dr. Belsito - Are we voted it off for priority list now we have to determine whether it goes back on.

Dr. Bergfeld - Well, I think the clarification that it is a cosmetic ingredient, I guess we can call for emotion. So Don, you want to do that motion?

Dr. Belsito - Yes, put it back on the priority list.

Dr. Bergfeld - Is there a second?

Dr. Cohen - Yeah, a second and Don, do you also as part of your motion wish to include table 2 in in that when we when we review it?

Dr. Belsito - Table three you mean with the list of all the other analogues or potential additions?

Dr. Cohen - No, no. I thought it was.

Dr. Belsito - OK too, yeah.

Dr. Bergfeld - 2.

Dr. Belsito - OK. Yes. I would like to include those. We also did include some others from Table 3 but.

Dr. Liebler - Yeah, all the prosteenoid structures.

Dr. Bergfeld - Yeah.

Dr. Belsito - Yeah. So of table two and Table 3, the only ones we knocked out were purano methylethyl, methylphenol triazole, which was the at the top of PDF page 6. And then we knocked out everything beginning with again PDF Page 6, glycerin it aconitic acid peg, four pentaerythritol, crosslink or crosspolymer, and the remaining 1,2,3,4 products 5,6 below that at the end of the table. But included all the process steps.

Dr. Cohen - So David, you're OK with that grouping as we second the motion for Belsito team? You know that they're appropriately grouped, that we should review those together.

Dr. Ross - So we're looking at tables 1,2?

Dr. Belsito - Table 2 and 3.

Dr. Ross - Structurally the no. Structurally looking similar. Yeah. I mean, I think you could bring those in?

Dr. Bergfeld - OK, Bart, the usual process is that you put it together, look at the chemistry and check with our chemists on the panel to make sure that the chemistry and appropriate ingredients are in it.

Dr. Heldreth (CIR) - Yeah. I mean I think that's whats been confirmed here just now. And so we will include in the draft final priorities list that comes back to the panel in September, we will include all of the ingredients in table one, table 2 and then the one ingredient from table 3 that isopropyl dimethyl norcargo prostate

Dr. Bergfeld - OK.

Dr. Ross - The *(inaudiable) was removed.

Dr. Heldreth (CIR) - Correct everything in Table 3 except for the isopropyl, dimethyl, nor carboprost state was removed.

Dr. Belsito - Yes.

Dr. Ross - Correct.

Dr. Bergfeld - OK, since we've had.

Dr. Heldreth (CIR) - The only reason that the only reason that I put that one in Table 3 instead of Table 2 is that it did not contain a phenylring like all of these structures and stable too.

Dr. Cohen - Yes.

Dr. Liebler - I think the relevant driver structure is at site that dihydroxypropyl entame that prostate piece so the others can be variable. I would expect at the at this point.

Dr. Rettie - Yeah.

Dr. Heldreth (CIR) - Works for me.

Dr. Bergfeld - Uh, is that OK? Alright, then I'm going to call the question. Then the question will go backwards that we're going to put back onto the priority list the prostaglandins with those that were noted earlier to be included and you want to oppose this? Abstaining? Approved. Alright, we're moving forward then. Now we come to the last administrative item and that is the use tables and there have been two proposed the old one and a new one, and Doctor Cohen's going to presently.

SEPTEMBER 2022 PANEL MEETING – PRIORITY LIST DISCUSSION

Cohen Team – September 26, 2022

Dr. Bergfeld - I think that I really want to look at the prostaglandin. So I'm glad everyone's agreeable to keeping them there. They're very much in the world of dermatology, in the topical agents that we use both in cosmetics as well as in prescription drugs. The other thing is that you might want to just briefly discuss that I think the lowest use in this is the hair dye which is 22 and the prostaglandin it is 3 and then 182 the ones following. So we're going to have to decide if there is a line that we can draw. I mean, 3 uses perhaps wouldn't make it if we decide to have a concentration of use minimum.

Dr. Cohen - Yeah, there's a number of things to unpack there, Wilma, to ponder.

Dr. Bergfeld - Yeah.

Dr. Cohen - Susan, any thoughts about the prostaglandin grouping from your from your end? We wanted to get it as comprehensive as possible, but are there any outliers?

Dr. Tilton - No, I don't see anything that I would consider an outlier.

Dr. Cohen - Good to, Wilma

Dr. Tilton - So I'm assuming that some, many of them don't have uses.

Dr. Ross - We don't have.

Dr. Cohen - Many of them don't have what?

Dr. Tilton - Three of them. Three of them have uses. Is that right? Yeah.

Dr. Cohen - Yeah.

Belsito Team – September 26, 2022

Minutes not available.

Full Panel – September 27, 2022

Dr. Belsito - And then just there's a another point that we did discuss was with the Nanumm,Sephora group. There's a flower oil that has a VCRP name but not an INCI name with 9 uses. Which we will include, we just brought that out as how do you deal with an ingredient that is not listed in the cosmetic dictionary? But we'll look at it just as a point of reference. The last and probably the most important was that it was. Recommended in terms of the prostaglandin analogues, of which there are many in the dictionary that we look at, only isopropyl cloprostenate because that's the one that VCRP had data on. However, I sort of felt strongly that we should look at tafluprostamide as well, since the Europeans looked at it specifically at a concentration of .018%, suggesting that it is on the EU, so market and more than likely on our market just not reported to be VCRP during that discussion John Bailey popped up and said there may be some other prostaglandins that industry wanted to add. So if he is online a John, do you want to say something about that?

Dr. Cohen - Someone just raised their hand.

Dr. Bailey (ECG) - OK.

Dr. Belsito - Yeah, it's John.

Dr. Bergfeld - John Bailey.

Dr. Bailey (ECG) - Yeah. No, I think that that's very accurately stated. I think that there is interest in supporting the safety review and that the number of prostaglandins that are established to be used in cosmetics is likely to expand by one or two and those should certainly be added and we will provide try to provide that information. As you know folks, I'm working with develop it and then and then provide that to you for your review. So I think I think it's good to be on there. I think your logic is very sound and we look forward to moving forward on this.

Dr. Belsito - Thanks John.

Dr. Bailey (ECG) - Yeah.

Dr. Belsito - That's all I our group had on the priorities.

Dr. Bergfeld - So it seems to me that we're endorsing the priority list with some addition and expansion of some of the different ingredients?

Dr. Belsito - Yes.

Dr. Bergfeld -Bart, we need to do anything else?

Dr. Heldreth (CIR) - No, I just also just making it quite clear that we're also decreasing the size of the grouping from prostaglandins down to the two.

Dr. Bergfeld - OK.

Dr. Belsito - Or possibly more depending upon industry Bart.

Dr. Heldreth (CIR) - Correct.

Dr. Cohen - Yeah, that's what you meant, right, Don?

Dr. Belsito - Yes.

Dr. Bergfeld - Yeah.

Dr. Cohen - Yeah.

Dr. Bergfeld - So it could be up to five or six maybe. OK. Well, thank you very much. We're going on to our last item of discussion, which is yeast Doctor Belsito and to remind everyone we did have a presentation by the French Group who outlined the class of Yeast that are in cosmetics primarily so Don do you want to carry on?

JUNE 2023 PANEL MEETING – DRAFT REPORT

Cohen Team – June 12, 2023

DR. COHEN: Okay. We can keep muscling through. Prostaglandins. I'm going to need your help here.

DR. ROSS: I would just note here that someone, you know, put prostaglandins, Amphocarboxylates and yeast together on this schedule.

DR. BERGFELD: This is before lunch though.

DR. COHEN: We don't have to -- well, no, we do need to follow that based on the writers. Yes, I know it's a tough run.

DR. ROSS: Let's do it.

DR. COHEN: All right. Ethyl Tafluprostamide and Isopropyl Cloprostenate. So, Priya, this is yours. And these are used as hair conditioning agents and also reported function in cosmetics as nail conditioning agents.

The Isopropyl Cloprostenate in two eyelash serums at 0.0044 and 0.0048 percent. We have a question from the staff. Does the Panel agree that the data on Cloprostenol are not appropriate for inclusion in this report because the data cannot be read across to Isopropyl Cloprostenate? And I'd have to throw that to you guys.

DR. ROSS: I think that concludes -- well, my opinion was that conclusion is valid.

DR. COHEN: That we cannot read across?

DR. ROSS: You cannot read across, yes.

DR. TILTON: Yes. For systemic uptake that would -- and absorption.

DR. COHEN: So we have some impurities, no method of manufacturing, some DART, no genotox. Why don't I open it up for you guys to comment and then we can put our group together.

Actually, before that, I just had a question. On PDF 19, when we have a discussion about the two eyelash serums, it says unknown if these are marked serums. I was determined that 0.0044 and 0.0048 percent, respectively, corresponding to a weight of 8.4 and 13 milligrams of Isopropyl Cloprostenate per usage of each serum, respectively, does that --

DR. ANSELL: No, it's an error.

DR. BAILEY: That's a mistake.

DR. ANSELL: It should be micrograms, not milligrams.

DR. COHEN: Yeah.

DR. TILTON: Okay.

DR. ANSELL: Or nanograms, not milligrams.

DR. COHEN: Okay.

DR. ROSS: Yeah, there was a sentence in there, one or two sentences that didn't quite come together based on the previous one.

DR. COHEN: Yeah, there's one other point where it's four milligrams per brush stroke. And I'm, like, well, that's a lot of -- yeah.

DR. ROSS: Yeah. It didn't come together.

DR. BAILEY: Hi. Can I come up and speak on the mic?

DR. COHEN: Sure.

DR. BERGFELD: John Bailey. I don't know if you all have met John.

DR. BAILEY: Hi. John Bailey.

DR. HELDRETH: Can you announced your affiliation on the microphone please.

DR. BAILEY: My name is John Bailey. I'm currently a consultant with EAS Consulting group. Prior to that I sat in the PCPC chair. Prior to that I sat in the FDA chair. So, I'm very familiar with the activities of this committee.

Regarding the prostaglandins, I think, you've got a report that was mostly extracted from the SCCS. We're fortunate to have some sponsors for this ingredient for use in cosmetics. And some of the data has been submitted in May, so it was late.

But keep in mind that the SCCS is currently reviewing the prostaglandins as well, and some of the data is being generated in conjunction with that, and some of it is not. But it's a work in progress. More data will be coming in that was captured in the reports. And so, it's something that I think will give you guys a much better basis for considering and making a decision regarding the safety.

This is a cosmetic use. It's not a drug use. The products are formulated with primarily two prostaglandins, the Isopropyl Cloprostenate and the DDDE. And the rest of them, as far as I know, are not used in cosmetics. But they may be. But we really have two sponsors.

The use in cosmetics is one where the products are specially formulated so that they're thicker, they're applied in a controlled way. They're applied according to very concise directions, and they're applied with applicators that control -- adding the product in a very controlled way. So, it really becomes an exposure issue mostly. And some of the data will show that the exposure does not cause pharmacological effects, like reducing IOP measurements in eyes.

So, I think that we have some good data coming in. We're going to have more data coming in. And that will be probably be in July and August. So, that can be incorporated in for discussion in a future meeting.

Also, sponsors are going to be bringing in experts, because I'm not an expert on all these different endpoints. And the experts can present data and then be available to answer questions when you get to your reviews.

DR. COHEN: So, should we table this report?

DR. ROSS: There was a list of data coming in on PDF Page 180, according to my notes. It was quite an extensive list.

DR. BAILEY: Yeah. And, of course, we're interested in the Panel's take on what data needs exist. But there is an extensive list of data coming in and that will include the normal toxicological assessment.

DR. COHEN: It sounds like -- because if we start just doing an IDA with needs, knowing that there's other material coming in, maybe we should just table it until that data dump comes and then start the adjudication process.

DR. ROSS: I think we need some direction.

DR. HELDRETH: I mean, you could certainly do that. It seems like we have a timeline for data coming in. That's one option. Another option is to put out your data needs and then John's company would know exactly what you're looking for. And then not bring this report back until, say, December, which would give our staff plenty of time to incorporate what's coming out in July, and any responses to your IDA. So, either way it can work.

DR. COHEN: My only concern is we don't know what's in that data load. And so our IDA will seem ignorant to all the data that we're going to be reviewing the next time. So, that means we'd go out with another IDA. It wouldn't be an IDA, it would be insufficient conclusion. Right?

DR. HELDRETH: Right. Well, I mean, if the data needs change, it's a second IDA.

DR. ROSS: Yeah. And I think there is a list, a fairly comprehensive list on that page. John, has that changed? Do you know?

DR. BAILEY: Pardon?

DR. ROSS: Has that changed? It's a list in your report of the data coming in and when it's about to come in. Has that changed? Is there more data coming?

DR. BAILEY: That list is accurate and there may be some more data coming in, in addition to that. Or additional assessments that -- reports that we added to the body of data that you have available.

DR. COHEN: What PDF is that again, David?

DR. ROSS: 180.

DR. COHEN: And that's specific to DDDE?

DR. ROSS: Yeah. I mean, I went through the report and I've got a list of things that I would consider insufficient, but you know, I don't know if you want to go down that road or not. Why do we have to make that decision first?

DR. COHEN: I think it would be cleaner to have -- I think we'd be better to have the data in the report and then figure out what our data needs are. As opposed to putting data needs on, getting this and then doing another data need. I don't -- that's my gut, but I'm open to suggestions.

DR. BERGFELD: John, do we have any of the medical data on the prostaglandins? How they're used in dermatology for hair growth and eyelash growth. Is that going to be coming in as well?

DR. BAILEY: It may be included in some of the --

DR. BERGFELD: I think that would be helpful, to have it at probably higher concentrations for glaucoma (inaudible).

DR. BAILEY: Yeah. Those analyses will be part of the report.

DR. COHEN: It sounds like these cosmetics are applied the same way a drug is applied,

DR. BERGFELD: Right. Of the new -- the current one, Latisse, in particular.

DR. COHEN: Yeah. It sounds like it's very specifically controlled.

DR. BAILEY: Not quite.

DR. ROSS: I don't think so. I think Latisse is applied to the eyelids. I think these are applied to the eyelashes. Isn't that correct?

DR. BAILEY: Right. That's the way they're applied. It's not to the base.

DR. COHEN: It's not to the base. Right.

DR. ROSS: Yeah.

DR. BAILEY: It's actually applied to the lashes themselves. So, that helps to control the exposure.

DR. COHEN: Yeah.

DR. BERGFELD: But the other thing, it's only applied to one lid. And then the people go like this and it gets on two lids. Both lids are growing hair.

DR. COHEN: Are you talking about Latisse?

DR. BERGFELD: Um-hmm.

DR. COHEN: Oh, people just apply it to both?

DR. BERGFELD: No.

DR. BAILEY: Well, there were some studies to address that exposure, so.

DR. BERGFELD: The original study was to the upper lid first.

DR. ROSS: I see.

DR. BERGFELD: And so, when they blotted, it got on the lower lid.

DR. ROSS: Yeah, I'm not apprised, this is very different, but it's interesting. I did notice -- I think your comment was, it's going to be all about the exposure and I think that's correct.

DR. BAILEY: Yes.

DR. ROSS: Because, you know, the DART studies in here, there were flags, obviously. And there was nothing on the Ethyl Tafluprostamide. You know, there's no repro, no developmental. And the Isopropyl Cloprostenate had some male reproductive effects but, again, no developmental tox. But again, that revolves around the actual exposure.

DR. COHEN: Would there be any objection to us tabling this until these reports come in?

DR. HELDRETH: No objection here. You could also do kind of a combined approach and say it's tabled, but also when we publish our post meeting announcement, put in there, here's things that the Panel is hoping to see based on their review of the documents that they have in front of them, you know, the list that they just went through of everything.

It's a possibility. I'm not saying it's what you have to do. I'm just saying. That may help steer the right data that you want to come in before you see this again.

DR. COHEN: Tom?

DR. SLAGA: Tabling it would be fine.

DR. BERGFELD: Well, I think tabling with the comment that Bart made, is if we already know there's some data that's a little bit weak, the data to point out for the tox, we should include that in our request.

DR. COHEN: Okay. So why don't we enumerate those things?

DR. ROSS: Okay.

DR. COHEN: So, Belsito is going to be presenting this tomorrow and we should just be ready to go. So, we don't have method of manufacturing.

DR. ROSS: That should be an easy one. That's on my list. Yeah.

DR. COHEN: We have impurities.

DR. TILTON: I noted we have predicted absorption rates for Isopropyl Cloprostenate.

DR. BERGFELD: Can't hear you.

DR. SLAGA: We need 28 dermal on both genotoxs.

DR. TILTON: Yeah, we need 28 day, but also experimental data on dermal absorption.

DR. COHEN: On both?

DR. TILTON: We have experimental data for DBTE (phonetic).

DR. BERGFELD: Isopropyl (inaudible).

DR. TILTON: And it looks like there's going to be some additional skin penetration data coming forward.

DR. COHEN: Well, let's see. We can -- it was -- I'm sorry, David, PDF what?

DR. ROSS: 180. You've got some --

DR. TILTON: It's just the prostaglandin. It's just Page 180.

DR. COHEN: It's page 180?

DR. TILTON: Yeah.

DR. COHEN: The IDA is page 180. Plus method of manufacturing and --

DR. ROSS: Yeah, but with respect to potential things that are missing, I think we have to have a discussion whether DART is needed given the exposure. And if DART is needed, then you would need those studies on repro and developmental for Ethyl Tafluprostamide. And you would need developmental on Isopropyl Cloprostenate.

DR. COHEN: What did you need on that, reproductive?

DR. ROSS: Yeah, reproductive toxicology. I think the discussion has to be whether it's needed depending on exposure.

DR. ANSELL: Right.

DR. ROSS: And then, if the answer to that is yes, then you need both repro and developmental of both compounds. Right now there is repro in the document from the Isopropyl Cloprostenate and that's it.

My other question I had on this was, you know, there's a lot of discussion on intraocular pressure. And there's some nice data in there with the Ethyl Tafluprostamide that looked okay, used in a specific product, which was an eyelash product. So, I don't think you need anything more there.

The Isopropyl Cloprostenate was done with a microgram applied directly into the eye. And that saw about a 39 percent decrease in the intraocular pressure.

I think that should be repeated with the eyelash prep to make sure that intraocular pressure is not actually adversely affected when you are using that concentration with the eyelash composition. My guess is, it probably isn't, but I don't know the answer to that.

DR. COHEN: So you want data on intraocular pressure for the eyelash preps?

DR. ROSS: For the Isopropyl Cloprostenate, not for the Ethyl Tafluprostamide. You have that already.

DR. COHEN: We have that. Okay. I think I have those all down now.

DR. BAILEY: Okay. Can I make one additional comment? And that would be, as far as read across goes, is to, you know, not close that door. I think there's going to be an opportunity to make some presentations to support read across later when we bring in the experts to talk about this. Just to keep that on our radar, and I think it'll be worthwhile.

DR. ROSS: Well, people do seem to get very nervous when we talk about read across for prostaglandins.

DR. BAILEY: They do. Yeah.

DR. ROSS: Because You know, the interaction as you know, with the receptors is so specific and stereospecific and so it's tricky to do.

DR. BAILEY: Right.

DR. COHEN: The steroids.

DR. ROSS: Yeah.

DR. COHEN: All right. So we can comment that we can't read across for now pending any new data.

DR. ROSS: And that may change, but right now that's how we see it.

DR. COHEN: Yeah.

DR. HELDRETH: Do you have an idea of what the read across source would be that the experts would talk about?

DR. BAILEY: Pardon? Can't --

DR. HELDRETH: Do you have an idea of which read across source? Like would it be medipros (phonetic) or --

DR. BAILEY: Not specifically at this point.

DR. HELDRETH: Okay. I was just curious. Thanks.

DR. BAILEY: I mean, Cloprostenate is maybe an outlier to some degree. So, I'm not sure how that one might work. But some of the others where we have data might be useful is what I'm hearing.

DR. COHEN: Okay. We'll see how it's presented tomorrow, but it looks like we're going to go for a table with commentary. There's just so much coming in it sounds like.

DR. BAILEY: Thank you very much.

DR. COHEN: Thank you.

DR. ROSS: Thank you.

DR. HELDRETH: Thanks.

DR. BERGFELD: Nice to see you again. You going to be here tomorrow?

DR. BAILEY: Yes.

DR. BERGFELD: Okay, John, thank you.

DR. COHEN: What do you want to do?

DR. ANSELL: It's 11:52.

DR. SLAGA: Break for lunch and come back.

DR. COHEN: Yeah. So what time should we come back? You want to do --

DR. SLAGA: Quarter to.

DR. COHEN: Yeah, I was going to say 12:45. Is that good, or you want 1:00?

DR. ROSS: No, I'm fine.

DR. COHEN: 12:45? 12:45 and we reopened with the Amphocarboxylates.

DR. ROSS: Let's see how many people come back.

DR. COHEN: But we got very far through the list.

DR. BERGFELD: That's nice especially.

DR. COHEN: The backend is not going to be terribly challenging, except for the first two.

Belsito Team – June 12, 2023

DR. BELSITO: Okay, Prostaglandins. Boy, Priya. Yeast and Prostaglandin, did someone not like you?

MS. CHERIAN: Monice.

DR. BELSITO: Okay. So, again, we got a Wave 2 on the prostaglandins. So, there were a few questions that were asked that I guess we should answer. Do we agree that the data on cloprostenol are not appropriate for inclusion in the report because the data cannot be read across to isopropyl cloprostenate and this is -- I have a question for the team, particularly Allan.

DR. RETTIE: Yeah, I would agree with that. The esters are quite a bit more lipophilic so date of distribution is going to be different. I mean, they're all ultimately going to have to observe the biological effect by being converted to acid to act as a receptor, but the distribution of the more lipophilic ester prodrug that I guess is the question here. So I would agree that the read across is not there for our purposes.

DR. BELSITO: Curt, Paul?

DR. KLAASSEN: Fine.

DR. BELSITO: I had a question on the developmental and reproductive tox study.

DR. SNYDER: What page?

DR. BELSITO: This is page PDF 20. So, it's just they -- you know SCCS is using in silico tools. And in this case, they used an in silico tool to predict that they could be a reasonable certainty of developmental or reproductive toxicity. And of course, we've got the notes of guidance. I don't know if you all read through that as to how the SCCS will be operating in this 2023 and going forward.

And we're not really using any of those tools and should we be using those tools? I mean, they're in silico tools. Can we not buy that software that would allow us to put in structure and look at structural activity relationship and do things like blue screen to give us, you know, genotox alerts and alerts for DART in the absence of data?

DR. RETTIE: I'm only concerned with having some evidence of robustness for the evolving in silico tools. I'm familiar with a few of them, not every one. I've heard that they are performing pretty well, and if we have that kind of information then I'd definitely agree with us using new technology in that regard.

DR. BELSITO: I mean, I think this raises the point and, I mean, we need to enter what will soon be the second quarter of the 21st century. And maybe we need someone to come in on a specific tox endpoint and talk to us. I

mean, we're not going to be doing DPRA's or KeratinoSens we need to understand them. But talk to us about what types of in silico tools can be used to help predict where data is not present or at least give us alerts.

I mean, we can decide what to do with it but, you know, update us on where we are. Because, I mean, I'm on other tox committees and these are being used and they're being accepted. I mean, the SCCS does not use tools that aren't being accepted within the tox world.

DR. KLAASSEN: I think it's a good idea that we look into these. I think I have a similar question, is how we end up using them. You know, if there is no data and we get an alert, and it's for carcinogenicity or something bad, you know really bad, do we then ask for more data or?

DR. SNYDER: Well, I think we want all data available. And then we make a weight of evidence approach to these reports based upon the use, concentration of use -- so it's like we're not just going to take anything standalone -- I mean, I would've bet that they would've flagged prostaglandins. I mean, I would just guess that they would flag it just because the nature of that group.

So, I'm not surprised they flagged it. We do have repo data. So, we look at the repo data and if we have good solid data then we're comfortable with based upon the --

DR. BELSITO: Absorption, use --

DR. SNYDER: -- absorption and use, concentration of use and things like that. So, I agree with both of you. I think we want to see the data because sometimes we don't have that data. And if there's no alerts then we have more confidence to support that we're not concerned for cosmetic of use, so.

DR. RETTIE: So, to some extent it's getting to be a brave new world when we confront new technologies, and I just wondered if it's worth having an expert --

DR. SNYDER: I think it's a great idea.

DR. RETTIE: -- or a group presentation at the beginning of one of our meetings down the line.

DR. SNYDER: Yeah. And have them give us specific examples , like, of how it's utilized. It's utilized in other arenas.

DR. RETTIE: And how it's validated, I'd be interested in that.

DR. BELSITO: I mean, because even if we don't have the software to do this ourselves, we're going to be seeing data that has used this software and we need to know how to interpret that.

I think there are lots of insufficiencies. So, we don't have any use concentrations. We have imputed use concentrations from the products that we were reported on use. The absorption from one of the eye products seems low but we don't have any good systemic toxicity data. We have no DART, no genotox. Sensitization and irritation, to me, seemed okay if those are the concentrations that are actually used.

But, you know, I think in the end my question is are these OTC drugs because I know what they're marketed for, but they do -- they're prostaglandins. We know that there's PGF2 alpha receptors on the hair bulbs. We know that there is some penetration. We know that penetration through hair follicles will be better. If these are getting to the bulb and are causing eyelash or eyebrow growth, then that's not a cosmetic, right? So, we don't have a dose response curve on growth of hair. And an analogue, bimatoprost at 0.03 percent is marketed as a drug.

The trade name is Latisse, it's by prescription. It does cause skin darkening and there's one report of periocular darkening in this paper and it also, in some individuals, has changed the color of the eye from blue to brown. So, I mean, I think we need a lot more data on this. We need dose response in terms of hair growth because we know some of these products can do that. We need DART, we need genotoxicity. Concentration of use. Did you have any other data needs? Allan you're chuckling?

DR. RETTIE: No. I mean, that's just like the whole list here. Yeah, I have the same thing.

DR. SNYDER: Yeah, and we have very little tox data and it's all IP. So, it's not -- there's a lot of data needs.

DR. BELSITO: In Wave 2, I -- that's the problem with Wave 2, I need to keep popping back and forth.

DR. SNYDER: PC comments on the report.

DR. BELSITO: Just -- that was it in Wave 2?

DR. BAILEY: Dr. Belsito?

DR. BELSITO: Yes.

DR. BAILEY: If I could just interject here for a couple minutes.

DR. BELSITO: Sure.

DR. BAILEY: Yeah, there are basically two analogues that are currently being used that we're aware of.

MS. FIUME: Dr. Bailey, would you mind coming to a microphone? Thank you.

DR. BAILEY: Thanks. There are two prostaglandins that we have in the market now that we're aware of. Certainly, that are a step forward to address the CIR review and these are isopropyl cloprostenol and DDDE. And the two companies that are marketing these have submitted data in May, so it was submitted close to the deadline. But more data's being developed, and more reports are going to be coming in over the next two or three months. So, I think that these questions will be addressed and filled out as that data becomes available.

The question of drug versus cosmetic is one that legally these are marketed as cosmetics because they don't make any drug claims and they're used in a very different way than the drugs are used. So, the way I view it is the task here is to look at those cosmetic uses and then determine whether or not they're safe within that context. And if you look at the submissions, the manner of application, the concentrations and things like that are different than the drug uses. And the exposures are going to be very different as well.

So, I think there'll be data forthcoming to answer your questions. I think what we really need is to just have an itemization of the questions that you have. And then, of course, you know this is being reviewed by the SCCS concurrently and some of that information's being developed, or maybe most of it's being developed, for the SCCS as well.

Thinking about the next meeting in September, there is an offer to bring in experts to present and talk about the data that's being developed. So that will give you an opportunity to ask questions and have the experts answer them and enter into a dialog on this. I think that this is kind of a work in progress at this point.

DR. RETTIE: As you're here, can I ask you a question about the cosmetic use or practice amongst cosmetic users across -- because I believe that Latisse when it's being given under the guidance of a practitioner and indeed the dosing recommendation is it's only applied once a day, is that the common practice amongst cosmetic users?

DR. BAILEY: Those are the directions of -- and these products are characterized by directions, clear directions for how to use them and how to apply them and the applicators are designed so that it's applied to the hair and not the skin. So, there are a number of factors that address the exposure. And those calculations, some of them were in the submissions that we made.

DR. RETTIE: I was just curious about it because I read this in a submission that the application, the way that you apply it would localize it to the upper eyelid.

DR. BAILEY: Yeah. On the hair above the base of the --

DR. RETTIE: I just was curious about advantageous application into the eye. The iris coloration. I kind of wondered how that could be avoided at least.

DR. BAILEY: Again, this is addressed in the submissions and the ones that are commenting to provide a level of confidence that's not getting into the eye. And some of the data talks about the IOP versus when you're using this product and that there's no decrease in the IOP during application.

DR. RETTIE: So that would speak to a limited --

DR. BAILEY: Very limited exposure. I think you can almost view this as a de minimis exposure, but a de minimis exposure with controls. And those controls are the directions for use and the applicator to make sure that the product is applied in a controlled way.

DR. BELSITO: So perhaps we need that kind of information as well into this report as some more information about application and consumer instructions and what the applicator looks like.

DR. BAILEY: And again, the two PGAs that people I'm working with are not drug active ingredients, they're not used in drugs. And I think that's because they're not as powerful as bimatoprost and some of the others. But again, I think it's a de minimis use of something that is a class of chemicals that some of which are drug active ingredients and approved by FDA.

DR. RETTIE: So, in terms of the relative potency, I was curious about that, too. I did try to look into it and found a few numbers for KIs against clone receptors, but it doesn't help, the clone receptors, without knowing who they are. The KIs that I found were for ethyl cloprostenate. I think they were -- I heard like 0.4 nanomolar. I mean, that's incredibly biological.

DR. BELSITO: Allan, I'm having trouble hearing you.

DR. RETTIE: Yeah.

DR. BELSITO: The KIs that you found for?

DR. RETTIE: Let me just see which one it was. I was talking about the potency, trying to pick up on your comment about potency because I was curious about the potency of these non-drugs. And there's one here, I actually have it somewhere. So cloprostenol, which we're not talking about --

DR. BAILEY: We're not talking about that.

DR. RETTIE: -- that's the one where the KIs down about one to two nanomolar. Yeah, yeah. So, it gets a bit confusing to kind of compare because you've got a prodrug for cloprostenol and then you've got the biological data from the acids.

DR. BAILEY: Right.

DR. RETTIE: So, I just didn't have a good feel about the potency comparisons.

DR. BAILEY: Maybe that can be explained better for the purposes of the report.

DR. BELSITO: And perhaps we can get that information for these specific compounds.

DR. RETTIE: If it's out there.

DR. BELSITO: You know, their binding affinity for the PGF2 alpha receptor.

DR. BAILEY: Also, one other comment if I may. And that is the idea of read across. I know there's some question about read across, but I think there may be some valuable data that can be mined, if you will, from read across. I wouldn't rule it out. And I think when we do have the experts here, maybe they can explain that better than I can.

But I think read across may be applicable, for example, carcinogenicity or something like that where there's been a study for one of the analogues because they should be similar.

DR. KLAASSEN: So, is the purpose as a cosmetic, is it to increase the hair growth?

DR. BAILEY: No, it's not. And in fact, I think some of the studies have shown that there's not a hair growth. I'm just drawing from memory on it, but I think that's the case. Especially the IOP measurements, I think they were important to understand that this is not an exposure that is systemic and enough to cause pharmacological effects.

DR. KLAASSEN: So even -- it won't increase the hairs on the eyelid to grow?

DR. BAILEY: Not -- not --

DR. KLAASSEN: I guess my question is, why are they putting this on the eyelid?

DR. BAILEY: It's listed in the dictionary as a hair conditioner. And apparently the function is to -- in conjunction with the other ingredients of the product -- to condition the hair and the eyelashes and eyebrows.

DR. BELSITO: And, John, do you have any idea of what kind of data we would expect to receive in August or September, which would be too late for our September meeting, so this would be pushing it to December, I presume, at the very earliest?

DR. BAILEY: Yeah. It's listed in the --

MS. FIUME: PDF page 180 has a list.

DR. BAILEY: And that's just one list. There's also another sponsor who maybe providing more data as well, so.

DR. BELSITO: So, we'll get in vitro skin penetration, we'll get a DPRA, HRIPT, KeratinoSens, some genotox, bacterial and mammalian, EpiDerm irritation, absorption, UV. So, with the tox analysis, again, are we going to get KIs, binding affinities, that type of stuff? Do you know?

DR. BAILEY: I don't know. But I can take that back and ask them and see what they have.

DR. BELSITO: And I think that would be helpful to, you know, if they can provide that vis-a-vie something like the bimatoprost so we could compare a drug to a cosmetic, give us some idea of potency.

DR. SNYDER: And 28-day dermal. I mean, we just got to ask for it. If we get other data that says, yes, we don't need it, but I think we should ask for it.

DR. BELSITO: Okay.

DR. SNYDER: Don't you think?

DR. BELSITO: Yeah.

DR. SNYDER: I mean, and then if it's, you know, and then it's the litany afterwards if it shows, you know, potential issues then we need to have the full gamut. I think with this group we don't assume anything other than there's going to be biological activity.

DR. BELSITO: Could be.

DR. SNYDER: Could be.

DR. BELSITO: Curt, Allan, any other comments? So, what I have here is concentration of use, need information on the application and packaging and instructions to consumers to prevent skin exposure. 28-day dermal and if positive, other data, DART may be needed. Genotox we're expecting to get. You're saying there's going to be an AMES and a mammalian. Sensitization and irritation, we've got an HRIPT and irritation studies coming.

And if industry could provide some idea of relative potency through KIs comparing it to bimatoprost I think that would be very helpful for us. And hopefully we can get that by September at the latest so it can be incorporated in December rather than a data dump a week before our December meeting.

DR. RETTIE: It may be difficult to get the KI data. I'm just reading around this and bimatoprost is prost so it hits the prostamide receptor. We don't know what that is, so how can you get KI against something you can't clone? I suspect we won't get that data, but we can certainly ask, who knows what industry might have.

DR. BELSITO: I mean, I'm not a chemist, is there some way of comparing potencies across classes of prostaglandin analogues?

DR. BAILEY: I mean, I don't know. I'd have to take it back.

MS. FIUME: Bart may be able -- or our chemist might be able to answer it. Or Curt, can you answer?

DR. RETTIE: Curt can probably talk to this. They're all terribly potent. I mean, is there a very weak --

DR. KLAASSEN: Well, the other problem is that there's more than one type of prostaglandin receptor. You know, there's a dozen of them.

DR. RETTIE: Sure. But they've cloned most of those. It's this amide receptor that's out of there.

DR. KLAASSEN: Yeah. I would think that that data might even be available. I mean, people that are really working in that area. I mean, that's kind of the first thing you do, you clone the receptor and then you take a bunch and do a SAR on it and see which ones inhibit it. So, yeah, I think we definitely should ask for it and hopefully they even have it.

DR. BELSITO: Presumably this is the PGF2 alpha receptor, which is specific on the hair bulb.

DR. RETTIE: That's true, I believe for the cloprostenol. I think everybody agrees it's the PGF2 alpha receptor.

DR. BELSITO: But that's what's on the hair bulb that allows bimatoprost, I believe, to cause increase lash growth.

DR. RETTIE: My reading is that that's maybe a little controversial and it probably does interact with that particular receptor. But the amides interact because they have different properties on different cell types compared to the acids, supposedly at this unknown prostamide receptor which might be some splice variant that's going to be hard to get a recombinant preparation to test against. That's my reading of it at least.

DR. BELSITO: Okay.

DR. RETTIE: But we surely can ask for data that is out there.

DR. BELSITO: Other comments? Paul?

DR. SNYDER: Mm-uhm.

MS. FIUME: Don, typically with an IDA we tend to try and skip a meeting. So normally when we were scheduling this it would be scheduled for December, so is that acceptable? It gives industry time and Priya time to -- yeah.

DR. BELSITO: Yeah.

MS. FIUME: Okay. So, yeah.

DR. BELSITO: What I said is hopefully we get the data in September so we're not getting a data dump right after thanksgiving for our December meeting.

MS. FIUME: I think Priya would very much appreciate that.

MS. CHERIAN: Yeah.

MS. FIUME: I'll be nice to Priya. And I just want to clarify, you said irritation/sensitization appears to be okay or it's part of the IDA?

DR. BELSITO: No, I mean, the irritation/sensitization, there was some irritation but it's part of the IDA because at this point, I don't know the concentration of use. I mean, I'm basing all of this off of -- I mean, I'm presuming if people are doing these studies that's what is being marketed out there, but we don't have that data. But, I mean, yeah.

DR. BAILEY: We can clarify that.

DR. BELSITO: You know, I -- we're told that that's part of the data that's being developed to give us in August or September, so yes, I'd like to see that data.

MS. FIUME: I just wanted to clarify that.

DR. BELSITO: These weren't clinical studies, right, they were just reports.

MS. FIUME: They were use studies.

DR. BELSITO: Yeah.

MS. FIUME: Yeah.

DR. SNYDER: And there is a nail conditioning use. Don't forget. It's not just eye lash.

DR. BELSITO: It's reported in the dictionary.

DR. SNYDER: Oh, okay.

DR. BELSITO: I didn't see any products listed here that were specific for nail.

DR. SNYDER: And we do have the application data instructions on page 181 for what product, product A there? On page 181?

DR. BELSITO: Yeah, I saw that, but I think John was saying that the way it's packaged is also -- was that not the case?

DR. BAILEY: Well, it's formulated, number one, to be thick so it doesn't drip into the eyes. And number two, its instructions are very clear for how to use it and where to apply it and the package is designed to make sure that that all works together. And some of that was submitted in the one report, but we can cull that out and make it much clearer.

DR. SNYDER: That'd be kind of unique to have in our summary that the explicit instructions need to be included. Yeah, I mean, I'm not quite certain how we're going to handle that.

DR. BELSITO: I'm sorry?

DR. SNYDER: I'm not quite certain how we're going to handle that if there's a requirement that it be packaged a certain way to minimize ocular exposure or adjacent skin exposure. I mean, I guess safe as used when packaged with application or whatever.

DR. BELSITO: I think we say, you know, safe as used and we refer to the application method in the paper, right? We've done that with hydroxyethyl methacrylate and the acrylates for nail products, right?

MS. FIUME: Alpha hydroxy acids had a very long --

DR. BELSITO: Alpha hydroxy acids and use with a sunscreen and --

MS. FIUME: Yes.

DR. BELSITO: We've done this before.

DR. SNYDER: Okay.

MS. FIUME: It just gets complicated in deciding -- well, not complicated. The decision comes down to is it something that's purely in the discussion or is it in the discussion and the conclusion. Because I think it may have gone both ways in the past.

DR. BELSITO: Well, with alpha hydroxy acids, it was in the conclusion that it be used with a sunscreen or recommendations. With the acrylates, it was not, it was in the discussion.

MS. FIUME: So that would be a decision the panel would need to make at that time if that was the route they were going to take.

DR. SNYDER: Okay.

DR. BELSITO: I mean, I think it would be nice to see this, right, then we can decide how exactly we want to handle it.

MS. FIUME: So then, Don, if that information comes in -- so currently, it has been maybe in the in-use study where the directions to the participants of the study, they were told what to do. So for the request it's for what the actual instructions are as it would be packaged so that it wasn't just an in-use study, it would actually be actual cosmetic use and instructions. Is that what the panel's requesting?

DR. BELSITO: I mean, I would just like to see what the applicator looks like and what the instructions to the consumer are.

MS. FIUME: And all that should go under the Use section, or would you like to see it somewhere else?

DR. BELSITO: No, I think as part of the Use section is enough.

MS. FIUME: Okay. That's what I thought. Thank you.

DR. BELSITO: Anything else?

DR. KLAASSEN: There is a risk assessment here which I --

DR. BELSITO: What PDF are you on?

DR. KLAASSEN: On Page 23. And it's not explained very well but it says there's a margin of safety --

DR. SNYDER: Two and a half.

DR. KLAASSEN: -- of 2.5. I mean, we're usually -- I mean, if that's really true that's, well, bad news. I don't understand how they got it. I mean, we usually are looking for a hundred, right?

DR. BELSITO: I think this was the SCCS calculation and it was based upon -- I forget what their point of departure was, but it's reference 29, is that to the SCCS?

DR. KLAASSEN: What page is that?

DR. SNYDER: 23.

DR. BELSITO: It says for non-allowed pharmacological substances present in food of animal origin and (inaudible).

MS. CHERIAN: Jinqiu added that reference because he did this part because we confused about the MOS calculation too.

DR. KLAASSEN: It might not be relevant but if it is relevant, it's very bad. So that definitely needs to be looked into what's really going on there.

DR. BELSITO: I mean, the problem here is that we know none of what they're talking about, right? We don't know the point of departure. I mean, what is the endpoint that they're using?

DR. KLAASSEN: Right. That's what I said, we need a lot of information there.

DR. BELSITO: Yeah.

DR. KLAASSEN: But they even go on to say here that a margin of safety greater than 1 is considered to be protective. That's a pretty strange statement in itself. I mean, that might be true for cancer chemotherapeutic drug but nothing else.

DR. BELSITO: I think that we need to -- that paper needs to be fleshed out a little bit more.

DR. KLAASSEN: Yes.

MS. FIUME: So, the risk assessment did come from the SCCS, that was the additional information I believe that Jinqiu added. I just asked Bart if Jinqiu was available to step over here. I don't know if he'll see the message. But the risk assessment was in the SCCS paper.

MS. CHERIAN: And it's referenced here, the first reference.

DR. KLAASSEN: Yeah.

DR. BELSITO: But why is it referenced as a Knutson article that references 29? Because that's what I thought it was from, was the SCCS paper.

MS. FIUME: This was Jinqiu's explanation, he delved into it a little more. Hopefully, Jinqiu can step into the room and give an explanation.

DR. BELSITO: Okay. So, we just want to move on and come back to this one when Jinqiu --

DR. KLAASSEN: Yes, let's do that.

MS. FIUME: That's fine. Yes.

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DR. BELSITO: This is the first time that we're seeing this report. And after reviewing the data and being told that there would be some data forthcoming in the fall, we thought that this was insufficient. First for use concentration, we only have imputed uses based upon some sensitization and irritation data.

We're told that the way that this product is packaged, it would prevent skin contact, so we wanted information on the packaging and the directions to consumers for use. We wanted a 28-day dermal and if absorbed other tox endpoints.

We wanted some type of information on KI or binding of these materials, particularly as it would compare to matoprost, which is a prescription medication that causes eyelash growth. And sensitization and irritation at concentration of use, if its reported concentration of use is higher than what we currently have data on.

DR. COHEN: Second. For discussion, we checked all the boxes together. Intraocular pressure for Isopropyl Cloprostenate, because it looked like we had it for ET, but in the eyelash prep, right.

DR. BELSITO: Yes.

DR. COHEN: And we're fine with your IDA. Our group had suggested that we table this report until all of that data on PDF 180 -- this is a wave of data coming at us. But, we went back and forth between an IDA and a table. And if we tabled it, we were still going to list the things anyways. So, we'll second your motion, and just, perhaps, add that intra-ocular pressure.

DR. BELSITO: Sure.

DR. BERGFELD: Any other discussion? David?

DR. ROSS: I just raise the point we did discuss -- the issue here, as you pointed out, was exposure, you know, what is going to be the exposure amount with the way it's applied. I think the clarification is a really good one.

We talked a fair bit about the DART data, and not being any developmental tables with either of these two compounds. We wanted your opinions on that, whether you had any discussion on it.

DR. BELSITO: Again, I think it's going to depend upon the 28-day dermal and what we see in terms of absorption.

DR. SNYDER: And I agree that in silico that it was not predicted to be a repro.

DR. BELSITO: Right.

DR. ROSS: I think they were both flagged in silico, were they not?

DR. BERGFELD: Can't hear you, David.

DR. ROSS: I thought they were both flagged as potential in silico, maybe I got that wrong.

DR. COHEN: It says SCCS flagged both as potential reproductive developmental toxicants.

DR. ROSS: Yeah, that's what I thought.

DR. COHEN: With a reasonable model certainty.

DR. SNYDER: We're also looking at .02 percent maximum concentration.

DR. ROSS: Yeah, that was the whole thing. It's a discussion about exposure.

DR. SNYDER: We just have to see when we get the rest of the data.

DR. COHEN: That's in the insufficiency with the absorption.

DR. ROSS: But let's not forget that one when we get the absorption.

DR. BERGFELD: All right, any other discussion? Because we can call the question to go insufficient on this ingredient. John Bailey, you want to come forward? Mic, please.

DR. BAILEY: I just wanted to add from yesterday, what I heard is to move forward thinking about tabling this until the December meeting. I just wanted to make sure that I heard it right.

DR. BELSITO: No, we weren't discussing tabling.

DR. COHEN: We discussed tabling at ours. Don's motion is for an IDA with all the insufficiencies. Our team talked about a table, listing the insufficiencies. I guess the question is if there are more insufficiencies we'd have to issue a new IDA, as opposed to tabling it, we would have a single IDA at the next go around.

DR. HELDRETH: I think in this particular case, since even with an IDA our plan is to wait until December to bring this back, it's a little bit semantics, IDA or table. It's going to achieve the same goal.

DR. BAILEY: Okay, that was semantics. Okay. That's fine. I just want to make sure I heard things right.

DR. BERGFELD: Thank you. So we'll call the question on this insufficient, all those in favor of going insufficient at this time? And Tom is yes? Unanimous then. All right, Dr. Cohen, you have the next big one, Yeast.

Safety Assessment of Ethyl Tafluprostamide and Isopropyl Cloprostenate as Used in Cosmetics

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The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Thomas J. Slaga, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D., and the Senior Director is Monice Fiume. This safety assessment was prepared by Priya Cherian, M.S., Senior Scientific Analyst/Writer, CIR.

1620 L Street, NW, Suite 1200 ♢ Washington, DC 20036-4702 ♢ ph 202.331.0651
cirinfo@cir-safety.org

ABBREVIATIONS

ADME	absorption, distribution, metabolism, and excretion
C	concentration
CAS	Chemical Abstracts Service
CIR	Cosmetic Ingredient Review
CLP	classification, labeling, and packaging
Council	Personal Care Products Council
CPSC	Consumer Product Safety Commission
DDDE	dechloro dihydroxy difluoro ethylcloprostenolamide
DMSO	dimethyl sulfoxide
DPRA	direct peptide reactivity assay
ECHA	European Chemicals Agency
ED_5	median effective dose
$E_{product}$	estimated daily exposure to a cosmetic product per kg bw
EU	European Union
FDA	Food and Drug Administration
HET-CAM	hen's egg test chorioallantoic membrane
HRIPT	human repeated insult patch test
IC_{30}	30% inhibitory concentration
IC_{50}	half maximal inhibitory concentration
log K_{ow}	n-octanol/water partition coefficient
MoS	margin of safety
NR	none reported
NTP	Notice to Proceed
OECD	Organisation for Economic Co-operation and Development
Panel	Expert Panel for Cosmetic Ingredient Safety
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PoD	point of departure
QSAR	quantitative structure-activity relationship
SAR	structure-activity relationship
SCCS	Scientific Committee on Consumer Safety
SED	systemic exposure dosage
TG	test guideline
TSV	toxicological screening value
US	United States
VCRP	Voluntary Cosmetic Registration Program
Dictionary	web-based *International Cosmetic Ingredient Dictionary and Handbook* (wINCI)

DRAFT ABSTRACT

The Expert Panel for Cosmetic Ingredient Safety (Panel) assessed the safety of Ethyl Tafluprostamide and Isopropyl Cloprostenate, which are reported to be used as hair conditioning agents in cosmetics. Ethyl Tafluprostamide is also reported to function in cosmetics as a nail conditioning agent. The Panel reviewed all relevant data and concluded that Ethyl Tafluprostamide and Isopropyl Cloprostenate… [to be determined].

INTRODUCTION

This assessment reviews the safety of Ethyl Tafluprostamide and Isopropyl Cloprostenate as used in cosmetic formulations. According to the web-based *International Cosmetic Ingredient Dictionary and Handbook (*wINCI; *Dictionary)*, both Ethyl Tafluprostamide and Isopropyl Cloprostenate are reported to function in cosmetics as hair conditioning agents.[1] Ethyl Tafluprostamide is also reported to function in cosmetics as a nail conditioning agent (Table 1). Ethyl Tafluprostamide is also known as dechloro dihydroxy difluoro ethylcloprostenolamide (DDDE).

This safety assessment includes relevant published and unpublished data that are available for each endpoint that is evaluated. Published data are identified by conducting an extensive search of the world's literature; a search was last conducted October 2023. A listing of the search engines and websites that are used and the sources that are typically explored, as well as the endpoints that the Panel typically evaluates, is provided on the Cosmetic Ingredient Review (CIR) website (https://www.cir-safety.org/supplementaldoc/preliminary-search-engines-and-websites; https://www.cir-safety.org/supplementaldoc/cir-report-format-outline). Unpublished data are provided by the cosmetics industry, as well as by other interested parties.

Much of the data included in this safety assessment were found on the Scientific Committee on Consumer Safety (SCCS) website.[2] Please note that the SCCS website provide summaries of information generated by industry, and it is those summary data that are reported in this safety assessment when SCCS is cited.

CHEMISTRY

Definition and Structure

Ethyl Tafluprostamide (CAS No. 1185851-52-8; Figure 1) and Isopropyl Cloprostenate (CAS No. 157283-66-4; Figure 2) are structurally related as prostaglandin analogues. Prostaglandins are a ubiquitous group of physiologically active lipids (a.k.a. eicosanoids or autacoids) known to demonstrate diverse hormone-like effects. In humans and other animals, prostaglandins are derived enzymatically from the fatty acid arachidonic acid.[3] However, both of these ingredients are synthetic analogues. The definitions of these ingredients are provided in Table 1.

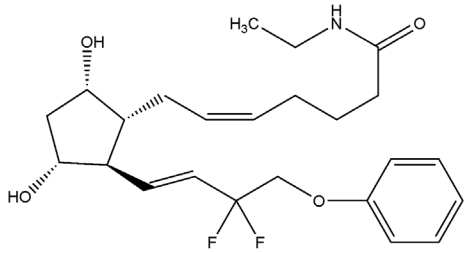

Figure 1. Ethyl Tafluprostamide

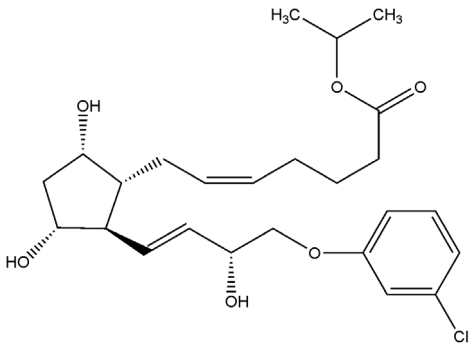

Figure 2. Isopropyl Cloprostenate

Chemical Properties

The ingredients reviewed in this report are hydrophobic, water-insoluble substances. [2] Ethyl Tafluprostamide is a colorless to pale yellow solution, with a reported water solubility of 1.05 g/l (at 20° C), and a high octanol/water partition coefficient (log K_{ow}; 2.74 ± < 0.01).[4,5] Other physical and chemical properties of Ethyl Tafluprostamide and Isopropyl Cloprostenate can be found in Table 2.

Method of Manufacture

Method of manufacture data were not found in the published literature, and unpublished data were not submitted.

Composition and Impurities

Ethyl Tafluprostamide

According to the SCCS and an unpublished data submission, Ethyl Tafluprostamide has a purity of no less than 99%.[2,6] In addition, according to the unpublished data submission, Ethyl Tafluprostamide should not contain more than 1% impurities.

An eyelash product containing 0.018% Ethyl Tafluprostamide reported to contain 18 other ingredients is referred to in several studies.[6] The additional ingredients include water, glycerin, biotin, cellulose gum, phenoxyethanol, chlorphenesin, disodium phosphate, phosphoric acid, butylene glycol, calendula officinalis flower extract, panax ginseng root extract, serenoa serrulata fruit extract, camellia sinensis leaf extract, triticum vulgar (wheat) protein, pentylene glycol, swertia japonica extract, biotinoyl tripeptide-1, and octapeptide-2. The concentrations of these ingredients were not stated.

Isopropyl Cloprostenate

The SCCS also reported that Isopropyl Cloprostenate has a purity level no less than 99.4%.[2] Impurities and accompanying contaminants in this ingredient include 15-epimer (0.25%), ethyl acetate (0.2%), and water (0.15%).

USE

Cosmetic

The safety of the cosmetic ingredients addressed in this assessment is evaluated based on data received from the US Food and Drug Administration (FDA) and the cosmetics industry on the expected use of these ingredients in cosmetics and does not cover their use in airbrush delivery systems. Data are submitted by the cosmetic industry via the FDA's Voluntary Cosmetic Registration Program (VCRP) database (frequency of use) and in response to a survey conducted by the Personal Care Products Council (Council) (maximum use concentrations). The data are provided by cosmetic product categories, based on 21CFR Part 720. For most cosmetic product categories, 21CFR Part 720 does not indicate type of application and, therefore, airbrush application is not considered. Airbrush delivery systems are within the purview of the US Consumer Product Safety Commission (CPSC), while ingredients, as used in airbrush delivery systems, are within the jurisdiction of the FDA. Airbrush delivery system use for cosmetic application has not been evaluated by the CPSC, nor has the use of cosmetic ingredients in airbrush technology been evaluated by the FDA. Moreover, no consumer habits and practices data or particle size data are publicly available to evaluate the exposure associated with this use type, thereby preempting the ability to evaluate risk or safety.

According to the 2023 VCRP survey data, Isopropyl Cloprostenate is reported to be used in 3 formulations, all of which are reported to be "other eye makeup preparations" (Table 3).[7] No uses were reported in the VCRP for Ethyl Tafluprostamide. No concentrations of use were reported for either Ethyl Tafluprostamide or Isopropyl in response to a survey initiated by the Council in 2022 (and for which results were submitted in 2023).[8] However, according to data submitted by industry as a submission separate from the concentration of use survey, the average concentrations of Isopropyl Cloprostenate in two eyelash serums were determined to be 0.0044 and 0.0048%, respectively (corresponding to a weight of 8.4 and 13 ng Isopropyl Cloprostenate per usage of each serum, respectively); it is unknown if these are marketed serums.[9] Another separate unpublished data submission (specifically stating concentration of use) reported that an eyelash serum contained 0.0075% Isopropyl Cloprostenate.[10]

In addition, according to another unpublished data submission, products intended for use on eyelashes, eyebrows, or scalp hair contain Ethyl Tafluprostamide in concentrations ranging from 0.012 – 0.02%; it is unknown if these are marketed products.[6] The amount of an eyelash product containing 0.018% Ethyl Tafluprostamide (the composition of this product can be found in the Composition and Impurities section above) applied per brush stroke was evaluated to be, on average, 2.4 mg of the product (maximum amount of 4 mg per brush stroke).[11] Accordingly, the average amount of Ethyl Tafluprostamide applied per brush stroke with use of the eyelash product was calculated to be 0.432 µg (maximum amount of 0.72 µg per brush stroke).

Although products containing these ingredients may be marketed for use with airbrush delivery systems, this information is not available from the VCRP or the Council survey. Without information regarding the frequency and concentrations of use of these ingredients (and without consumer habits and practices data or particle size data related to this use technology), the data are insufficient to evaluate the exposure resulting from cosmetics applied via airbrush delivery systems.

The ingredients named in the report are not restricted from use in any way under the rules governing cosmetic products in the European Union.[12] The SCCS is not able to conclude on the safety of Ethyl Tafluprostamide and Isopropyl Cloprostenate when used up to the intended use concentrations (0.018% for Ethyl Tafluprostamide and 0.006% and 0.007% for Isopropyl Cloprostenate).[2] The SCCS noted concerns about the safety of Ethyl Tafluprostamide and Isopropyl Cloprostenate when used in cosmetic products, particularly those used near the eye, as these are pharmacologically active substances that may have effects at low concentrations.

Eyelash Product Information/Consumer Use Instructions

Ethyl Tafluprostamide

An eyelash product containing 0.018% Ethyl Tafluprostamide is reported to be a thickened solution provided in an aluminum, tube-like container.[6] A multi-use applicator wand is attached to the container's screw-on cap. The tip of the applicator consists of a very fine brush that is designed to optimize precise application of a small amount of the product to the eyelashes. The tube neck removes excess solution from the applicator when the applicator is removed from the container.

This product is to be used once per day, directly to the eyelashes, near the base, above the eyelash line, and should be dried completely prior to the application of other products. This product includes caution statements that inform consumers to avoid contact with the eye, rinse eyes if eye contact occurs, reduce and/or discontinue frequency of product use if irritation occurs, and to keep out of reach of children. The caution statement also informs users of potential skin discoloration of the eyelash base following use (predominantly excessive use) of this product, and to discontinue use of the product if this discoloration is of concern to the consumer.

Isopropyl Cloprostenate

Eyelash serums containing 0.004 and 0.005% Isopropyl Cloprostenate are to be applied once a day, as a thin line on the eyelid, just above the upper lash line.[13,14] These serums are reported to be packaged similarly to the eyelash product containing 0.018% Ethyl Tafluprostamide described above. Serums should be applied to a clean, dry lash line, using a single stroke (similar to application of liquid eyeliner). Users are instructed to use one dip into the bottle for both eyes, and to allow 1 - 2 min for the serum to dry. Applications should occur nightly for a duration of approximately 3 mo. After 3 mo, users should apply every other day or 2 - 3 times per week to maintain benefits. Caution statements on these products inform users to rinse eyes with cold water if eye contact occurs, and to discontinue use if irritation occurs. Statements also suggest certain populations avoid use of the product (e.g., those who are pregnant, under the age of 18, undergoing chemotherapy, or with previous history of eye disorders or illnesses).

Non-Cosmetic

No FDA-approved prescription or over-the-counter drug uses for these ingredients were found in the literature. However, it should be noted that while these prostaglandin analogues are not reported to be used in FDA-approved drug formulations, other prostaglandin analogues are used in FDA-approved pharmaceuticals to treat glaucoma (e.g., bimatoprost, latanoprost, travoprost).[15] Aside from cosmetics, no other types of industrial uses were found for these ingredients.

TOXICOKINETIC STUDIES

Dermal Absorption

In Vitro

A percutaneous absorption study performed according to Organisation for Economic Co-operation and Development (OECD) test guidelines (TG) 428, using human skin samples (n = 3 replicates/dose), was performed using different eyelash products containing radiolabeled Ethyl Tafluprostamide ([^{3}H]Ethyl Tafluprostamide) at concentrations of 0.012, 0.018, 0.020, and 0.024%.[16] The test formulations were applied to skin samples (approximately 1 µCi at 10 µg/cell) for 24 h. Following application of test substances containing 0.012, 0.018, 0.020, and 0.024% Ethyl Tafluprostamide, the absorbed fraction was reported to be 6.44 ± 2.14, 6.51 ± 2.16. 9.12 ± 7.23, and 10.68 ± 7.18% of the applied dose, respectively

Computational

Ethyl Tafluprostamide

According to unpublished data, the estimated maximum amount of Ethyl Tafluprostamide that would be dermally absorbed from an eyelash product containing 0.018% Ethyl Tafluprostamide was determined to be 0.144 µg per use.[6] This calculation was based on a conservative dermal absorption of 20% and maximum single brush stroke application of the product (corresponding to maximum amount of 0.72 µg Ethyl Tafluprostamide, per brush stroke).

Isopropyl Cloprostenate

Dermal absorption of Isopropyl Cloprostenate was estimated using a quantitative structure-activity relationship (QSAR) model.[2] The estimated dermal absorption was determined to be 10% (based on a molecular weight of 476 g/mol and a log K_{ow} of 5.15 for Isopropyl Cloprostenate; no other information provided). Assuming the maximum amount of eyelash product applied per application is 4 mg,[6] the amount of Isopropyl Cloprostenate applied per brush stroke is 0.005% × 4.0 mg eyelash product = 0.2 µg. Thus, based on the estimated dermal absorption of 10%, the estimated amount of Isopropyl Cloprostenate

that would be dermally absorbed from an eyelash product containing 0.005% Isopropyl Cloprostenate was calculated to be 0.02 µg per use.

Absorption, Distribution, Metabolism, and Excretion (ADME)

In Vitro

Ethyl Tafluprostamide

An in vitro percutaneous metabolism study was performed according to OECD TG 428 using human skin samples (n = 3 replicates/dose; 0.5 cm^2 skin area).[17] Ethyl Tafluprostamide (6.0 µg/cm^2 diluted in 50% ethanol) was applied to skin samples for up to 24 h on a static transwell system. Identification and quantification of Ethyl Tafluprostamide, its metabolite tafluprost (free acid), and the reference substance (caffeine) were evaluated using an ultra-high-performance liquid chromatography system. Ethyl Tafluprostamide was found to be extensively metabolized into the free acid (tafluprost; 68.5 ± 2.7%) after 24 h. Bioavailabilites of Ethyl Tafluprostamide in the skin model were 12.3 ± 2.2 and 42.4 ± 23.1% after 4 and 24 h, respectively. Penetration of the marker compound (caffeine) was comparable with existing data from the literature.

Human

Oral

Ethyl Tafluprostamide

No oral toxicokinetic studies on Ethyl Tafluprostamide were found in the literature; however, based on reported physical and chemical properties, Ethyl Tafluprostamide is estimated to have a moderate oral absorption potential.[11] This estimation is based on a molecular weight of 452.5 g/mol, water solubility of 1.05 g/l, and a log K_{ow} of 2.74. Of note, however, the molecular weight of Ethyl Tafluprostamide is not 452.5 g/mol, but is 437.5 g/mol. Tafluprost, a chemical used by the study authors as a read-across source to target Ethyl Tafluprostamide, has a molecular weight of 452.5 g/mol.

TOXICOLOGICAL STUDIES

Acute Toxicity Studies

Parenteral

Isopropyl Cloprostenate

White albino Swiss mice (20/group; sex not stated) were administered a single dose of Isopropyl Cloprostenate (50, 75, or 100 mg/kg bw; dissolved in 1:19 dimethyl sulfoxide (DMSO) and water) via intraperitoneal injection, and observed for 14 d.[18] Two control groups were treated with physiological solution or DMSO and water. No adverse effects regarding clinical parameters, mortality, or body weight were observed.

Short-Term Toxicity Studies

Parenteral

Isopropyl Cloprostenate

Hematological evaluations were performed on white Wistar rats (10/group; sex not stated) treated with Isopropyl Cloprostenate (15 mg/kg bw/d) for 7 d via intraperitoneal injection.[18] Control groups received a solution of DMSO and water. Parameters evaluated include red blood cell count, hemoglobin, hematocrit, and red/white cell indices. Two hours after the last administration, animals were killed, and blood was examined. Results were similar among control and treated groups.

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY STUDIES

In Silico

Ethyl Tafluprostamide and Isopropyl Cloprostenate

The SCCS flagged both Ethyl Tafluprostamide and Isopropyl Cloprostenate as potential reproductive/developmental toxicants with a reasonable model certainty, based on an in silico assessment.[2] The systems used included QSAR-based systems (VEGA-QSAR and US EPA-TEST) and read-across (TOXREAD). No other details were provided.

Parenteral

Isopropyl Cloprostenate

The effect of Isopropyl Cloprostenate on the apoptosis of male mice (20/group; strain not stated) and Wistar rat (20/group) testicular cells was evaluated in a 28-d study.[19] Intraperitoneal injections of the test substance were given to mice in a dose of 25 µg/kg bw/d, and to rats in doses of either 25 or 100 µg/kg bw/d. Control groups of mice and rats were left untreated. Animals were killed at different time intervals (after 7, 14, and 28 d of treatment), and histological examinations of the gonads were performed. Normal structures of the testicular cells were observed in control groups. In rats treated with 100 µg/kg bw/d, enlarged blood vessels were noted. Blood vessel diameter increased in a time-dependent manner. This effect was also noted in rats treated with 25 µg/kg bw/d; however, the increase in blood vessel diameter was smaller. After 14 and 28 d of treatment, hyaline-like material was observed in the interstitial space surrounding the seminiferous tubules in

rats treated with 100 µg/kg bw/d. Also observed in this group was accumulation of polymorphonuclear neutrophils and macrophages, reduced spermatozoa, affected spermatogenesis, and nuclear condensation of the testicular cells. Macrophages, decreased spermatozoa, and affected spermatogenesis were observed in treated mice.

A similar study was performed in male mice (12 mice/group; strain of mice not specified).[20] Mice were treated with Isopropyl Cloprostenate (25 µg/kg bw/d) for 28 d via intraperitoneal injection. A control group of mice was left untreated. After 7, 14, or 28 d, animals were killed and effects on the gonads were examined. Results revealed swollen endothelial cells, macrophages with residual bodies, a large number of fibroblasts in interstices, lysosome-like dense bodies in the cytoplasm of Sertoli cells, clumped erythrocytes in capillaries, spermatocytes with condensed cytoplasm, and nuclei with a high chromatin condensation.

GENOTOXICITY STUDIES

In Silico

Isopropyl Cloprostenate

A QSAR model and a statistical-based model of an Ames test on Isopropyl Cloprostenate predicted no genotoxicity.[2] No details were provided.

In Vitro

Ethyl Tafluprostamide

No mutagenicity was observed in a 2-part Ames assay performed using Ethyl Tafluprostamide (purity: 99.78%; vehicle: DMSO; tested at up to 5000 µg/plate; OECD TG 471; performed with and without metabolic activation) on *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100, and *Escherichia coli* WP2 uvrA (pKM101).[21] Similarly, no genotoxicity was observed in a 3-part micronucleus assay using Ethyl Tafluprostamide (purity 99.78%: vehicle: DMSO; tested at up to 350 - 500 µg/ml in main experiments; OECD TG 487; performed with and without metabolic activation) on human lymphocytes.[22]

CARCINOGENICITY STUDIES

In Silico

Ethyl Tafluprostamide and Isopropyl Cloprostenate

According to a structure-activity relationship (SAR) analysis conducting using OECD QSAR Toolbox v.4.6 and Derek Nexus v.6.2.1, no structural alerts were found on Ethyl Tafluprostamide indicating a potential for carcinogenicity.[11] However, according to an in silico analysis of Ethyl Tafluprostamide and Isopropyl Cloprostenate performed by the SCCS, both Ethyl Tafluprostamide and Isopropyl Cloprostenate were flagged for potential carcinogenicity with a reasonable model certainty, raising the concern that these ingredients may be non-genotoxic carcinogens.[2] QSAR analysis conducted using the VEGA v.1.2 and liver specific cancer (rat/mouse in vivo) Danish QSAR model platforms gave mixed results (which included negative, positive, and inconclusive predictions of carcinogenicity for Ethyl Tafluprostamide).[11] However, predictions by these platforms were outside the applicability domain.

OTHER RELEVANT STUDIES

Characterization of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) Receptors in Human Eyelids

The following study has been included in this report as it may provide insight regarding the potential sites of toxicity of Isopropyl Cloprostenate.

Isopropyl Cloprostenate

The distribution and presence of $PGF_{2\alpha}$ receptors in human hair follicles was evaluated in excised lower eyelid specimens.[23] Analysis was performed on 37 samples examining 17 eyes using 15 patients. Samples were stained with hematoxylin and eosin prior to analysis. All specimens contained hair follicles in the anagen phase, while only 4 samples had specimens in the catagen phase, and staining was only present in hair follicles on the anagen stage. Among the four parts of the hair follicle (bulb, stem/suprabulbar, isthmus, and infundibulum), only the bulb and stem/suprabulbar areas displayed positive staining for $PGF_{2\alpha}$ receptors. In the bulb, the strongest staining occurred in the matricular cells and in the inner sheath layer. Within the inner sheath of the bulb (consisting of Henley, Huxley, and cuticle layers), the presence of $PGF_{2\alpha}$ receptors was observed mainly in the Huxley layer. Generally, when staining was apparent, it occurred predominantly in the cytoplasm of cells with slight membranous staining.

Evaluation of Conjunctival Hyperemia

The following studies on conjunctival hyperemia, pupil constriction, intraocular pressure, ocular pigmentation, and periorbital volume have been included in this report as they may provide insight on ocular effects following exposure to prostaglandin analogues.

Isopropyl Cloprostenate

Conjunctival hyperemia was evaluated in New Zealand albino rabbits.[24] The dose of Isopropyl Cloprostenate estimated to produce conjunctival hyperemia in 15% of the tested rabbits over a 4 h period was 0.3 µg. No other details were provided for this study.

Pupil Constriction

Isopropyl Cloprostenate

The effect of Isopropyl Cloprostenate on the constriction of pupils was evaluated in cats.[24] Potency was expressed as an ED_5 value which represents the dose estimated to produce a 5 unit area (mm*h) in a graph of the difference in pupil diameter in the dosed eye versus time (or median effective dose). The ED_5 for Isopropyl Cloprostenate was determined to be 0.013 µg. No other details were provided in this study.

Intraocular Pressure

Ethyl Tafluprostamide

The effect of an eyelash product containing 0.018% Ethyl Tafluprostamide on intraocular pressure was evaluated in 19 subjects.[6] Subjects were instructed to use the product for 28 d, and were evaluated at baseline and on day 28. No changes in intraocular pressure were observed in subjects after 28 d of product use. The within-eye differences in intraocular pressure from the beginning to the end of the study were not statistically significant ($t > 0.05$). A similar assay was performed in 19 subjects using an eyelash product containing 0.025% Ethyl Tafluprostamide.[11,25] Subjects applied the product to eyelashes, once per day for 28 d, with evaluations occurring at baseline and day 28. No statistically significant reduction in intraocular pressure was observed over the 28-d study. The results of the ocular irritation evaluation performed during these studies can be found in the Ocular Irritation section of this report.

Isopropyl Cloprostenate

The intraocular pressure lowering efficacy of Isopropyl Cloprostenate was evaluated in conscious ocular-hypertensive cynomolgus monkeys.[24] A 39% reduction in intraocular pressure was observed following application of Isopropyl Cloprostenate (1 µg) to lasered right eyes. No other details were provided for this study.

The potential for an eyelash serum containing 0.005% Isopropyl Cloprostenate to affect intraocular pressure was evaluated in a 28-d study on 21 subjects.[26] Subjects were instructed to apply the serum to the eyelashes of both eyes, nightly. Intraocular pressure measurements were taken at baseline and at day 28. No statistically significant differences in intraocular pressure was observed in either the left or right eyes after 28 d of use.

Ocular Pigmentation and Periorbital Volume

Isopropyl Cloprostenate

The effect of an eyelash serum containing 0.0044% Isopropyl Cloprostenate on ocular pigmentation and periorbital volume was evaluated in 120 subjects for 8 mo.[13] Imaging was performed at baseline and at 1, 2, 4, and 8 mo intervals to measure the potential change in ocular pigmentation and periorbital volume. No statistically significant differences in visible eye color of the iris from baseline to after 8 mo of use was observed. In-depth photography indicated a statistically significant increase in overall color change of the iris over the length of the study; however, this was attributed to effects that were not relevant to the issue of ocular pigmentation (e.g., redness). There was no change in periorbital fat volume after 8 mo of use. Ocular irritation evaluated in this study can be viewed in the Ocular Irritation section of this report.

Endocrine Effects

Ethyl Tafluprostamide

The endocrine activity potential of Ethyl Tafluprostamide was evaluated using several in silico tools (OECD QSAR Toolbox v.4.6, Derek Nexus version 6.2.1, Danish QSAR models, VEGA v.1.2.3, Endocrine Disruptome).[11] Mixed results were obtained, indicating that Ethyl Tafluprostamide may have some endocrine disruption activity.

DERMAL IRRITATION AND SENSITIZATION STUDIES

Details on the dermal irritation and sensitization studies summarized below can be found in Table 4.

Ethyl Tafluprostamide (98.5% purity; tested neat) was determined to be non-irritating in one EpiDerm™ assay (1-h exposure);[27] however, Ethyl Tafluprostamide (99.78% purity; tested neat) was determined to be irritating in a different EpiDerm™ assay (15-min exposure).[28] A negative prediction for sensitization was determined in a direct peptide reactivity assay (DPRA) using Ethyl Tafluprostamide (98.5% purity) in acetonitrile (100 mM; cysteine peptides only used in assay).[29] In a DPRA performed using Ethyl Tafluprostamide (99.78% purity) in acetonitrile (100 mM; cysteine and lysine peptides used in assay), a negative prediction for sensitization was determined according to the cysteine 1:10/lysine 1:50 prediction model; however, precipitation was observed in the lysine-peptide assay (conclusion of lack of reactivity could not be drawn with sufficient confidence).[30] Ethyl Tafluprostamide (98.5% purity; up to 2000 µM) in DMSO was not predicted to induce sensitization in a KeratinoSens™ assay.[31] However, inconclusive results were obtained in a KeratinoSens™ assay with Ethyl Tafluprostamide (99.78% purity; up to 250 µM) due to no clear dose-dependent results (increase in luciferase induction at

250 M; all lower test concentrations showed induction values in range of solvent control).[32] An eyelash product containing 0.018% Ethyl Tafluprostamide (n = 51; tested neat),[6] an eyelash conditioner containing 0.025% Ethyl Tafluprostamide (n = 51; tested neat),[33] and 7.5% Ethyl Tafluprostamide in phenoxyethanol (n = 54; final test concentration of 0.267% Ethyl Tafluprostamide) were considered to be non-sensitizing in human repeat insult patch tests (HRIPTs).[6,33,34] HRIPTs were also performed using eyelash serums containing Isopropyl Cloprostenate (0.0044% and 0.005%; tested neat; n = 50-56).[35-38] Three of the four assays were performed under semi-occlusive conditions. The serums tested were considered to be non-irritating and non-sensitizing in all assays.

Phototoxicity

Ethyl Tafluprostamide

Although no photo-induced toxicity studies were available in the literature on these ingredients, an ultraviolet-visible study with Ethyl Tafluprostamide (neat oil) performed in accordance with OECD TG 101 revealed an absorption band in the range of 210 - 240 nm, with maximum absorption at 226 nm, and an absorption band in the range of 250 - 285 nm, with three maxima at 265, 258, and 276 nm.[39] Molar extinction coefficients for these three maxima were within the range of 1046.2 – 1306.1 l/(mol * cm). Because these maxima are above the cut-off limit (> 1000 l/(mol * cm), photoreactivity cannot be ruled out. However, according to SCCS notes of guidance, because the maximum absorbance wavelength is below 313 nm, further in vitro toxicity testing is not required.[40]

OCULAR IRRITATION STUDIES

Details on the in vitro and human ocular irritation studies summarized below can be found in Table 5.

An eyelash product containing 0.018% Ethyl Tafluprostamide (test concentration not stated),[6] an eyelash product containing 0.025% Ethyl Tafluprostamide (tested neat),[41] and Ethyl Tafluprostamide (99.78% purity; tested neat)[42] were not predicted to be an ocular irritant in a hen's egg chorioallantoic membrane (HET-CAM) assays. Eyelash serums containing Isopropyl Cloprostenate (0.0044[43,44] and 0.005%[45-47]) were evaluated in HET-CAM assays (tested at 10 - 50% dilutions resulting in actual test concentrations of 0.00044% - 0.0025% Isopropyl Cloprostenate). All test substances were predicted to be slightly or non-irritating. Similarly, Isopropyl Cloprostenate (0.1%) was predicted to be non-irritating in a HET-CAM assay (tested at a 50% dilution resulting in an actual test concentration of 0.05% Isopropyl Cloprostenate).[48]

Several use studies were performed with eyelash products. With an eyelash product containing 0.018% Ethyl Tafluprostamide, the majority of subjects displayed no signs of ocular irritation when the product was applied to the eyelashes of 19 subjects for 28 d (4 subjects reported minor allergic reactions).[6] Similar results were observed in a use study performed in 19 subjects using an eyelash product containing 0.025% Ethyl Tafluprostamide for 28 d.[11,25] No ocular irritation was observed in 29 subjects after use of an eyelash serum containing 0.0044% Isopropyl Cloprostenate for 6 wk and of an eyebrow serum containing 0.0044% Isopropyl Cloprostenate for 7 wk.[49] Reversible ocular irritation was observed in 2 subjects in a 12-wk assay in which 32 subjects applied an eyelash serum containing 0.0044% Isopropyl Cloprostenate. Slight, transient ocular irritation was observed in an 8-mo use study performed in 120 subjects using an eyelash serum containing 0.0044% Isopropyl Cloprostenate.[13] No ocular irritation, other than slight bulbar conjunctival irritation in one assay, was observed in two ocular irritation assays performed in humans (n = 30; 32) using eyelash and eyebrow serums containing 0.005% Isopropyl Cloprostenate.[50,51] No ocular irritation was observed in a 4-wk assay in which an eyelash formulation containing 10% Isopropyl Cloprostenate was applied near the eyes of 27 subjects.[2]

CLINICAL STUDIES

Clinical Trial

Isopropyl Cloprostenate

The effect of an eyewash containing Isopropyl Cloprostenate (0.01%) in a phosphate buffered saline was evaluated in 23 patients with glaucoma.[2] The eye wash was applied to the eyes once daily for 3 mo. Over the treatment period, no changes in visual acuity or papilla appearance were observed. Mild hyperemia of the bulbar conjunctiva was observed; however, this was reported to disappear after 2-3 d of treatment. No other adverse effects were observed.

Case Report

Isopropyl Cloprostenate

A 32-yr-old woman presented to an outpatient department due to periocular discoloration for 4 mo.[52] The patient denied the use of medications other than a Chinese tea mixture for acne treatment. The patient reported the use of an eyelash serum containing Isopropyl Cloprostenate which resulted in irritated periorbital skin after a month of treatment. Approximately 1 yr later, greenish discoloration appeared, which worsened over time; however, the patient continued use of the product. No pathological changes were found, and no ocular abnormalities were observed other than hyperemia of the eyelids, upon assessment. Confocal laser canning microscopy revealed small white spots in the perifollicular dermis and in the surrounding dilated vessels. A significant reduction of the discoloration was observed at a follow-up appointment at 17 mo later. (The study does not clearly state if serum use was discontinued prior to follow-up appointment.)

Periocular effects following the use of an eyelash product containing Isopropyl Cloprostenate were also observed in a 35-yr-old woman who reported use of the product for 10 mo.[53] During use period, the patient reported hollowing, thinning, wrinkling, and darkening of the skin of the periorbital region. Six months after discontinued use, the patient reported extensive improvement of symptoms.

Adverse Event Reports

Ethyl Tafluprostamide

According to an unpublished data submission, a company evaluated undesirable effects that were reported by consumers of an eyelash product containing 0.018% Ethyl Tafluprostamide over the course of 2 yr (2011 – 2013).[6] The number of reported undesirable effects for this product, during this time period, was 0.00717% of the number of sold units. The reported adverse effects were described as typical in nature to those associated with cosmetic products near the eyes, specifically mascara and eyeliner.

RISK ASSESSMENT

True margins of safety for these ingredients could not be calculated as systemic toxicity data on these ingredients are not available. However, margin of safety (MoS) calculations have been performed using systemic points of departure (POD derived from chemicals similar to Ethyl Tafluprostamide and Isopropyl Cloprostenate (tafluprost and travoprost, respectively). The MoS for an eyelash product containing 0.018% Ethyl Tafluprostamide was calculated to be 481 when the average amount of product is applied, and 288 for when the maximum amount or product is applied.[11] An MoS of an eyelash serum containing 0.005% Isopropyl Cloprostenate was calculated to be 1029.[54] Each of these MoS values is considered to be protective. Explanations of the parameters used for these calculations can be found in Table 6.

SUMMARY

The safety of 2 prostaglandin analogues, Ethyl Tafluprostamide and Isopropyl Cloprostenate, is reviewed in this safety assessment. According to the *Dictionary*, these ingredients are reported to function as hair conditioning agents in cosmetics. Ethyl Tafluprostamide is also reported to function in cosmetics as a nail conditioning agent.

According to 2023 VCRP data, Isopropyl Cloprostenate is used in 3 “other eye makeup preparation” formulations, and no uses were reported to Ethyl Tafluprostamide. No concentrations of use were reported for either Ethyl Tafluprostamide or Isopropyl Cloprostenate in response to a survey initiated by the Council in 2022. However, unpublished data submitted separately from the survey state that Isopropyl Cloprostenate is used at up to 0.0075% in eyelash serums. In addition, an unpublished data submission indicated products used on eyelashes, eyebrows, or scalp hair contain Ethyl Tafluprostamide in concentrations ranging from 0.012% - 0.020%.

User instructions on an eyelash product containing 0.018% Ethyl Tafluprostamide state that the product is to be applied once per day, directly to the eyelashes, near the base, above the eyelash line, and should be dried completely prior to the use of other products. Eyelash serums containing 0.004 and 0.005% Isopropyl Cloprostenate are also to be applied once per day; however, these products are applied in a thin line on the eyelash line (similar to application of liquid eyeliner). Caution statements are provided on these products informing users to rinse eyes and discontinue use if irritation occurs.

According to unpublished data, the estimated maximum amount of Ethyl Tafluprostamide that would be dermally absorbed was calculated to be 0.144 µg (based on maximum use of a product containing 0.018% Ethyl Tafluprostamide and dermal absorption rate of 20%). The estimated maximum amount of Isopropyl Cloprostenate that would be dermally absorbed was calculated to be 0.02 µg (based on estimated maximum use of a product containing 0.005% Isopropyl Cloprostenate and a dermal absorption rate of 10%). In an in vitro percutaneous absorption study, the absorbed fraction of an eyelash product containing 0.018% Ethyl Tafluprostamide was determined to be 6.51 ± 2.16% of the applied dose (after a 24 h exposure period). An estimated dermal absorption of Isopropyl Cloprostenate was determined to be 10%, according to a QSAR model. In an in vitro percutaneous metabolism study, Ethyl Tafluprostamide (50% in ethanol) was found to be extensively metabolized into tafluprost (i.e., free acid) after 24 h. Ethyl Tafluprostamide was estimated to have a moderate oral absorption potential based on the reported physical and chemical properties of this ingredient.

An acute toxicity assay was performed in rats given Isopropyl Cloprostenate in DMSO and water (up to 100 mg/kg bw) via intraperitoneal injection. No adverse effects were observed throughout the 14-d observation period.

A hematological analysis was performed in rats given Isopropyl Cloprostenate (15 mg/kg bw/d), via intraperitoneal injection, for 7 d. No hematological abnormalities were observed.

Based on an in silico analysis, the SCCS flagged Ethyl Tafluprostamide and Isopropyl Cloprostenate as potential reproductive/developmental toxicants. The effect of Isopropyl Cloprostenate (25 or 100 µg/kg bw/d) on gonads and testicular cells was evaluated in mice and rats. In these assays, animals were treated for 28 d, and killed at different time intervals prior to evaluation. Time- and dose-dependent adverse effects (e.g., enlarged blood vessels, macrophages, reduced spermatozoa, reduced spermatogenesis, dense bodies in cytoplasm of Sertoli cells, clumped erythrocytes) were observed in treated animals.

A QSAR model and a statistical-based model of an Ames test on Isopropyl Cloprostenate predicted no genotoxicity. Negative results were obtained in a multi-part Ames assay and micronucleus assay using Ethyl Tafluprostamide (purity: 99.78%; concentrations of up to 5000 µg/plate in Ames assay; concentrations of up to 350-500 µg/ml in micronucleus assay). Both assays were performed with and without metabolic activation.

No structural alerts were observed for Ethyl Tafluprostamide according to SAR analyses performed using OECD QSAR Toolbox v.4.6 and Derek Nexus v.6.2.1. However, both Ethyl Tafluprostamide and Isopropyl Cloprostenate were flagged for potential carcinogenicity by the SCCS due to in silico analyses. QSAR analysis conducted using the Vega v.1.2 and liver specific cancer (rat/mouse in vivo) Danish QSAR model platforms gave mixed results (negative, positive or inconclusive predictions of carcinogenicity for Ethyl Tafluprostamide). However, predictions by these platforms were outside the applicability domain.

The distribution and presence of $PGF_{2\alpha}$ receptors in human hair follicles was evaluated using excised lower eyelid samples. Receptors were only found in hair follicles in the anagen stage and were primarily present in the matricular cells of the bulb and inner sheath layer of the hair follicle.

The dose estimated to produce conjunctival hyperemia in 15% of test rabbits over a 4 h period was determined to be 0.3 µg Isopropyl Cloprostenate. The ED_5 for Isopropyl Cloprostenate was determined to be 0.013 µg in an assay performed in cats evaluating pupil constriction potential.

No statistically-significant changes in intraocular pressure were observed in 19 subjects after a 28-d use period of an eyelash product containing 0.018% Ethyl Tafluprostamide or an eyelash product containing 0.025% Ethyl Tafluprostamide. A 39% reduction in intraocular pressure was observed in ocular hypertensive monkeys treated with 1 µg Isopropyl Cloprostenate (in lasered right eyes). No statistically-significant changes in intraocular pressure were observed in a 28-d study in which 21 subjects applied an eyelash serum containing 0.005% Isopropyl Cloprostenate nightly.

The potential for an eyelash serum containing 0.0044% Isopropyl Cloprostenate to cause changes in ocular pigmentation and periorbital volume was evaluated in an 8-mo study involving 120 subjects. No serum-induced changes in ocular pigmentation or periorbital volume were observed.

Mixed results were observed in several in silico models evaluating the potential endocrine disruption activity of Ethyl Tafluprostamide. This suggests that this ingredient may have some endocrine disruption activity.

Ethyl Tafluprostamide (98.5% purity; tested neat) was determined to be non-irritating in one EpiDerm™ assay (1-h exposure); however, Ethyl Tafluprostamide (99.78%) was determined to be non-irritating in a different EpiDerm™ assay (15-min exposure). A negative prediction for sensitization was determined in a DPRA using Ethyl Tafluprostamide (98.5% purity) in acetonitrile (100 mM); however, precipitation was observed in the lysine-peptide assay of a different DPRA using Ethyl Tafluprostamide (99.78% purity) in acetonitrile (100 mM). Ethyl Tafluprostamide (98.5% purity; up to 2000 µM) in DMSO was not predicted to induce sensitization in a KeratinoSens™ assay. However, inconclusive results were obtained KeratinoSens™ assay using Ethyl Tafluprostamide (99.78% purity; up to 250 µM) due to no clear dose-dependent effects. An eyelash product containing 0.018% Ethyl Tafluprostamide (tested neat), an eyelash conditioner containing 0.025% Ethyl Tafluprostamide (tested neat), and 7.5% Ethyl Tafluprostamide in phenoxyethanol (final test concentration of 0.267% Ethyl Tafluprostamide) were considered to be non-sensitizing in HRIPTs. HRIPTs were performed using serums containing Isopropyl Cloprostenate (0.0044% and 0.005%; tested neat). The serums tested were considered to be non-irritating and non-sensitizing in all assays.

No phototoxicity studies on these ingredients were found in the literature; however, according to an ultraviolet-visible study with Ethyl Tafluprostamide, photoreactivity could not be ruled out as calculated molar extinction coefficients were above the cut-off limit. Because the maximum absorbance wavelength was below 313 nm, no further in vitro toxicity testing is required, according to SCCS notes of guidance.

An eyelash product containing 0.018% Ethyl Tafluprostamide (test concentration not stated), an eyelash product containing 0.025% Ethyl Tafluprostamide, and Ethyl Tafluprostamide (99.78% purity; tested neat), were not predicted to ocular irritants in HET-CAM assays. Eyelash serums containing Isopropyl Cloprostenate (0.0044 and 0.005%) were evaluated in HET-CAM assays (tested at 10 - 50% dilutions resulting in actual test concentrations of 0.00044 - 0.0025% Isopropyl Cloprostenate). All test substances were predicted to be slightly or non-irritating. Similarly, Isopropyl Cloprostenate (0.1%; tested at a 50% dilution, resulting in an actual test concentration of 0.05%) was predicted to be non-irritating in a HET-CAM assay.

The majority of subjects displayed no signs of irritation in two use assays in which eyelash products containing either 0.018% Ethyl Tafluprostamide or 0.025% Ethyl Tafluprostamide were applied daily for 28 d (n =19 in both assays). No ocular irritation was observed in 29 subjects after use of an eyelash serum containing 0.0044% Isopropyl Cloprostenate for 6 wk and of an eyebrow serum containing 0.0044% Isopropyl Cloprostenate for 7 wk. Slight ocular irritation was observed in an 8-mo use study in which 120 subjects used an eyelash serum containing 0.0044% Isopropyl Cloprostenate. Reversible ocular irritation was observed in 2 subjects in a 12-wk assay in which 32 subjects applied an eyelash serum containing 0.0044% Isopropyl Cloprostenate. No ocular irritation, other than slight bulbar conjunctival irritation in one assay, was observed in ocular irritation assays performed in humans (n = 30; 32) using eyelash and eyebrow serums containing 0.005%

Isopropyl Cloprostenate. No ocular irritation was observed in a 4-wk assay in which an eyelash formulation containing 10% Isopropyl Cloprostenate was applied near the eyes of 27 subjects.

The effect of an eyewash containing Isopropyl Cloprostenate (0.01%) was evaluated in 23 glaucoma patients (treatment once daily for 3 mo.). No adverse effects other than reversible mild hyperemia of the bulbar conjunctiva were observed.

A 32-yr-old woman experienced periocular discoloration following the use of an eyelash serum containing Isopropyl Cloprostenate. The patient reported that discoloration began after 1 mo of treatment, which continued to worsen over time. Discoloration was significantly reduced at a 17-mo. follow-up appointment. A 35-yr-old woman reported hollowing, thinning, wrinkling, and darkening of the skin around the periorbital region following the use of an eyelash product containing Isopropyl Cloprostenate. Symptoms were significantly improved 6 mo after discontinued use.

A company evaluated undesirable effects that were reported by consumers of an eyelash product containing 0.018% over the course of 2 yr (2011 – 2013). The number of reported undesirable effects for this product, during this time period, was 0.00717% of the number of sold units.

The MoS for an eyelash product containing 0.018% Ethyl Tafluprostamide was calculated to be 288 based on a maximum daily amount of 8 mg of the product. An MoS of an eyelash serum containing 0.005% Isopropyl Cloprostenate was calculated to be 1029.

DRAFT DISCUSSION

[Note: This Discussion is in the draft form, and changes will be made following the Panel meeting.]

This assessment reviews the safety of 2 prostaglandin analogues, Ethyl Tafluprostamide and Isopropyl Cloprostenate, as used in cosmetic formulations. The Panel concluded [TBD].

The Panel's respiratory exposure resource document (https://www.cir-safety.org/cir-findings) notes that airbrush technology presents a potential safety concern, and that no data are available for consumer habits and practices thereof. As a result of deficiencies in these critical data needs, the safety of cosmetic ingredients applied by airbrush delivery systems cannot be determined by the Panel. Therefore, the Panel has concluded the data are insufficient to support the safe use of cosmetic ingredients applied via an airbrush delivery system.

CONCLUSION

To be determined.

TABLES

Table 1. Definitions, structures, and reported functions[1, CIR STAFF]

Ingredient (CAS No.)	Definition	Function
Ethyl Tafluprostamide (1185851-52-8)	Ethyl Tafluprostamide is a synthetic analogue of a prostaglandin. It conforms to the structure in Figure 1.	hair conditioning agents; nail conditioning agent
Isopropyl Cloprostenate (157283-66-4)	Isopropyl Cloprostenate is a synthetic analogue of a prostaglandin. It conforms to the structure in Figure 2.	hair conditioning agent

Table 2. Chemical properties

Property	Value	Reference
Ethyl Tafluprostamide		
Physical Form	liquid	[2]
Color	colorless to pale yellow	[2]
Molecular Weight (g/mol)	437.5	[2]
Density (g/ml)	1.21	[11]
Vapor pressure (Pa at 25 ºC)	1.25 x 10^{-13}	[11]
Melting Point (°C)	95.08	[11]
Boiling Point (°C)	503.76	[11]
Water Solubility (g/l @ 20ºC)	1.05	[5]
log K_{ow} (@ 25° C)	2.74 ± < 0.01	[4]
UV Absorption (nm; symmetric peak)	226 - 276	[39]
Isopropyl Cloprostenate		
Molecular Weight (g/mol)	467	[2]
Water Solubility (mg/l @ 25ºC)	0.047	[2]
log K_{ow}	5.15	[2]

Table 3. Frequency and concentration of use (2023) by product category[6-10]

	Isopropyl Cloprostenate		Ethyl Tafluprostamide	
	# of Uses	Max Conc of Use (%)	# of Uses	Max Conc of Use (%)
Eye Makeup Preparations				
Other Eye Makeup Preparations	3	0.0044 - 0.0075[a]	NR	0.012 – 0.02[b]
Hair Preparations (non-coloring)				
Other Hair Preparations	NR	NR	NR	0.012 – 0.02[c]

NR = not reported

[a]average concentration of Isopropyl Cloprostenate in eyelash serums according to unpublished data sources

[b]concentration of Ethyl Tafluprostamide in products used on eyelashes, eyebrows, and scalp hair, according to an unpublished data submission

Table 4. Dermal irritation/sensitization

Test Article	Vehicle	Concentration/Dose	Test Population	Procedure	Results	Reference
IRRITATION						
In Vitro						
Ethyl Tafluprostamide (purity: 98.5%)	NR	100%; 30 µl	3 samples	EpiDerm™ assay; reconstructed human epidermis; OECD TG 439; 1h exposure period; negative control: phosphate-buffered saline; positive control: 5% sodium dodecyl sulfate	Non-irritating Tissue viability in 3 replicates was 100, 106, and 82% at end of test Control substances gave expected results	[27]
Ethyl Tafluprostamide (purity: 99.78%)	NR	100%; 30 µl	3 samples	EpiDerm™ assay; reconstructed human epidermis; OECD TG 439; 15-min exposure period; negative control: phosphate-buffered saline; positive control: 5% sodium dodecyl sulfate	Irritating Tissue viability in 3 replicates determined to be 3, 17.2 and 27.7% at end of test. The mean value of relative tissue viability was 16.0% after the treatment. This value is below the threshold for skin irritation (50 %). Thus, the test item is considered to be an irritant to skin. Control substances gave expected results.	[28]
SENSITIZATION						
In Chemico/In Vitro						
Ethyl Tafluprostamide (purity: 98.5%)	acetonitrile	100 mM; 50 µl	cysteine peptides	Direct peptide reactivity assay; OECD TG 442C; cys- peptides assay; solvent used as negative control; positive control: cinnamic aldehyde	Negative prediction for sensitization Negative and positive controls gave expected results	[29]
Ethyl Tafluprostamide (purity: 99.78%)	acetonitrile	100 mM; 50 µl	lysine and cysteine peptides	Direct peptide reactivity assay; OECD TG 442C; cys- and lys- peptides assay; solvent used as negative control; positive control: cinnamic aldehyde	Negative prediction for sensitization according to cysteine 1:10/lysine 1:50 prediction model; however, observed precipitation in lys-peptide assay; conclusion on lack of reactivity could not be drawn from conditions of this study Mean peptide depletion in the cys-peptide assay, which showed no precipitation for test item was 3.2%, suggesting no or minimal reactivity Control substances gave expected results	[30]

Table 4. Dermal irritation/sensitization

Test Article	Vehicle	Concentration/Dose	Test Population	Procedure	Results	Reference
Ethyl Tafluprostamide (purity: 98.5%)	DMSO	Test 1: 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µM Test 2: 55.80, 72.54, 94.30, 122.59, 159.37, 207.18, 269.33, 350.13, 455.17, 591.72, 769.23, and 1000 µM Test 3: 67.29, 80.75, 96.90, 116.28, 139.54, 167.45, 200.94, 241.13, 289.35, 347.22, 416.67, and 500 µM All concentrations tested at a dose volume of 50 µl	KeratinoSens™ cell line	KeratinoSens™ assay; OECD 442D; solvent used as negative control; positive control: trans-cinnamaldehyde; test 2 and 3 performed to determine IC_{30} and IC_{50} values more precisely since strong cytotoxicity was observed at high concentrations	Negative prediction for sensitization Control substances gave expected results	[31]
Ethyl Tafluprostamide (purity: 99.78%)	DMSO	0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µM; 50 µl (luciferase induction activity only observed at concentrations up to 250 µM)	KeratinoSens™ cell line	KeratinoSens™ assay; OECD 442D; solvent used as negative control; positive control: cinnamic aldehyde; experiment repeated due to lack of dose-response in first experiment	Inconclusive results In experiment 1, a statistically significant increase in luciferase induction >1.5-fold was observed at 250 µm; all lower concentrations showed induction values in the range of the solvent control In experiment 2, a statistically significant increase in luciferase induction to exactly 1.5-fold was observed at 250 µM; induction values at lower concentrations were all in range of solvent control No clear dose-dependent results were observed – result was considered inconclusive Control substances gave expected results	[32]

Table 4. Dermal irritation/sensitization

Test Article	Vehicle	Concentration/Dose	Test Population	Procedure	Results	Reference
			Human			
eyelash product containing 0.018% Ethyl Tafluprostamide	NR	100%; dose not stated	51	HRIPT; level of occlusion not stated; nine 24-h applications to the upper back over a 3-wk period for induction; 2 test challenge patches after a 10 - 14 d rest period; challenge patches were applied to a previously untreated site adjacent to the test site (48- and 96-h exposures)	Two of 561 total evaluations were scored "1" (indicating erythema throughout at least ¾ of patch area; unknown which stage of study these effects were seen); study reported no adverse effects or signs or symptoms of sensitization throughout study	6
eyelash conditioner containing 0.025% Ethyl Tafluprostamide	NR	100%; 0.02 – 0.05 ml	51	HRIPT; occlusive conditions; nine applications to the upper back over a 3-wk period for induction (1st patch 24-h exposure; remaining patches 48-h exposures); test challenge patch after a 10 - 14 d rest period; challenge patch applied to a previously untreated site adjacent to the test site (48- and 96-h exposures)	Non-irritating; non-sensitizing	33
7.5% Ethyl Tafluprostamide in phenoxyethanol (final test concentration of 0.267% Ethyl Tafluprostamide)	deionized water	3.55%; 0.02 – 0.05 ml	54	HRIPT; semi-occlusive conditions; eight to nine applications to the upper back over a 3-wk period for induction (1st patch 24-h exposure; remaining patches 48-h exposures); test challenge patch after a 10 - 14 d rest period; challenge patch applied to a previously untreated site adjacent to the test site (48- and 96-h exposures)	Non-irritating; non-sensitizing	6,34
eyelash serum containing 0.0044% Isopropyl Cloprostenate	NR	100%; 0.2 ml	53	HRIPT; semi-occlusive conditions; nine 24-h applications to the upper back over a 3-wk period for induction; challenge phase after a minimal 10-d rest period; challenge patches were applied to a previously untreated site adjacent to the test site, and the site was evaluated immediately after removal and 72 h after patch removal	Non-irritating; non-sensitizing	35
eyelash serum containing 0.0044% Isopropyl Cloprostenate	NR	100%; dose volume not stated	56	HRIPT; semi-occlusive conditions; nine 24-h applications to the upper back over a 3-wk period for induction; challenge phase after a 10 - 21-d rest period; 24-h challenge patches were applied, and the site was evaluated immediately and 24 and 48 h after patch removal	Non-irritating; non-sensitizing	36
eyelash serum containing 0.005% Isopropyl Cloprostenate	NR	100%; 0.2 ml	50	HRIPT; occlusive conditions to the infrascapular region of the back; nine 24-h applications over a 3-wk period for induction; challenge phase after a 10 - 14-d rest period; challenge patches were applied to a previously untreated site for 24 h, and the site was evaluated immediately and 48 h after patch removal	Non-irritating; non-sensitizing	37

Table 4. Dermal irritation/sensitization

Test Article	Vehicle	Concentration/Dose	Test Population	Procedure	Results	Reference
eyelash serum containing 0.005% Isopropyl Cloprostenate	NR	100%; dose not stated	53	HRIPT; semi-occlusive conditions; nine applications to the upper back over a 3-wk period for induction; challenge phase after a 10 - 21-d rest period; challenge patches were applied to the lower back and the site was evaluated immediately, 24, and 48 h after patch removal	Non-irritating; non-sensitizing	[38]

DMSO = dimethyl sulfoxide; HRIPT = human repeated insult patch test; IC_{30} = 30% inhibitory concentration; IC_{50} = half maximal inhibitory concentration; OECD = Organisation for Economic Co-Operation and Development TG = test guideline

Table 5. Ocular irritation studies

Test Article	Vehicle	Concentration/Dose	Test Population	Procedure	Results	Reference
IN VITRO						
Eyelash product containing 0.018% Ethyl Tafluprostamide	NR	NR	hen's egg chorioallantoic membranes (n = 4)	HET-CAM assay; reference test articles include a one-coat mascara and waterproof eyeliner (details regarding these substances not stated); evaluations performed 0.5, 2, and 5 min after test article exposure	Irritation potential score: 0.0 (mean scores of 0.0 - 4.9 indicate an irritation potential of practically none) Reference test articles have historically been shown to be practically non-irritating. Study author concluded the test substance would have practically no ocular irritation potential in vivo	[6]
Eyelash product containing 0.025% Ethyl Tafluprostamide	NR	100%; 0.3 ml	hen's egg chorioallantoic membranes (n = 4)	HET-CAM assay; reference test articles include a one-coat mascara and waterproof eyeliner (details regarding these substances not stated); evaluations performed 0.5, 2, and 5 min after test article exposure	Non-irritating Mean irritation score of 0.0 at all test points Reference test articles have historically been shown to be practically non-irritating.	[41]
Ethyl Tafluprostamide (purity: 99.78%)	NR	100%; 0.03 ml	reconstructed human corneal epithelium (n = 2)	EpiOcular™ assay; OECD TG 492: negative control: phosphate-buffered saline; positive control: sodium dodecyl sulfate	Non-irritating Control substances gave expected results	[42]
Eyelash serum containing 0.0044% Isopropyl Cloprostenate	saline	10%: 0.3 ml	hen's egg chorioallantoic membranes (n = 6)	HET-CAM assay; vehicle control: saline; positive controls: sodium hydroxide and sodium dodecyl sulfate	Irritation potential score: 0.0 Threshold concentration (lowest concentration at which slight reactions occur) for this test substance was greater than 10% Control substances gave expected results Study author concluded that the irritation potential of the test substance was determined to be none to slight	[43]
Eyelash serum containing 0.0044% Isopropyl Cloprostenate	NR	50%*; 0.3 ml	hen's egg chorioallantoic membranes (n = 4)	HET-CAM assay; reference test articles include a one-coat mascara and waterproof eyeliner (details regarding these substances not stated); evaluations performed 0.5, 2, and 5 min after test article exposure	Irritation potential score for eyelash serum: 1.25 (mean scores of 0.0 - 4.9 indicate an irritation potential of practically none) Reference test articles have historically been shown to be practically non-irritating. Study author concluded that the test substance, at 100%, would have practically no ocular irritation in vivo.	[44]
Eyelash serum containing 0.005% Isopropyl Cloprostenate	NR	50%*; 0.3 ml	hen's egg chorioallantoic membranes (n = 4)	HET-CAM assay; reference test articles include a one coat mascara and waterproof eyeliner (details regarding these substances not stated); evaluations performed 0.5, 2, and 5 min after test article exposure	Irritation potential score for eyelash serum: 2.50 (mean scores of 0.0 - 4.9 indicate an irritation potential of practically none) Reference test articles have historically been shown to be practically non-irritating. Study author concluded that the test substance, at 100%, would have practically no ocular irritation in vivo.	[45]

Table 5. Ocular irritation studies

Test Article	Vehicle	Concentration/Dose	Test Population	Procedure	Results	Reference
Eyelash serum containing 0.005% Isopropyl Cloprostenate	saline	10%: 0.3 ml	hen's egg chorioallantoic membranes (n = 6)	HET-CAM assay; vehicle control: saline; positive controls: sodium hydroxide and sodium dodecyl sulfate	Irritation potential score: 0.0 Threshold concentration (lowest concentration at which slight reactions occur) for this test substance was greater than 10% Control substances gave expected results Study author concluded that the irritation potential of the test substance was determined to be none to slight	[46]
Eyelash serum containing 0.005% Isopropyl Cloprostenate	saline	10%; 0.3 ml	hen's egg chorioallantoic membranes (n = 6)	HET-CAM assay; vehicle control: saline; positive controls: sodium hydroxide and sodium dodecyl sulfate	Irritation potential score: 2.6 Threshold concentration (lowest concentration at which slight reactions occur) for this test substance was greater than 10% Control substances gave expected results Study author concluded that the irritation potential of the test substance was determined to be none to slight	[47]
0.1% Isopropyl Cloprostenate	NR	50%*; 0.3 ml	hen's egg chorioallantoic membranes (n = 6)	HET-CAM assay; reference test articles include a one coat mascara and waterproof eyeliner (details regarding these substances not stated); evaluations performed 0.5, 2, and 5 min after test article exposure	Irritation potential score for eyelash serum: 1.50 (mean scores of 0.0 - 4.9 indicate an irritation potential of practically none) Reference test articles have historically been shown to be practically non-irritating Study author concluded that 0.1% Isopropyl Cloprostenate would have practically no ocular irritation potential in vivo	[48]
HUMAN						
Eyelash product containing 0.018% Ethyl Tafluprostamide	NR	100%	19 subjects	Home use study. Subjects applied product to eyelashes for 28 d. Eyes were assessed by ophthalmologist at baseline and on day 28 (slit-lamp examinations)	The majority of subjects displayed no signs of irritation; however, one patient was scored a "2" (moderate intolerance to product). Four subjects reported minor adverse reactions consistent with allergic reactions.	[6]
Eyelash product containing 0.025% Ethyl Tafluprostamide	NR	100%	19 subjects	Home use study. Subjects applied product to eyelashes for 28 d. Intraocular pressure was measured in each eye of each subject at beginning and end of study. Eyes were assessed by ophthalmologist at baseline and on day 28 (slit-lamp examinations).	Minor ocular effects self-reported by 4/19 volunteers (slight dryness, slight itching, slight stinging, slight watering and redness, moderate to high burning) Study authors determined that the formulation did not produce an eye irritation or hypersensitivity of clinical magnitude.	[11,25]

Table 5. Ocular irritation studies

Test Article	Vehicle	Concentration/Dose	Test Population	Procedure	Results	Reference
Eyelash serum containing 0.0044% Isopropyl Cloprostenate and eyebrow serum containing 0.0044% Isopropyl Cloprostenate	NR	100%	29 subjects	Home use study. Subjects applied eyelash serum to the top eyelash line once daily for 6 wk; questionnaires completed after 2, 4 and 5 wk of eyelash serum use; photos taken at baseline, and after 4 wk of serum use. Subjects also instructed to apply the eyebrow serum for 7 wk; questionnaires completed after 6 and 7 wk of eyebrow serum use; photos taken at baseline and after 6 wk of serum use	No adverse effects observed relating to product use	[49]
Eyelash serum containing 0.0044% Isopropyl Cloprostenate	NR	100%	32 subjects	Home use study. Subjects applied eyelash serum daily for 12 wk; subjects completed questionnaires after 6 and 12 wk of use; subjects evaluated at testing facility at baseline and after 12 wk of serum use	Overall, the eyelash serum was considered to be well-tolerated, with at most, mild effects that are short-term and reversible One subject reported slight stinging in both eyes if product was applied too close to the corner of the eye One subject reported ocular pruritis 20 min after application for 2 wk after an unspecified number of applications; at the end of the 2-wk period, itching stopped and did not recur for the remainder of the study	[49]
Eyelash serum containing 0.0044% Isopropyl Cloprostenate	NR	100%	120 subjects	Home use study. Subjects applied eyelash serum daily for 8 mo. Slit-lamp evaluations occurred at baseline, 1 mo, 2 mo, 4 mo, and 8 mo intervals.	Slight transient ophthalmological irritation observed. The serum was determined to be safe for use by both contact lens and non-contact lens wearers.	[13]
Eyelash serum containing 0.005% Isopropyl Cloprostenate	NR	100%	32 subjects	Serum applied to eyelid, above upper lash line (lash root area), on both eyes, once per day, each evening; eyes evaluated for irritation from baseline to 3 mo of product use	Non-irritating Subjective evaluations by the test population were favorable	[50]
Eyelash serum containing 0.005% Isopropyl Cloprostenate and eyebrow serum containing 0.005% Isopropyl Cloprostenate	NR	100%	30 subjects	In- use study. Subjects applied eyelash serum to left eye lashes and eyebrow serum to right eyebrow; evaluations performed at baseline and 8 h after application; slit-lamp examination of bulbar conjunctival irritation, palpebral conjunctival irritation, and lid disease	Eyelash serum results: mean irritation score: 0.0 (non-irritating) at baseline; slight bulbar conjunctival irritation observed at 8 h observation (mean irritation score of 0.4/3) Eyebrow serum results: Mean irritation score of 0.0 (non-irritating) at baseline and at 8 h observation	[51]
Eyelash formulation containing 10% Isopropyl Cloprostenate	NR	100%	27 subjects	Application of test substance for 4 wk; applications in both contact lens users and non-contact lens users; no details were provided	non-irritating	[2]

HET-CAM = hen's egg test chorioallantoic membrane; NR = not reported; OECD TG = Organisation for Economic Co-Operation and Development Test Guidelines

*study author states that a 50% dilution of the test and reference articles may be used to approximate in vivo irritation potential at 100%, as the hen's egg is more sensitive to liquid irritants than the rabbit eye

Table 6. Margin of safety calculation parameters

	Isopropyl Cloprostenate[54]*	**Ethyl Tafluprostamide[11]****
estimated daily exposure to lash serum	0.28 mg/d	0.04 mg/d (average) 0.067 mg/d (maximum)
concentration of ingredient	0.005%	0.018%
dermal absorption	50%	8.67% (based on in vitro percutaneous study provided in this report; the mean absorbed mean absorbed fraction (6.51 ± 2.16%) plus 1 standard deviation was used)
dermal retention	100%	100%
body weight	60 kg	60 kg
systemic exposure dose	1.17×10^{-7} mg/kg/d	6.24×10^{-7} mg/kg/d (average) 1.04×10^{-6} mg/kg/d (max)
systemic point of departure	0.00012 mg/kg/d (derived from a systemic toxicity assay on travoprost)	0.0003 mg/kg bw/d (derived from a systemic toxicity study on tafluprost)
margin of safety	1029	481 (average) 288 (max)

*calculation for an eyelash serum containing 0.005% Isopropyl Cloprostenate
**calculation for an eyelash product containing 0.018% Ethyl Tafluprostamide

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Data Demonstrating the Safe Use of Ethyl Tafluprostamide in Cosmetics

Supplemental Report: *Dossier on the Human Safety Evaluation of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE) or Ethyl Tafluprostamide in Cosmetic Products*

Prepared By: ToxMinds BVBA, Brussels, Belgium

Submitted to CIR By:

John Bailey, PhD
EAS Consulting Group
1700 Diagonal Road, Suite 750
Alexandria, VA 22314
Jbailey@easconsultinggroup.com

Submitted On Behalf Of:

COMPANY

Submitted To:

Bart Heldreth, PhD
Executive Director
Cosmetic Ingredient Review
Washington, DC
heldrethb@cir-safety.org

October 18, 2023

ToxMinds BVBA
Avenue de Broqueville 116
1200 Brussels, Belgium
Phone: +32 (0)2 762 91 45
Email: info@toxminds.com
TVA: BE 0877.628.383

4 October 2023

Mr. Hans Ingels
Head of Unit, Unit F2 - Bioeconomy
Chemicals & Cosmetics, DG GROW

Subject: **Dechloro dihydroxy difluoro ethylcloprostenolamide ('DDDE'; CAS# 1185851-52-8)**
Human safety evaluation of DDDE in cosmetic products

Dear Mr. Ingels,

On behalf of the cosmetic product manufacturer [redacted], ToxMinds BVBA wishes to submit a dossier on the human safety of dechloro dihydroxy difluoro ethylcloprostenolamide ('DDDE') or ethyl tafluprostamide in cosmetic products.

In February 2022, the EU's Scientific Committee on Consumer Safety (SCCS) released its opinion on the safety of prostaglandins and prostaglandin-analogues ('PGAs') used in cosmetic products identifying toxicological data shortcomings preventing the SCCS to complete a full safety evaluation of the use of PGAs in cosmetic products. From the beginning on, [redacted] has been in close contact with the Commission services and supported SCCS's safety evaluation of PGAs by conducting additional toxicological testing of DDDE in accredited European CROs.

Following completion of all studies, [redacted] wishes now to further support SCCS's evaluation by submitting a dossier on the human safety of DDDE at use levels of up to 0.018% in cosmetic eyelash products. This evaluation has been conducted according to the SCCS Notes of Guidance 12th Revision (NoG) (SCCS, 2023) and is based on compositional, physico-chemical, and/or newly conducted in vitro toxicological data for DDDE provided by the dossier submitter [redacted] In line with the safety assessment of DDDE by the German Risk Assessment Institute 'BfR', any existing data gaps for DDDE were addressed by means of read across to the toxicological data available for the close structural analogue tafluprost. The available chemical, toxicokinetic and toxicological information for tafluprost has been reviewed and found to be adequate for read across purposes, in line with existing read across guidance provided by regulatory authorities including the OECD and the European Chemicals Agency (ECHA).

The applicant would be grateful if you find it acceptable to forward this contribution to the SCCS in view of its future evaluation of the human safety of DDDE in cosmetic applications. Please do not hesitate to contact us in case you require any further information or clarification.

Yours sincerely,

Dr. Thomas Petry, ERT, DABT
Managing Director

Dossier on the Human Safety Evaluation of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE) or Ethyl Tafluprostamide in Cosmetic Products CAS No. 1185851-52-8/EC No. 867-521-0

17 October 2023

For Submission to the Scientific Committee on Consumer Safety

Table of Contents

1. BACKGROUND

In 2018, the German Federal Institute for Risk Assessment (BfR) informed the European Commission that they were concerned that the use of prostaglandins and their analogues as ingredients in cosmetic products may pose health risks for consumers (BfR, 2018; SCCS, 2022).

As a result of this communication, EU countries' competent authorities were invited in 2019 to participate in a survey of products for eyelash growth containing prostaglandins and their analogues. In December 2019, the sub-working group on borderline products assessed this topic and considered that a Scientific Committee on Consumer Safety (SCCS) opinion would be useful to assess the safety of those products. Following a call for data, which was conducted in 2020[1], the European Commission requested the SCCS to carry out a safety assessment of uses of prostaglandins or their analogues in cosmetic products in view of the information provided (SCCS, 2022).

SCCS released its opinion on prostaglandins and prostaglandin-analogues (in the following abbreviated as 'PGAs') used in cosmetic products in February 2022 (SCCS, 2022). The SCCS stated that it could not advise on safe use concentrations of PGAs in cosmetic products due to a scarcity/lack of toxicology data on the individual PGAs. The SCCS also stated that they will be ready to assess any evidence provided to support safe use of PGAs in cosmetic products (SCCS, 2022).

The objective of the present dossier is to assess the safety of ethyl tafluprostamide (i.e., dechloro dihydroxy difluoro ethylcloprostenolamide, in the following abbreviated as 'DDDE') at use levels of up to 0.018% in a cosmetic eyelash product formulation. This evaluation has been conducted according to the SCCS Notes of Guidance 12th Revision (NoG) (SCCS, 2023) and is based on compositional, physico-chemical, and/or newly conducted *in vitro* toxicological data for DDDE provided by the dossier submitter [redacted]., referred to herein as 'applicant', supplemented with the *in silico* predictions as well as publicly available toxicological information on tafluprost, a close structural analogue, by means of read across.

[1] https://ec.europa.eu/newsroom/growth/items/680681/en

2. EXECUTIVE SUMMARY

This dossier has been prepared in accordance with the SCCS 12th Notes of Guidance (SCCS, 2023) and evaluates the safety of Ethyl tafluprostamide or dechloro dihydroxy difluoro ethylcloprostenolamide used at concentrations up to 0.018% in a cosmetic eyelash product formulation manufactured and sold by applicant. In this dossier, the ingredient is abbreviated and referred to as DDDE to be consistent with the chemical descriptors used by the applicant in recently conducted toxicology studies and previous submissions to the SCCS ([redacted] 2020).

DDDE is used in a cosmetic eyelash product formulation, named [redacted] (hereafter referred to as the 'cosmetic eyelash product'). The toxicological profile of DDDE has been assessed on the basis of data available from the product manufacturer (the applicant) and complemented by information provided from (Q)SAR modelling. Relative to the SCCS Notes of Guidance (NoG), toxicological data gaps were identified for acute, repeated dose toxicity, developmental and reproductive toxicity endpoints as well as carcinogenicity. In line with assessment on DDDE by the German Risk Assessment Institute 'BfR', these data gaps were addressed by means of read across to the toxicological data available on the structural analogue tafluprost. The analogue has been reviewed and found to be adequate for read across purposes in line with existing read across guidance provided by regulatory authorities including the OECD and the European Chemicals Agency (ECHA).

The safety assessment has been conducted using a Margin of Safety (MoS) approach, according to which a level considered to be safe for human health, expressed as the systemic point of departure (PoD_{sys}), is compared with the estimated systemic exposure dose (SED).

Considering all available information, an intravenous No Observed Adverse Effect Level (NOAEL) of 0.3 µg/kg bw/day from a prenatal developmental toxicity study in rats conducted with the analogue tafluprost has been selected as the PoD. No bioavailability correction is required for deriving a systemic dose **(PoDsys)** as the PoD has been derived from the intravenous route study, resulting in a systemic PoD (**PoDsys**) of 0.3 µg/kg bw/day.

In a recent, OECD guideline compliant *in vitro* percutaneous penetration study, the dermal penetration of DDDE was determined to be **8.67%** (mean value + 1SD). This data has been used along with the measured amount of the applicable eyelash product from the applicant, to determine the systemic exposure dose (SED) of DDDE when present in eyelash cosmetic products at a concentration of 0.018%.

Dividing the determined SED by the PoDsys revealed an MoS value of greater than 100. According to the SCCS NoG, an ingredient used in a cosmetic product is considered safe if the calculated MoS for systemic toxicity is equal to or greater than 100. The present assessment, therefore, supports the safe use of DDDE in eyelash cosmetic products when used at a level of 0.018%.

The applicant hereby confirms that the information contained in this dossier complies with the provisions on animal testing as laid down in Article 18(1) of the Cosmetic Products Regulation (EC) No 1223/2009.

3. DOSSIER OF DATA FOR DDDE

Data have been compiled according to the structure specified in the SCCS 12th Notes of Guidance (SCCS, 2021).

3.1. CHEMICAL AND PHYSICAL SPECIFICATIONS

INCI Name	Ethyl tafluprostamide
Chemical name	Tafluprost ethyl amide (TEA); Dechloro dihydroxy difluoro ethylcloprostenolamide (DDDE)[2]; Taflpostamide (SCCS, 2022)
Trade name	Not available
IUPAC name	(Z)-7-[(1R,2R,3R,5S)-2-[(E)-3,3-difluoro-4-phenoxybut-1-enyl]-3,5-dihydroxycyclopentyl]-N-ethylhept-5-enamide (SCCS, 2022)
CAS#	1185851-52-8
EINECS#	Not available
Molecular weight	437.52 g/mol
Molecular formula	$C_{24}H_{33}F_2NO_4$
SMILES	CCNC(=O)CCCC=CCC1C(CC(C1C=CC(COC2=CC=CC=C2)(F)F)O)O
Structural Formula	-
Physical state or appearance	DDDE is a colourless to pale yellow solution (SCCS, 2022)
Partition coefficient (Log Pow)	2.74 ± < 0.01 at 25°C (Experimental) (Moller, 2023a) 5.03 (Estimated using EPI Suite™ v.4.11) SCCS, 2022)
Solubility in water	1.05 g/L at 20°C (Experimental) (Moller, 2023b) 0.09 mg/L at 25°C (Estimated using EPI Suite™ v.4.11) SCCS, 2022)
Melting point	95.08 °C (Estimated using US EPA TEST v.5.1.2)
Boiling point	503.76 °C (Estimated using US EPA TEST v.5.1.2)
Relative density	1.21 g/cm^3 (Estimated using US EPA TEST v.5.1.2)
Vapour pressure	1.2E-13 Pa at 25°C (Estimated using EPI Suite™ v.4.11)
UV Absorbance	226-276 nm (Experimental) (Johannes, 2023)
Purity	Not less than 99% (see **Annex I**)
Typical impurities	Not more than 1% (see **Annex I**)

3.2. FUNCTION AND USES

DDDE is used in a cosmetic eyelash product formulation, at 0.018% concentration. It is intended to be used as an eyelash conditioner that "helps to strengthen eyelashes while protecting against breakage and brittleness" while "improving flexibility, moisture and shine for bold, beautiful, more dramatic looking lashes". The cosmetic eyelash product is not marketed to grow eyelashes. The concentration of DDDE in the cosmetic eyelash product is 0.018%.

([redacted] 2020; SCCS, 2022)

[2] The study investigators referred to the chemical identifier 'TEA' (Tafluprost ethyl amide) which is chemically identical DDDE. The applicant also referred to "TEA" until June 2010, when the INCI name of *Dechloro Dihydroxy Difluoro Ethylcloprostenolamide* (DDDE) was assigned to the molecule [redacted]).

3.3. TOXICOLOGICAL EVALUATION

An exhaustive literature search for toxicological information on DDDE was carried out. The search using 'chemical name/structure' and specific toxicology-relevant keywords was conducted in databases in ChemEXPERT™, PubMed Toxicology, and Google Scholar. ChemEXPERT™ is a commercial expert database which covers the toxicological data inventories of key global regulatory databases in the European Union, United States, Canada, Australia and Asia. Priority was given to regulatory reviews conducted in recent years.

The available data from the applicant, complemented with the literature search in publicly available and commercial sources listed above, revealed data for DDDE on the following endpoints:

- Dermal absorption
- Skin irritation
- Eye irritation
- Skin sensitisation
- Genotoxicity

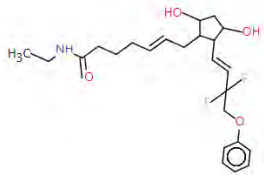
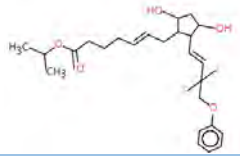
Hence, relative to the endpoint requirements discussed in the SCCS NoG, toxicological data gaps for DDDE were identified for acute toxicity, repeated dose toxicity, carcinogenicity, and developmental and reproductive toxicity endpoints. These endpoints were addressed by means of read across to data from an analogue, which was identified using the ECHA recommended tools for analogue identification such as OECD v.4.5 (Q)SAR Toolbox (OECD, 2022) and the US EPA AIM model (US EPA, 2023) according to the process described by Wu *et al.* (Wu *et al.*, 2010) and Blackburn and Stuard (Blackburn and Stuard, 2014).

Rationale for read across justification

The identified analogues with relevant toxicological data were evaluated for their suitability in accordance with the analogue justification guidance which exists under the European Chemicals Agency read-across assessment framework (RAAF) (ECHA, 2017), based on the following criteria:

- Common functional groups and structure
- Common structural alerts or reactivity
- Common physico-chemical properties
- Likelihood of common breakdown products via biological/degradation processes

Among several candidates, **tafluprost** (CAS No. 209860-87-7) was identified as one of the structurally closest analogues with available toxicological data. The toxicological profile of tafluprost was reviewed in several regulatory reports, such as the US FDA and Australian public assessment reports (CDER, 2011; TGA, 2012).

Substance	Chemical Name	CAS No.	Reference
Target 	DDDE	1185851-52-8	(NCBI, 2022a)
Analogue 	Tafluprost	209860-87-7	(NCBI, 2022b)

With regard to the four criteria related to the analogue evaluation, tafluprost contains similar features as the target substance, with some differences which were evaluated for their relevance for hazard assessment of the target substance DDDE:

- Tafluprost presents a high Dice index[3] (0.86), indicative of high structural similarity.
- It shares key functional groups like alcohol, cycloalkane, ether moiety, alkyl halide and aryl groups. The analogue presents the carboxylic acid ester group instead of the amide group and it contains the isopropyl group.
- Regarding the structural alerts, (Q)SAR analysis, as provided by the OECD QSAR Toolbox v.4.5 (OECD, 2022), revealed that tafluprost is classified as Cramer Class III[4] (high toxicity) like for DDDE. The analogue also presents the same structural alerts identified by the 'Estrogen Receptor Binding' and 'Oncologic Primary Classification' profilers.
- It presents physicochemical properties in the same range[5] as compared to the target substance with a slightly higher log Kow and lower water solubility. These differences in log Kow and water solubility suggest the analogue to be less bioavailable under oral exposure conditions compared to the target substance.
- Finally, data available for tafluprost indicate that the substance is rapidly hydrolysed to form tafluprost acid and isopropanol; tafluprost acid is further metabolized via fatty acid β-oxidation, hydroxylation and phase II conjugation. This is in agreement with the (Q)SAR analysis of the target substance. DDDE is predicted by Meteor™ Nexus (Judson *et al.*, 2015) v.3.1 to mainly undergo hydrolysis of acyclic carboxylic amides with the formation of tafluprost acid and ethyl amine, and to a lesser extent aromatic hydroxylation (see also **Annex II**). The hydrolysis reaction of DDDE is further supported by the results of the *in vitro* skin metabolism study (Weghuber, 2022) (see **Section 3.3.1.3**).

Based on the above (Q)SAR analysis, the analogue **tafluprost** has been ranked as 'suitable with interpretation' according to the criteria set by (Wu *et al.*, 2010). The interpretation is related to the fact that (1) the target gives rise to ethyl amine as a result of hydrolysis, and (2) the analogue seems to be less bioavailable as compared to the target substance (see **Annex III**). This will need to be considered when assessing the systemic toxicity of DDDE based on tafluprost data.

The formation of the uncommon metabolites, ethyl amine for DDDE and isopropanol for tafluprost, was not shown to have an impact on the read across strategy. In fact, tafluprost was found to have effects on developmental toxicity at low doses. Isopropanol is not a developmental toxicant and presents significantly higher NOAELs in systemic toxicity studies (see **Annex IV**). Likewise, in the case of DDDE, the hydrolysis product ethylamine does not show critical adverse effects (see **Annex IV**), further supporting the consideration that tafluprost acid is the toxicity driving moiety in DDDE. Thus, assessing the toxicity of DDDE based on tafluprost data is considered suitable.

The assumed difference in bioavailability between the analogue and DDDE is not considered to have an impact on the assessment, because the point of departure (PoD) study is based on a developmental toxicity study in rats using intravenous dosing where 100% bioavailability is assumed (unlike dermal or oral routes), by representing the worst case. Therefore, no bioavailability correction is required for the risk assessment of DDDE which is based on read across to tafluprost data (see **Section 3.4.1**).

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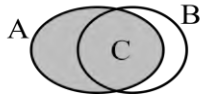
[3] The Dice index or Dice coefficient measures the similarity between two molecules on the basis of structure fingerprints, by counting the number of bits 'on' in both molecules (C) and the number of bits 'on' in each molecule separately (A, B).



The Dice index is calculated with the following formula: 2C/A+B.

[4] Cramer Class III chemicals refer to substances with chemical structures that permit no strong initial presumption MOS or may even suggest significant toxicity or have reactive functional groups.

[5] Log Kow and water solubility of the target = 5.03 and 0.091 mg/L; Log Kow and water solubility for the analogue = 6.51 and 0.0039 mg/L estimated via EPI Suite v.4.11 for comparison purposes.

Taking all information into consideration, the toxicology data available for tafluprost is considered suitable for the safety assessment of DDDE using a read across approach. This is further supported by the fact that also the German Risk Assessment Institute 'BfR' assessed risks associated with DDDE based on tafluprost data (BfR, 2017).

To summarise, the safety evaluation of DDDE in a cosmetic eyelash product formulation will be based on the following endpoint assessment strategy:

Endpoints	Endpoint assessment strategy
Dermal absorption	Study available on DDDE
Acute toxicity	Read across to Tafluprost
Skin irritation	Study available on DDDE
Eye irritation	Study available on DDDE
Skin sensitisation	Study available on DDDE
Phototoxicity	UV/VIS absorbance study available on DDDE
Repeated dose toxicity	Read across to Taflurprost
Genotoxicity	Study available on DDDE
Carcinogenicity	(Q)SAR predictions for DDDE and read across to Taflurprost
Reproductive toxicity	Read across to Taflurprost
Developmental toxicity	Read across to Taflurprost

The following sections summarise the available experimental data on DDDE and tafluprost.

3.3.1. Toxicokinetics

The following section presents data on the dermal/percutaneous absorption of DDDE as it is the most important exposure route in the context of cosmetic applications. Information on the toxicokinetic properties of DDDE is also provided.

3.3.1.1 Dermal/ percutaneous absorption

One guideline compliant *in vitro* dermal penetration study using human skin was available on DDDE.

In vitro percutaneous absorption (human skin)

Guideline:	OECD Test Guideline 428 (2004)			
Test system:	Human skin			
Test substance:	DDDE and ^{3}H-radiolabelled DDDE			
Theoretical concentration of reference DDDE at 7.5% in phenoxyethanol (%)	0.16	0.24	0.27	0.32
Theoretical concentration of active substance DDDE (%)	0.016	0.018	0.020	0.024
Theoretical amount of reference substance applied on skin (µg)	1.6	1.8	2	2.4
Dose of test formulation:	Approximately 1 µCi on each cell (10 µL of test substance/cell)			
Batch:	TAF-F-0122-01 and 22-0809-93			
Purity:	Non labelled-99.42% and radiolabelled->98%			
Specific activity:	138.971 mCi/mg (by calculation)			

Route:	Topical application
Trans epidermal water loss (TEWL):	1.85 – 10.12 g/m²/h (closed chamber)
Number of donors:	8
Number of cells/donor (replicates):	3
Total number of cells/formulations:	12
Total cells:	48
Thickness of skin:	310 - 400 µm
Receptor fluid:	5% w/w Bovine serum albumin, 0.9% NaCl in water
Washing of test formulation:	0.5 mL Tween 80® 5%; 1 half cotton bud 3.5 mL of UHQ water (0.5 mL, 7 times) 3 dried half cotton swabs A maximum of 20 strips was performed.
Strips:	The strips were pooled as follows: 1-2, 3-6, 7-11, 12-15, 6-20.
Separation Epidermis/Dermis:	Yes (separation by heat)
Extraction solvent for rinsing donor compartment (RCD) and rinsing of receptor compartment (RCR), tape strips and cotton-swabs:	Ethanol
Exposure time:	0.5, 2, 4, 8, 12 and 24 hours
Good laboratory practice (GLP):	Yes
Study period:	2022-23

The *in vitro* absorption potential of DDDE (99.42% purity) present in a representative cosmetic eyelash product formulation[6] was determined in healthy human skin mounted on dynamic cells in an OECD Test Guideline 428-compliant study. The human skin samples were obtained from abdominal surgery and dermatomed to a thickness of 310-400 µm. The integrity of the skin was confirmed by measuring the trans-epidermal water loss. Only skin samples exhibiting values between 1.85 and 10.12 g/m²/h were included into the study.

Four test concentrations of the cosmetic eyelash product containing radiolabelled DDDE (^{3}H-DDDE) were applied to the surface of human skin samples mounted on dynamic cells. The % of DDDE in the cosmetic eyelash product is 0.018%. For comparison, one test substance with a lower % of DDDE (0.012%) and two with higher % of DDDE (0.020 and 0.024%) were also tested. A total of 8 donors were used, and each donor was used for 2 test substances. Donors 1-4 were used for the two highest concentrations (i.e., 0.020%, 0.024%), and donors 5-8 for the two lower concentrations (i.e., 0.012%, 0.018%). The test formulations, approximately 1 µCi at 10 µg/cell, were applied for 24 hours. Skin absorption rates were measured at various time intervals by assessing the activity of radiolabelled ^{3}H-DDDE. After the 24-hour exposure period, skin samples were washed with a mild soap solution, rinsed and dried. The upper layers of the stratum corneum were removed by tape stripping. The remaining skin was separated into epidermis and dermis. The stability of the test formulation was performed at the start and after 24 hours, at 32°C, of the experiment.

Results

The mean results obtained for test formulation containing DDDE and ^{3}H-radiolabelled DDDE are presented in the following tables:

[6] Representative cosmetic eyelash product formulation refers to the product whose dose and vehicle/formulation represent the in-use conditions of the intended cosmetic product (SCCS, 2023).

Table 1. *In vitro* percutaneous absorption of DDDE through human skin

Formulations	DDDE							
	DDDE Dilution 0.012%		DDDE Dilution 0.018%		DDDE Dilution 0.020%		DDDE Dilution 0.024%	
	n=12		n=12		n=12		n=12	
Number of strips (mean by test substance)	9		11		9		9	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Strips 1-2	5.53	1.57	5.18	2.11	4.24	2.19	5.75	1.99
Strips 3-20	9.02	4.12	9.27	3.08	15.78[#]	4.06[#]	15.86	6.58
Total strips	14.55	4.48	14.45	4.47	18.71	7.05	21.61	7.22
Skin Excess*	71.01	5.92	70.06	6.6	67.7	5.03	66.82	6.39
Epidermis	3.63	1.95	4.17	1.02	5.93	5.11	6.63	4.56
Dermis	0.61	0.64	0.4	0.3	0.21	0.19	0.43	0.44
Receptor fluid	2.2	1.27	1.94	1.25	2.98	2.29	3.61	3.36
Epidermis + dermis + receptor fluid**	6.44	2.14	6.51	2.16	9.12	7.23	10.68	7.18
TOTAL RECOVERY	92	3.06	91.02	2.18	95.53	4.26	99.11	3.13

*Skin excess corresponds to: Washing + Donor compartment rinsing + Remaining skin

**Absorbed fraction of the applied DDDE according to SCCS guideline

[#] Mean and SD realized on 11 cells since Cell X has only 1 strip.

Table 2. Distribution of DDDE after application to human skin ($\mu g_{eq}/cm^2$)

Reference item	DDDE							
Formulations	DDDE Dilution 0.012%		DDDE Dilution 0.018%		DDDE Dilution 0.020%		DDDE Dilution 0.024%	
	n=12		n=12		n=12		n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Test substance applied ($\mu Ci/cm^2$)	0.92	0.02	0.91	0.03	0.88	0.03	0.87	0.02
Strips 1-2	0.07	0.02	0.1	0.04	0.09	0.04	0.14	0.05
Strips 3-20	0.11	0.05	0.17	0.05	0.32[#]	0.08[#]	0.37	0.15
Total strips	0.18	0.06	0.27	0.08	0.38	0.14	0.51	0.17
Skin Excess*	0.9	0.06	1.3	0.14	1.38	0.1	1.57	0.13
Epidermis	0.05	0.02	0.08	0.02	0.12	0.11	0.16	0.11
Dermis	0.008	0.008	0.007	0.006	0.004	0.004	0.01	0.01
Receptor fluid	0.028	0.016	0.04	0.02	0.06	0.05	0.09	0.08
Epidermis + dermis + receptor fluid**	0.08	0.03	0.12	0.04	0.19	0.15	0.25	0.17

*Skin excess corresponds to: Washing + Donor compartment rinsing + Remaining skin

**Absorbed fraction of the applied DDDE according to SCCS guideline

[#] Mean and SD realized on 11 cells since Cell X has only 1 strip.

For each test substance, the number of strips is variable, ranging from 1 to 20. This intra- and inter-formulation heterogeneity is also found in the results of the dermis, the epidermis and the receptor fluid.

The absorption results were presented according to SCCS guideline by adding the amounts of DDDE found in the receptor fluid, rinsing receptor compartment, dermis and epidermis during 24 hours:

- 6.44 ± 2.14% of applied dose corresponding to 0.08 ± 0.03 µg/cm² for DDDE Dilution 0.012%
- 6.51 ± 2.16% of applied dose corresponding to 0.12 ± 0.04 µg/cm² for DDDE Dilution 0.018%
- 9.12 ± 7.23% of applied dose corresponding to 0.19 ± 0.15 µg/cm² for DDDE Dilution 0.020%
- 10.68 ± 7.18% of applied dose corresponding to 0.25 ± 0.17 µg/cm² for DDDE Dilution 0.024%

The absorbed fraction of the applied test substance following topical application of **0.018%** DDDE in a representative cosmetic eyelash product formulation was **6.51±2.16%** of the applied dose after 24 hours of exposure.

The mean total recovery was within the SCCS acceptance criteria (i.e., 85-115%), validating the results obtained.

Donors 1-4, which were used to test the two highest concentrations (i.e., 0.020%, 0.024%) of DDDE, show a substantial greater variability (SD = 7.18 and 7.23%) compared to the donors 5-8, which were used to test the two lowest concentrations of DDDE (0.012%, SD = 2.14%; 0.012%, SD = 2.16%).

Conclusion

In conclusion, following topical application of **0.018%** DDDE in a representative cosmetic eyelash product formulation to human skin *in vitro*, the absorbed fraction of the applied test substance was determined to be **6.51±2.16%**.

(Klock, 2023a)

Note: The above GLP-OECD-compliant *in vitro* dermal absorption study met the required criteria specified in the SCCS NoG (2023) on dermal absorption and is considered to be scientifically acceptable. Considering that the donor variation in the 0.018% test group was low, a dermal absorption value of **8.67%** (i.e., **mean + 1 SD**) will be used for the Margin of Safety (MoS) calculations.

3.3.1.2 Oral absorption/bioavailability

No relevant *in vivo* or *in vitro* toxicokinetic studies could be identified for DDDE in the public literature. The oral absorption or bioavailability of DDDE can therefore be assessed based on its physicochemical properties.

Considering its molecular weight of 452.5 g/mol, water solubility of 1.05 g/L and log K_{OW} of 2.74, DDDE is assessed to have moderate oral absorption potential.

3.3.1.3 Metabolism, distribution and excretion

One guideline compliant *in vitro* skin metabolism study using human skin was available on DDDE.

In vitro percutaneous metabolism (human skin)

Guideline:	OECD Test Guideline 428 (2004)
Test system:	Human skin
Test substance:	DDDE
Batch:	Not specified
Purity:	Not specified
Route:	Topical application
Number of donors:	3
Thickness of skin:	4 mm
Receptor fluid:	Hank's balanced salt solution containing glucose[1g/L], supplemented with 5 g/L albumin
Vehicle	50% ethanol
Exposed skin area	0.5 cm^2

Exposure time:	2, 4, and 24 hours
GLP:	Yes
Study period:	2022

The skin metabolism of the test substance DDDE (purity not specified) was investigated in an OECD Test Guideline 428-compliant *in vitro* dermal penetration study. Methodologies for analytical detection using fresh human skin were established. Test substance was applied at 3.0 µg (6.0 µg/cm^2 diluted in 50% ethanol) for 2, 4 or 24 hours to human skin on a static transwell system using a physiological buffer as receptor fluid. The identification and quantification of DDDE, its metabolite tafluprost free acid (TPFA) and the reference substance caffeine were carried out using a Vanquish ultra-high-performance liquid chromatography (UHPLC) system. For the quantification of DDDE and the metabolite TPFA a six-point, weighted (1/x), external calibration in the concentration range from 0.05 µg/mL to 2.5 µg/mL was applied. The analytes were identified according to their specific masses in individual mass traces.

Results

The identification and quantitation of DDDE and its metabolite, TPFA, by HPLC-MS were sensitive and reproducible. The skin models were viable, and the penetration of the marker compound caffeine was comparable with data from the literature. DDDE was found to be extensively metabolized into TPFA (68.5 ± 2.7%) after 24 hours.

Conclusion

In conclusion, following topical application of DDDE to human skin *in vitro*, about 65.8-71.2% of the absorbed fraction of DDDE was found to be metabolised to tafluprost free acid.

(Weghuber, 2022)

Note: The results of the *in vitro* skin metabolism study are in line with the metabolism prediction for DDDE using Meteor^{TM} Nexus (Judson *et al.*, 2015) v.3.1 to which shows hydrolysis of acyclic carboxylic amides with the formation of tafluprost acid, and to a lesser extent aromatic hydroxylation (see above **section** and **Annex I**).

3.3.2. Acute toxicity

3.3.2.1 Acute oral toxicity

No acute oral studies could be identified for DDDE. The acute oral toxicity endpoint has therefore been assessed on the basis of an acute toxicity study available for the structural analogue tafluprost.

Guideline:	Not specified
Species/strain:	Rats/Sprague-Dawley
Number of animals:	5/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Vehicle:	Not specified
Batch:	Not specified
Purity:	Not specified
Dose levels:	0, 10, 30 and 100 mg/kg bw
Dose volume:	Not specified
Route:	Oral
Administration:	Not specified
Observation	14 days
GLP:	Not specified
Study period/year:	Not specified (pre-2009)

The test substance tafluprost (purity not specified) was investigated for acute oral toxicity in rats. Sprague-Dawley rats (5/sex/group) were administered single doses of 0, 10, 30 and 100 mg/kg bw. After dosing, the animals were observed daily for clinical signs of toxicity and mortality for 14 days. Body weights, food intake and water intake were determined at defined intervals.

Results

No mortalities and no marked changes in body weight, food intake, water intake or gross pathology were observed. The hunched posture and wasted appearance beginning one day after dosing and chest sores or loss of chest fur at Day 7 were observed in one animal of the 10 mg/kg bw dose group and one animal of the 100 mg/kg dose group. The chest sores or loss of chest fur was reversed by Day 14.

Conclusion

Under the conditions of the study, the LD_{50} of test substance was considered to be ≥100 mg/kg bw in rats.

(CDER, 2011; TGA, 2012)

3.3.2.2 Acute dermal toxicity

No acute dermal studies could be identified for DDDE or the analogue tafluprost.

3.3.2.3 Acute inhalation toxicity

No acute inhalation studies could be identified for DDDE or the analogue tafluprost.

3.3.2.4 Acute intravenous (IV) toxicity

Two acute intravenous studies are available for the read across substance tafluprost. As the PoD is based on an intravenous study, available acute intravenous studies for the analogue tafluprost in rats and dogs should also be considered.

1st study: Acute intravenous toxicity study in rats

Guideline:	Not specified
Species/strain:	Rats/Sprague-Dawley
Number of animals:	5/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Vehicle:	Not specified
Batch:	Not specified
Purity:	Not specified
Dose levels:	0, 1, and 3 mg/kg bw
Dose volume:	Not specified
Route:	Intravenous
Observation	14 days
GLP:	Not specified
Study period/year:	Not specified (pre-2009)

Tafluprost (purity not specified) was investigated for acute intravenous toxicity in rats. In this study, Sprague-Dawley rats (5/sex/group rats) were administered single intravenous doses of the test substance at 0, 1 and 3 mg/kg bw. Following exposure, the animals were observed daily for clinical signs of toxicity, and mortality for 14 days. Body weights, food intake and water intake were determined at defined intervals.

Results

Neither mortalities or clinical signs nor marked changes in body weight, food intake, water intake or gross pathology were observed by the study investigators.

Conclusion

Under the conditions of the study, the LD_{50} of test substance was determined to be >3 mg/kg bw in rats.

2nd study: Acute intravenous toxicity study in dogs

Guideline:	Not specified
Species/strain:	Beagle dogs
Number of animals:	2 males/group
Test substance:	Tafluprost
Product name:	AFP-168
Vehicle:	Not specified
Batch:	Not specified
Purity:	Not specified
Dose levels:	0, 0.3, 3, and 30 µg/kg bw (i.e., 0.0003, 0.003 and 0.03 mg/kg bw)
Dose volume:	Not specified
Route:	Intravenous
Observation	14 days
GLP:	Not specified
Study period/year:	Not specified (pre-2009)

Tafluprost (purity not specified) was investigated for acute intravenous toxicity in dogs. In this study, Beagle dogs (2 males/group) were administered single intravenous doses of the test substance at 0, 0.3, 3, and 30 µg/kg bw. Following exposure, the animals were observed daily for clinical signs of toxicity and mortality for 14 days. Body weights and food intake were determined at defined intervals. Haematology, urinalysis, body temperature, blood pressure or ophthalmologic findings were also evaluated.

Results

No mortalities or marked changes in body weight, haematology parameters, urinalysis, body temperature, or ophthalmologic findings were observed.

Clinical signs including salivation, vomiting, moderate miosis, irregular respiration and increased heart rate were observed at doses ≥0.003 mg/kg bw. The miosis was severe, and elevated blood pressure was also observed at 0.03 mg/kg bw. No adverse effects were noted at 0.0003 mg/kg.

Conclusion

Under the conditions of the study, the LD_{50} of test substance was determined to be >0.03 mg/kg bw in dogs.

(CDER, 2011; TGA, 2012)

Overall conclusion on acute toxicity potential

Based on the acute toxicity data available for the analogue tafluprost, DDDE is not expected to show severe toxicity at acute, single doses up to 3 mg/kg bw via the intravenous route or 100 mg/kg bw via the oral route.

3.3.3. Irritation and corrosivity

3.3.3.1 Skin irritation

Two guideline compliant *in vitro* skin irritation studies using reconstructed human epidermis (RhE) skin with neat DDDE and one *in vivo* human repeated insult patch test (HRIPT) with a cosmetic eyelash formulation containing 0.025% DDDE are available to assess the irritation potential of DDDE.

3.3.3.1.1 In vitro tests

1st study: *In vitro* skin irritation (EpiDerm™ RhE test method) with neat DDDE

Guideline:	OECD Test Guideline 439
Test system:	*In vitro* EpiDerm™ model / RhE
Test substance:	DDDE (neat oil)
Vehicle:	Unchanged
Batch/Lot:	TAF-10-1122-01
Purity:	99.78%
Dose applied:	30 µL
Negative control:	Dulbecco's phosphate buffered saline (DPBS)
Positive control:	5% sodium dodecyl sulphate (SDS)
Duration of exposure:	1 hour
GLP:	Yes
Study period	2023

The skin irritation potential of the test substance DDDE (99.78% purity) was investigated in an *in vitro* EpiDerm™ RhE assay conducted according to OECD Test Guideline 439, in compliance with GLP.

In the in vitro EpiDerm™ assay, skin irritant materials are identified by their ability to produce a decrease in cell viability, which is measured by dehydrogenase conversion of MTT (3-(4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazolium bromide), present in cell mitochondria, into a blue formazan salt. The formazan production is quantitatively evaluated after extraction, by measuring the optical density (OD) of the resulting solution. The percentage reduction of cell viability compared to untreated negative controls is used to predict the skin irritant potential. According to the OECD guideline, substances with > 50% cell viability compared to the negative control are assessed to be 'non-irritant to the skin' and those with ≤50% cell viability compared to the negative control are considered to be 'corrosive/irritant to skin'.

In this study, the test substance DDDE, was applied to a three-dimensional human epidermis tissue model in triplicate for an exposure period of 60 minutes. Dulbecco's Phosphate Buffered Saline (DPBS) buffer was used as a negative control, and 5% Sodium dodecyl sulphate (SDS) solution was used as a positive control. After treatment, the tissues were thoroughly rinsed with DPBS buffer, blotted on absorbent material and transferred into the pre-filled wells containing 0.9 mL fresh assay medium. Afterwards, the tissues were incubated for 25 hours at 37 ± 1 °C, 5 ± 1 % CO2 and ≥ 95% relative humidity. The viability of each disk was assessed by incubating the tissues for 3 hours with MTT solution at 37±1 °C, in 5±1 % CO2, ≥95 % relative humidity. The precipitated formazan was then extracted using acidified isopropanol and quantified spectrophotometrically.

Results

After the treatment with the test substance, the tissue viability in the three replicates was determined to be 3, 17.2 and 27.7% at the end of the test (see **Table 3**). The tissue fluctuations were attributed to an incomplete washing due to the high viscosity of the test substance or normal biological fluctuation. The mean value of relative tissue viability was determined to be 16.0 ± 12.4%, which is below the threshold for skin irritation potential (50%) and suggested that the test substance was at least irritant to the skin.

All study validity criteria were met. The mean absorbance value determined in the negative controls was 1.717, which was within the required acceptability criterion of '0.8 ≤ mean OD ≤ 2.8'. The positive control showed clear irritating effects, and the mean value of relative tissue viability was 2.6 % (required: ≤20 %). The variation within the tissue replicates of negative control, positive control and test substance was acceptable (required: ≤18 %). Further, the values for negative control and positive control were within the range of historical data of the test facility. Therefore, the study was considered valid.

Table 3. OD values and viability percentages of the positive control and the test substance

Designation	Replicate	OD	Viability (%)
Test substance	1	0.296	17.2
	2	0.475	27.7
	3	0.052	3.0
	Mean	**0.274**	**16.0**
	Standard deviation (SD)		12.4
Positive Control	1	0.044	2.6
	2	0.043	2.5
	3	0.047	2.7
	Mean	**0.045**	**2.6**
	Standard deviation (SD)		0.1

Conclusion

Under the conditions of the test, the test substance DDDE was considered at least irritant to the skin in the EpiDerm™ RhE test.

(Brandt, 2023b)

2nd study: *In vitro* skin irritation (EpiSkin™ RhE test method) with DDDE

Guideline:	OECD Test Guideline 439
Test system:	*In vitro* EpiSkin™ small model / Reconstructed human Epidermis (RhE)
Test substance:	DDDE
Vehicle:	Unchanged
Batch/Lot:	0652603-2
Purity:	98.5 %
Negative control:	Phosphate buffered saline (1xPBS)
Positive control:	5% SDS
Duration of exposure:	15 minutes
GLP:	Yes
Study period	2022

The skin irritation potential of the test substance DDDE (98.5% purity; note: study investigator described the test substance as 'tafluprost ethyl amide' which is chemically the same as DDDE) was investigated in an *in vitro* EpiSkin™ RhE assay conducted according to OECD Test Guideline 439, in compliance with GLP.

Undiluted test substance DDDE was applied to the EpiSkin™ tissue samples in triplicate for an exposure period of 15 min. DPBS buffer was used as a negative control, and 5% SDS solution was used as a positive control. After treatment, the tissues were thoroughly rinsed with DPBS buffer followed by incubation for 42 hours at 37±1 °C, 5 ± 1 % CO2 and ≥ 95% relative humidity. The viability of each disk

was assessed by incubating the tissues for 3 hours (± 5 min) with MTT solution at 37±1 °C in 5±1 % CO2, ≥95 % relative humidity protected from light. The precipitated formazan was then extracted using acidified isopropanol and quantified spectrophotometrically.

Results

After the treatment with the test substance, the mean value of cell viability was 96.0% (see **Table 4**), which is above 50% when compared to the viability values obtained from the negative control. Therefore, the test substance was considered to be non-irritant to skin.

All study validity criteria were met. The mean absorbance value determined in the negative controls was 0.967, which was within the required acceptability criterion of '0.6 ≤ mean OD ≤ 1.5'. The positive control showed clear irritating effects, and the mean value of relative tissue viability was 11 % (required: ≤40 %). The variation within the tissue replicates of negative control, positive control and test substance was acceptable (required: ≤18 %). The mean OD value of the blank sample acidified isopropanol was 0.0341, below the threshold value of 0.1. Therefore, the study was considered valid.

Table 4. OD values and viability percentages of the positive control and the test substance

Designation	Replicate	OD	Viability (%)
Test substance	1	0.965	100
	2	1.024	106
	3	0.792	82
	Mean	0.927	**96**
	Standard deviation (SD)		12.48
Positive Control	1	0.108	11
	2	0.133	14
	3	0.069	7
	Mean	0.103	**11**
	Standard deviation (SD)		3.30

Conclusion

Under the conditions of the test, the test substance DDDE was considered to be non-irritant to skin in the EpiSkin™ RhE test.

(Buda, 2022)

3.3.3.1.2 Human data

An HRIPT with a cosmetic eyelash formulation containing 0.025% DDDE, was not found to be irritating in 51 human volunteers. (See **Section 3.3.4.2** for study details).

Overall conclusion on skin irritation

The results of the two-guideline compliant *in vitro* RhE studies in combination with the absence of a skin irritation response following exposure to a cosmetic eyelash product formulation containing 0.025% in an HRIPT with 51 panellists (see **Sections 3.3.4**) does not raise a skin irritation concern for DDDE at its intended use concentration of 0.018%.

3.3.3.2 Mucous membrane irritation / eye irritation

One *in vitro* eye irritation study with neat DDDE using reconstructed human cornea-like epithelium (RhCE) and one *in vitro* Hen's Egg Test-Chorio Allantoic Membrane (HET-CAM) study with a cosmetic eyelash product formulation containing 0.025% DDDE are available. In addition, an *in vivo* study with cosmetic eyelash product containing 0.025% DDDE, in humans is available.

3.3.3.2.1 In vitro tests

In vitro eye irritation (RhCE test method) with neat DDDE

Guideline:	OECD Test Guideline 492
Test system:	*In vitro* EpiOcular™ model/ RhCE
Test substance:	DDDE (neat oil)
Vehicle:	Unchanged
Batch/Lot:	TAF-10-1122-01
Purity:	99.78%
Dose applied:	30 µL
Negative control:	Dulbecco's phosphate buffered saline (DPBS)
Positive control:	5% sodium dodecyl sulphate (SDS)
Duration of exposure:	1 hour
GLP:	Yes
Study period	2023

The eye irritation potential of the DDDE (99.78% purity) was investigated in an *in vitro* EpiOcular™ RhCE assay conducted according to an OECD Test Guideline 492-compliant study.

In the in vitro EpiOcular™ assay, eye irritant materials are identified by their ability to produce a decrease in cell viability, which is measured by dehydrogenase conversion of MTT (3-(4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazolium bromide), present in cell mitochondria, into a blue formazan salt. The formazan production is quantitatively evaluated after extraction, by measuring the optical density (OD) of the resulting solution. The percentage reduction of cell viability compared to untreated negative controls is used to predict the eye irritant potential. According to the OECD guideline, substances with > 60% cell viability compared to the negative control are assessed to be 'non-irritant to the eye' and those with ≤60% cell viability compared to the negative control are considered to be 'eye irritant'.

In this study, the test substance DDDE, was applied to a three-dimensional human cornea tissue model in duplicate for an exposure period of 28 minutes. Sterile demineralised water was used as a negative control, and methyl acetate was used as a positive control. After treatment, the tissues were thoroughly rinsed with DPBS buffer, blotted on absorbent material and transferred into the pre-filled wells containing 1 mL fresh assay medium. Afterwards, the tissues were incubated for 120 minutes at 37 ± 1 °C, 5 ± 1 % CO2 and ≥ 95% relative humidity. The viability of each disk was assessed by incubating the tissues for 3 hours with MTT solution at 37±1 °C, in 5±1 % CO2, ≥95 % relative humidity. The precipitated formazan was then extracted using acidified isopropanol and quantified spectrophotometrically.

Results

After the treatment with the test substance, the tissue viability in the two replicates was determined to be 98.4 and 99.3% at the end of the test (see **Table 5**). The mean value of relative tissue viability was determined to be 98.8%, which is above 50% when compared to the viability values obtained from the negative control. Therefore, the test substance was considered to be non-irritant to eye.

All study validity criteria were met. The mean absorbance value determined in the negative controls was 1.927, which was within the required acceptability criterion of '0.8 ≤ mean OD ≤ 2.8'. The positive control showed clear irritating effects, and the mean value of relative tissue viability was 22.6 % (required: <50 %). The variation within the tissue replicates of negative control, positive control and test substance was acceptable (required: <20 %). Further, the values for negative control and positive control were within the range of historical data of the test facility. Therefore, the study was considered valid.

Table 5. Comparison of tissue viability

Designation	Replicate	OD	Viability (%)
Test substance	1	1.896	98.4%
	2	1.914	99.3%
		Mean	**98.8%**
Positive Control	1	0.505	26.2%
	2	0.368	19.1%
		Mean	**22.6%**

Conclusion

Under the conditions of the test, the test substance was considered non-irritant to eyes in the *in vitro* EpiOcular™ RhCE test method.

(Brandt, 2023c)

***In vitro* eye irritation (HET-CAM assay) with cosmetic eyelash formulations containing 0.025% DDDE**

Guideline:	Not specified
Test system:	Hen's egg test - chorioallantoic membrane assay (CAM)
Number of CAM	4
Test substance:	Cosmetic eyelash product containing 0.025% DDDE
Batch/Lot:	090309-2
Dose levels:	0.3 mL or 0.3 g
Observation	0, 30 sec, 2 min and 5 min after exposure
Reference substance:	Almay one coat mascara Maybelline waterproof ultra-eyeliner
GLP:	Yes
Study year:	2009

A cosmetic eyelash product formulation containing 0.025% DDDE was investigated for its eye irritation potential in a HET-CAM assay by measuring its ability to induce toxicity in the chorioallantoic membrane of a chicken. The CAM is a complete tissue that is used extensively in toxicology tests and is accepted as an alternative to animal testing. Published studies have shown that the hen's egg CAM is more sensitive to liquid irritants than the rabbit eye (Bailey, 2023).

Two commercially available cosmetic products, AlmayOne (coat mascara 50%) and Maybelline waterproof eyeliner (ultra-eyeliner 50%), were used as reference substances. 0.3 mL or 0.3 g of test and reference substances were administered to each of four CAM's followed by rinsing with 5 mL of physiological saline after twenty seconds. All CAMs were observed immediately prior to test substance administration and at 30 seconds, two and five minutes after exposure to the test substance. The reactions of the CAM, the blood vessels, including the capillaries and the albumin were examined and scored for irritant effects as detailed below.

Table 6. Scores for the different reaction types

Effect	Score		
	0.5 mins	2 mins	5 mins
Hyperaemia	5	3	1
Minimal haemorrhage (feathering)	7	5	3
Haemorrhage (obvious leakage)	9	7	5
Coagulation and / or thrombosis	11	9	7

The numeric and time dependent scores were totalled for each CAM followed by determination of mean score. Each reaction type was recorded only once for each CAM, therefore the maximum score per CAM was 32. Based on the CAM scores the test substance was classified as: 0-4.9 (practically none), 5-9.9 (slight), 10-14.9 (moderate) and 15-32 (severe).

Results

A CAM score of 0 (zero) was noted at all test points (30 seconds, 2 and 5 minutes after exposure), indicating that the test substance had no irritation potential. The mean CAM scores for the reference substances, were determined to be 0.5 and 0.75 for mascara and eyeliner respectively, indicating practically no irritation potential. Historically, the reference substances were categorised as being practically non-irritating, eliciting scores approaching 0, at 24 hours, following dosing at 100% concentration in the Draize ocular irritation tests.

Conclusion

Under the conditions of the study, the cosmetic eyelash product formulation containing 0.025% DDDE, did not show eye irritation potential in the HET-CAM assay.

(Nitka, 2009)

Note: While the scores of the reference substances are acceptably low scores for cosmetic products, it is significant for the safety assessment of the use of DDDE in the cosmetic eyelash product. This is because this *in vitro* assay objectively showed that cosmetic eyelash product has less ocular irritation potential than representative mascara and eyeliner products (Bailey, 2023).

3.3.3.2.2 Human data

***In vivo* ocular irritation study in humans with eyelash cosmetic formulation containing 0.025% DDDE**

Guideline:	No guideline available
Species:	Healthy female volunteer
Group size:	22; 19 volunteers completed the study.
Test substance:	Cosmetic eyelash product formulation containing 0.025% DDDE
Batch/Lot:	Lot#: 100109-3 (10-7-09)
Duration:	28 days
Good clinical practice (GCP):	Yes
Study year:	2010

The cosmetic eyelash product containing 0.025% DDDE was investigated for the eye irritation potential in 19 human volunteers. The test substance was applied daily to the eyelid margin at the base of the upper eyelashes in accordance with the directions in the package insert for 28 days. The study was conducted under the supervision of an ophthalmologist. The IOP was measured in each eye of each subject at the beginning and end of the study. Apart from IOP measurements, ocular irritation

was assessed by the supervising ophthalmologist who queried each subject about adverse reactions and also conducted an ophthalmic examination on Day 0 and 28. The ophthalmic examination was performed with a slit lamp and included the subject's eyelids, cornea, conjunctive, anterior chambers, papillary reactions, and visual acuity. Any reaction in the eyes and or in the eye contour area was evaluated by the ophthalmologist and observations (if any) were recorded. Tolerance to the test substance was evaluated based on the observed reactions and their degree of severity, as well as on the reproducibility from one volunteer to another. The ophthalmologist scored any observed intolerance to the test substance as 0 (none), 1 (slight), 2 (moderate) and 3 (high).

Results

No ocular irritation was reported during the entire length of the study by the ophthalmologist. Also, there was no statistically significant reduction in IOP over the 28-day study.

Only minor ocular effects were self-reported by 4 of 19 volunteers including slight dryness sensation around the eye areas (5%), slight itching sensation (21%), slight stinging sensation (16%), slight eye watering and redness (11%) and moderate to high burning sensation (5%). These minor self-reported adverse reactions were consistent with allergic reactions.

Overall, the test substance was considered to be moderately tolerated by the majority of the volunteers.

Conclusion

Under the conditions of the study, the cosmetic eyelash product formulation containing 0.025% DDDE, did not produce an eye irritation or hypersensitivity of clinical magnitude and was considered safe for use in the human volunteers.

(Sebesten, 2010)

Overall conclusion on eye irritation

Based on the absence of irritation results in a guideline compliant *in vitro* RhCE study with neat DDDE and in a human volunteer's study with a cosmetic eyelash formulation containing 0.025% DDDE, no ocular irritation concern is considered for DDDE at its intended use concentration of 0.018%.

3.3.4. Skin sensitisation

Four *in vitro* skin sensitisation tests including two Direct Peptide Reactivity Assay (DPRA) and two KeratinoSens™ studies with neat DDDE, one *in vivo* HRIPT with a dilution of DDDE in phenoxyethanol and one *in vivo* HRIPT study with a cosmetic eyelash product formulation containing 0.025% DDDE are available.

3.3.4.1 In chemico and in vitro tests

1st study*: In chemico* skin sensitisation (DPRA) with neat DDDE

Guideline:	OECD 442C
Test system:	Synthetic model Cys- and Lys-peptides assay
Test substance:	DDDE (neat oil)
Batch/Lot:	TAF-10-1122-01
Vehicle:	Acetonitrile
Purity:	99.78%
Replicates:	Triplicate
Molar ratio:	1:10 and 1:50 molar ratio of the test substance with the Cys- and Lys-peptides
Solvent control:	Yes, without test substance
Positive control:	Cinnamic aldehyde
Study period	2023

The skin sensitisation potential of the DDDE (99.78% purity) was investigated in a DPRA according to the OECD Test Guideline 442C, in compliance with GLP.

The direct peptide reactivity assay (DPRA) addresses the first molecular key event (KE1) of the adverse outcome pathway (AOP) of skin sensitisation. It is an *in chemico* assay addressing epidermal protein binding and reactivity towards proteins, by mimicking the reaction with artificial peptides followed by quantification of the depleted peptides using HPLC. According to the guideline, the following reactivity classes are assessed based on the mean peptide depletion percentage values from the 'cysteine 1:10/lysine 1:50 prediction model' and 'cysteine1:10 prediction model' respectively:

- No or minimal reactivity: 0% < mean % depletion ≤6.38% and 0% ≤Cys % depletion ≤13.89%;
- Low reactivity': 6.38% < mean % depletion ≤22.62% and 13.89% < Cys % depletion ≤ 23.09%;
- Moderate reactivity': 22.62% < mean % depletion ≤42.47% and 23.09% < Cys % depletion ≤ 98.24%;
- High reactivity': 42.47% < mean % depletions 100% and 98.24% < Cys % depletion ≤ 100%.

Test substance assigned to the 'no or minimal reactivity' category are classified as 'non-sensitisers' whereas test substances assigned to the low, moderate or high reactivity categories are to be classified as 'sensitisers'.

In this study, the test substance was incubated for 22 hours at 25°C together with Cys-peptide and Lys-peptide, respectively. The peptide concentration after the incubation period was measured using HPLC-UV. Three replicates were prepared using 1:10 and 1:50 molar ratio of the test substance with the Cys- and Lys-peptides, respectively. Triplicate samples of the solvent without test substance were incubated and measured simultaneously. One valid experiment was performed.

The test substance showed turbidity right after mixing the test substance solution with buffer and the Lys-peptide-solution. After the incubation period, precipitation was observed and therefore only the supernatant was used for measurement.

Results

The test substance showed minimal reactivity towards the Cys-peptide (mean depletion 3.20) and a very low reactivity towards the Lys-peptide (mean depletion 0.08). Therefore, the DPRA prediction was considered "negative" with "no or minimal" reactivity. The mean peptide depletion in the Cys-peptide and Lys-peptide assay was 1.64%. The DPRA prediction was "negative" according to the Cysteine 1:10/Lysine 1:50 prediction model.

However, the test substance showed precipitation in the Lys-peptide-assay and according to guideline OECD 442C, the peptide depletion in these samples may be underestimated as the amount of the test substance in solution able to react with the peptides- is not well defined.

It is, however, important to note that the mean peptide depletion in the Cys-peptide assay, which showed no precipitation for the test substance, was 3.2%. This is well below the threshold of 6.38% - in the case of cysteine1:10/lysine 1:50 prediction model- and the threshold of 13.89% - in the case of cysteine1:10 prediction model - suggesting 'no or minimal reactivity'. In general, the cysteine1:10 prediction model is used for the interpretation of results, if there is coelution observed only with the lysine peptides. As the test substance does not show co-elution, the interpretation of the Cys-peptide assay does not allow a final conclusion. Nevertheless, it supports 'no or minimal reactivity'. In addition, the percentage peptide depletion value in the Cys-peptide assay was not close to the threshold for positive (≥23.09%) and negative (≤13.89%) interpretation.

Further, all acceptance criteria were fulfilled: (1) the criteria of the calibration curve (r2 > 0.99) and reference controls (mean peptide concentration 0.50 ± 0.05 mM) were fulfilled; (2) the mean peptide depletion of the positive control cinnamaldehyde was within the range 60.8 % - 100.0 %, the peptide depletion of the positive control 2,3-Butanedione was within 10.0 % - 45.0 %; (3) the standard

deviation of the replicates of the positive control and test substance was < 14.9 % in the Cys-peptide assay and < 11.6 % in the Lys-peptide assay, respectively. Therefore, the study was considered valid.

Table 7. Cysteine and lysine peptide depletion values for the positive control and the test substance

Sample	Cys-Peptide Depletion [%]	Mean Peptide Depletion [%]	Standard deviation (SD) for peptide depletion (%)	Lys-Peptide Depletion [%]	Mean Peptide Depletion [%]	Standard deviation (SD) for peptide depletion (%)
Positive control, rep I	82.18	82.42	0.23	21.49	22.47	0.96
Positive control, rep 2	82.44			23.41		
Positive control, rep 3	82.63			22.50		
Test substance, rep I	2.95	3.20	0.24	0 (-0.06) *	0.08	0.14
Test substance, rep II	3.25			0 (-0.04) *		
Test substance, rep III	3.42			0.24		
Mean depletion of both peptides after incubation with the test substance DDDE: **1.64 %**						

* Note: Negative depletion values were considered as “zero” when calculating the mean.

Conclusion

Under the conditions of the study, the DPRA prediction was “negative” according to the Cysteine 1:10/Lysine 1:50 prediction model. However, due to the observed precipitation in the Lys-peptide-assay, a conclusion on the lack of reactivity cannot be drawn with sufficient confidence. Nevertheless, the mean peptide depletion value in the Cys-peptide assay, which did not show precipitation, supports no or minimal reactivity.

(Brandt, 2023a)

2nd study*: In chemico* skin sensitisation (DPRA) with DDDE

Guideline:	OECD 442C
Test system:	Synthetic model Cysteine peptides assay
Test substance:	DDDE
Batch/Lot:	0652603-2
Vehicle:	Acetonitrile
Purity:	98.5%
Replicates:	Triplicate
Molar ratio:	1:10 molar ratio of the test substance with the Cys-peptides
Solvent control:	Yes, without test substance
Positive control:	Cinnamic aldehyde, ≥95 %, FG
Study period	2022

The skin sensitisation potential of DDDE (98.5% purity; note: study investigator described the test substance as ‘tafluprost ethyl amide’ which is chemically the same as DDDE) was investigated in another DPRA conducted according to the OECD Test Guideline 442C, in compliance with GLP.

The solubility of the test substance was tested in a non-GLP preliminary solubility test. The compatibility of the formulation with phosphate buffer was proven, no precipitate was observed in case of phosphate buffer (for cysteine analysis) after vertexing and homogenous, clear solution was obtained. In case of acetate buffer (for lysine analysis), opalescent formulation was obtained, and precipitation was observed. This formulation was not suitable for the high-performance liquid chromatography (HPLC) analysis. Because of the opalescent formulation, only cysteine run, and the evaluation were performed according to cysteine 1:10 prediction model.

The test substance was incubated for 24±2 hours at 25°C together with Cysteine peptide. The peptide concentration after the incubation period was measured using HPLC. Three replicates were prepared using 1:10 molar ratio of the test substance with the Cysteine peptide. Triplicate samples of the solvent without test substance were incubated and measured simultaneously. One valid experiment was performed.

Results

No co-elution was observed with the peptide. The mean cysteine peptide depletion value of the test substance was 5.61 % ± 0.44 % indicative for negative DPRA prediction.

Further, all acceptance criteria were fulfilled: (1) the criteria of the calibration curve (r^2 > 0.99) and reference controls (mean peptide concentration 0.50 ± 0.05 mM) were fulfilled; (2) the mean peptide depletion of the positive control cinnamaldehyde was within the range 60.8 % - 100.0 %; (3) the standard deviation of the replicates of the positive control and test substance was < 14.9 % in the Cys-peptide assay. Therefore, the study was considered valid.

Table 8. Cysteine peptide depletion values for the positive control and the test substance

Sample	Cys-Peptide Depletion [%]	Mean Peptide Depletion [%]	Standard deviation (SD) for peptide depletion (%)
Positive control, rep I	69.88	70.67	0.69
Positive control, rep 2	70.99		
Positive control, rep 3	71.14		
Test substance, rep I	5.11	5.61	0.44
Test substance, rep II	5.93		
Test substance, rep III	5.79		

Conclusion

The mean peptide depletion in the Cys-peptide assay for the test substance was 5.61%. This is well below 13.89% suggesting no or minimal reactivity under the experimental conditions of the DPRA method and was therefore concluded to be negative according to the prediction criteria.

(Sagi, 2022)

1st study: *In vitro* skin sensitisation (KeratinoSens™ assay) with neat DDDE

Guideline:	OECD 442D
Test system:	KeratinoSens™ Cell Line
Test substance:	DDDE (neat oil)
Batch/Lot:	TAF-10-1122-01

Purity:	99.78%
Test concentrations:	0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µM
Solvent control:	Dimethyl sulfoxide (DMSO)
Positive control:	Cinnamic aldehyde
Study period	2023

An *in vitro* study was performed to assess the potential of the DDDE (99.78% purity) to activate the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor by using the genetically modified keratinocyte cell-line "KeratinoSens™" in an OECD Test Guideline 442C-compliant study.

The KeratinoSens addresses the second key event (KE2) of the AOP of skin sensitisation assessing the induction of the luciferase gene by the test substance in keratinocytes. The luciferase gene induction is then quantitatively measured by using well established light producing luciferase substrates, as an indicator of the activity of the Nrf2 transcription factor.

Cytotoxicity and the relative luminescence intensity of luciferase substance in the lysates are measured and luciferase induction values compared to solvent/vehicle control are calculated. Test substances are considered positive in the KeratinoSens™ test method if they induce a statistically significant induction of the luciferase activity above a given threshold (i.e., ≥ 1.5 fold, or 50% increase), below a defined concentration which does not significantly affect cell viability (i.e., below 1000 µM and at a concentration at which the cellular viability is above 70%).

The assay was performed in two independent repetitions (I and II). 12 concentrations of the test substance were evaluated. The exposure time was 48 h. The following nominal concentrations of the test substance were investigated in repetition I and II: 0.98 µM, 1.95 µM, 3.91 µM, 7.81 µM, 15.63 µM, 31.25 µM, 62.5 µM, 125 µM, 250 µM, 500 µM, 1000 µM, 2000 µM.

A test substance concentration inducing a viability below 70% was considered as cytotoxic and therefore not considered further for evaluation of luciferase induction.

DMSO (final concentration: 1 %) was used as solvent control and cinnamic aldehyde (5 concentrations ranging from 4 to 64 µM) as positive control.

Results

Precipitation of the test substance was not visible in any of the repetitions. None of the real treatment concentrations in all repetitions deviated more than 10 % from the nominal concentration.

Cytotoxic effects were observed at doses ≥500 µM in repetition I and II. At the next lower test concentration (i.e., 250 µM), the cell viability rose sharply to a viability above 80%. Therefore, the lower concentrations ranging from 0.98 to 250 µM, that showed a viability ≥70%, were evaluated for the luciferase induction in repetitions I and II. The overall concentration values for 50% (IC_{50}) and 30% (IC_{30}) reduction of cellular viability were determined as 363.4 and 308.7 µM respectively (see **Table 10**).

In repetition I, a statistically significant increase in luciferase induction >1.5 fold was observed at the test substance concentration 250 µM (see **Table 9**). All lower concentrations showed induction values in the range of the solvent control. Since no dose-dependence was detected, this repetition was considered as "inconclusive" and was repeated.

In repetition II again, a statistically significant increase in luciferase induction to exactly 1.5-fold was observed at the test substance concentration of 250 µM (see **Table 9**). Like in repetition I, the induction values at the lower test substance concentrations were all in the range of the solvent control. For this reason, no clear dose-dependent effect was observed, and the result was considered inconclusive according to the criteria of OECD TG 442D. The overall maximal fold induction (I_{max}) value was determined as 1.8-fold (see **Table 10**).

Table 9. Results of test substance concentrations in repetitions I and II

Conc [µM]	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250
Repetition I									
Precipitates	-	-	-	-	-	-	-	-	-
Viability [%]	**95**	**104**	**96**	**100**	**103**	**106**	**106**	**106**	**81**
Induction [Fold]	**1.0**	**1.1**	**1.1**	**1.1**	**1.1**	**1.2**	**1.1**	**1.0**	**2.1**
Standard Deviation	0.02	0.06	0.08	0.05	0.03	0.15	0.11	0.16	0.36
p-value	0.67	0.48	0.20	0.09	0.08	0.19	0.48	0.80	0.01
Repetition II									
Precipitates	-	-	-	-	-	-	-	-	-
Viability [%]	**89**	**91**	**92**	**94**	**103**	**109**	**115**	**120**	**108**
Induction [Fold]	**0.9**	**1.0**	**0.9**	**0.9**	**0.9**	**0.9**	**1.0**	**0.8**	**1.5**
Standard Deviation	0.08	0.07	0.08	0.08	0.12	0.11	0.20	0.02	0.18
p-value	0.49	0.54	0.52	0.40	0.36	0.19	0.81	0.14	0.00

Table 10. Summary of Imax, IC_{50} and IC_{30} values

	Imax [Fold]	$EC_{1.5}$ [µM]	IC_{50} [µM]	IC_{30} [µM]
Value of Repetition I	2.1	184.2	348.4	285.2
Value of Repetition II	1.5	247.1	379.0	334.2
Mean	1.8	213.3	363.4	308.7

As the absence of a clear dose response in repetition I could be verified in repetition II, no further repetition was considered necessary. Therefore, the study was considered “inconclusive” under the experimental conditions chosen.

An additional evaluation according to OECD Test Guideline 497 indicates that the results of the two repetitions are not congruent - repetition I: inconclusive (due to lack of dose response), repetition II: borderline (due to the induction fold not >1.5) – therefore, the final assessment would lead to a ‘borderline’ conclusion, irrespective of the outcome of repetition III. Thus, no further repetition was considered necessary.

All study validity criteria were met. The coefficient of variation of the luminescence reading for the solvent control (i.e., DMSO) was below 20% in each repetition (after potential outlier removal of solvent control). In addition, the positive control cinnamic aldehyde was tested in a series of 5 concentrations ranging from 4 to 64 µM and it fulfilled all the acceptability criteria. Furthermore, the luciferase induction values remained well within the historical control range. Therefore, the study was concluded to be valid.

Conclusion

Under the experimental conditions of this study, no clear assessment of the potential to activate the Nrf2 transcription factor could be made due to the lack of a clear dose response. The results of the KeratinoSens™ test were therefore considered "inconclusive".

(Fruhmesser, 2023)

2nd study: *In vitro* – KeratinoSens assay with DDDE

Guideline:	OECD 442D
Test system:	KeratinoSens™ Cell Line
Test substance:	DDDE
Batch/Lot:	0652603-2
Purity:	98.5%
Test concentrations:	First test: 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µM Second test: 55.80, 72.54, 94.30, 122.59, 159.37, 207.18, 269.33, 350.13, 455.17, 591.72, 769.23 and 1000 µM Third test: 67.29, 80.75, 96.90, 116.28, 139.54, 167.45, 200.94, 241.13, 289.35, 347.22, 416.67, 500.00 µM
Solvent control:	DMSO
Positive control:	Trans-cinnamaldehyde
Study period	2022

An *in vitro* study was performed to assess the potential of the DDDE (98.5% purity; note: study investigator described the test substance as 'tafluprost ethyl amide (TEA)' which is chemically the same as DDDE) to activate the Nrf2 transcription factor by using the genetically modified keratinocyte cell-line "KeratinoSensTM" in an OECD Test Guideline 442C-compliant study.

Three valid independent tests were conducted, with a treatment period of 48 hours. For the test substance, twelve doses ranging from 0.98 to 2000 µM and 2-fold dilution factor were used in the first test. In order to be able to determine IC_{30} and IC_{50} values more precisely (since strong cytotoxicity was observed at the higher tested concentrations) and to investigate the possible positive effect of the test substance, lower top concentration and narrower dilution factor was used in the second and third tests. Thus, twelve doses ranging from 55.8 to 1000 µM and 1.3-fold dilution factor were used in the second test, while twelve doses - ranging from 67.29 to 500.00 µM and 1.2-fold dilution factor - were used in the third test.

After exposure, luciferase activity and cytotoxicity were measured. A test substance concentration inducing a viability below 70% was considered as cytotoxic and was not allowed to be evaluated for luciferase induction. For each individual test, four parallel plates were used: three replicates were used for the luciferase activity induction measurements, and one was needed for the MTT cell viability assay to measure the cytotoxicity induced by the test substance.

DMSO (final concentration: 1%) was used as solvent control and trans-cinnamaldehyde (5 concentrations ranging from 4 to 64 µM) as positive control.

Results

The test substance induced cytotoxicity in KeratinoSens™ cells compared to the solvent/vehicle control in all tests at the higher tested concentrations (≥500 µM in the first test, ≥350.13 µM in the second test and ≥289.35 µM in the third test). Thus, IC_{30} and IC_{50} values were determined for each independent test. The overall IC_{30} was determined as 278.51 µM, while the overall IC_{50} was 311.38 µM (see **Table 12**).

The induction values of the test substance did not exceed the 1.5-fold threshold at any tested concentrations compared to the respective negative control in either independent test. Thus, an $EC_{1.5}$ value could not be determined for any of the tests.

Moreover, according to the OECD Test Guideline 497 prediction model, there was only one induction value of the test substance which exceeded the lower limit of the borderline threshold (1.35-fold) compared to the respective negative control at the interim concentration of 250 µg/mL in the first test (see **Table 11**).

Table 11. Results of test substance concentrations in Test 1, 2 and 3

First test									
Conc [µM]	**0.98**	**1.95**	**3.91**	**7.81**	**15.63**	**31.25**	**62.5**	**125**	**250**
Precipitates	-	-	-	-	-	-	-	-	-
Viability [%]	**100**	**95**	**98**	**94**	**94**	**97**	**96**	**120**	**94**
Induction [Fold]	**0.83**	**1.00**	**0.87**	**0.94**	**0.90**	**1.07**	**1.12**	**1.03**	**1.48**
p-value	0.023	0.977	0.015	0.123	0.379	0.461	0.087	0.814	0.03
Second test									
Conc [µM]	**55.80**	**72.54**	**94.30**	**122.59**	**159.37**	**207.18**	**269.33**	**350.13**	**455.17**
Precipitates	-	-	-	-	-	-	-	-	-
Viability [%]	**114**	**113**	**119**	**130**	**125**	**116**	**75**	**1**	**0**
Induction [Fold]	**1.04**	**1.09**	**1.03**	**0.90**	**0.96**	**0.92**	**1.20**	**0.0**	**-0.01**
p-value	0.809	0.433	0.867	0.255	0.604	0.411	0.153	0.000	0.000
Third test									
Conc [µM]	**67.29**	**80.75**	**96.90**	**116.28**	**139.54**	**167.45**	**200.94**	**241.13**	**289.35**
Precipitates	-	-	-	-	-	-	-	-	-
Viability [%]	**103**	**105**	**103**	**106**	**103**	**94**	**105**	**77**	**41**
Induction [Fold]	**1.04**	**0.92**	**1.04**	**0.81**	**0.84**	**0.78**	**0.87**	**1.08**	**0.73**
p-value	0.769	0.098	0.335	0.006	0.022	0.062	0.023	0.510	0.004

The overall maximal fold induction (I_{max}) value was determined as 1.25-fold. The induction values of the test substance did not exceed the 1.5-fold threshold at any tested concentrations compared to the respective negative control in either independent test. Thus, an EC1.5 value could not be determined for any of the tests. In addition, no dose-response-relationship was observed in any of the tests (see below **Table 12 and 13**).

Table 12. Summary of Imax, IC_{50} and IC_{30} values

Test No.	Imax [Fold]	$EC_{1.5}$ [µM]	IC_{50} [µM]	IC_{30} [µM]
1	1.48	-	367.02	313.83
2	1.2	-	274.79	296.63
3	1.08	-	277.30	250.51
Mean	1.25	-	311.38	278.51

Table 13. Summary of the results

Test No.	Significant induction above 1.5-fold (yes/no)	Viability ≥ 70 % at lowest concentration with ≥ 1.5-fold (yes/no)	$EC_{1.5}$ < 1000 µM or 200 µg/ml (yes/no)	Clear dose response (yes/no)	Outcome based on OECD 442D	Outcome based on OECD 497
1	No	No	-	No	Negative	borderline*
2	No	No	-	No	Negative	Negative
3	No	No	-	No	Negative	Negative
Overall conclusion					**Negative**	

*At the concentration of 250 µg/mL the induction value is higher than 1.35 with cell viability > 70 %.

The coefficient of variation of the luminescence reading for the solvent control (i.e., DMSO) was below 20% in all tests. In addition, each individual test met all the acceptance criteria for the negative and positive controls. Therefore, the study was concluded to be valid.

Conclusion

Under the experimental conditions of this study, the test substance is concluded negative for skin sensitisation potential when tested up to cytotoxic concentrations.

(Hummel-Kocsi, 2022)

3.3.4.2 Human data

1st study: Human repeated insult patch test with 7.5% DDDE in phenoxyethanol

Guideline:	No guideline available
Test system	Healthy volunteers
Group size:	54 volunteers completed the study (12 males and 42 females).
Test substance:	7.5% DDDE in phenoxyethanol
Batch/Lot:	TAF-F-0522-01
Route:	Dermal
Administration:	Semi-occlusive (diluted to 0.267% in deionised water)
Exposure period:	48 hours (after 1st application), 24 hours after subsequent patches
Application frequency:	3 times per week for 3 consecutive weeks
Positive irritation control:	1% sodium lauryl sulphate
Negative control:	Distilled water
Resting period:	10-14 day
Scoring scale:	International contact dermatitis research group scoring scale

GCP:	Not specified
Study period	October to November 2022

The skin sensitisation and cumulative irritation potential of DDDE (7.5% in phenoxyethanol) was investigated in an HRIPT in 54 human volunteers (ages 18 – 64 years). In the induction phase, the test substance, diluted to 0.267% in deionised water, was applied via semi occlusive patches to the skin on the back (intrascapular area) of the human volunteers for 24 hours, 3 times/week for 3 consecutive weeks. Prior to each patch application, the test sites were evaluated by trained laboratory personnel for any signs of skin reactions (see **Table 14**). Fourteen days after removal of the last induction patches, a challenge and re-challenge patch was applied to each of the study participants. After 48 and 96 hours of application, the test sites were evaluated. Each evaluation was scored as per the following:

Table 14. Scoring for the evaluation of skin reactions

Score	Reaction
0	No reaction
1	Erythema throughout at least ¾ of the patch area
2	Erythema and induration throughout at least 2/4 of patch area
3	Erythema, induration and vesicles
4	Erythema, induration and bullae

Results

All 486 evaluations were scored "0". No adverse reactions of any kind were reported during the course of this study.

Eighteen subjects showed a Grade 1 reaction to the positive irritation control and one with a Grade 2 reaction. No subjects showed any signs of reaction to the negative control.

Conclusion

Under the conditions of the HRIPT, the test substance did not reveal any signs or symptoms of a skin sensitisation (contact allergy) or a cumulative irritation response in any of the 54 panellists completing the study.

(Martin, 2022)

2nd study: Human repeated insult patch test with a cosmetic eyelash formulation containing 0.025% DDDE

Guideline:	No guideline available
Species:	Healthy volunteer
Group size:	52; 51 volunteers completed the study (4 males and 48 females).
Test substance:	Eyelash cosmetic product containing 0.025% DDDE.
Batch/Lot:	090309-2
Route:	Dermal
Administration:	Occlusive epicutaneous
Exposure period:	24 and 48 hours
Application frequency:	3 times per week for 3 consecutive weeks
Resting period:	10-14 day
Scoring scale:	International contact dermatitis research group scoring scale
GCP:	Not specified
Study period	October to November 2009

A cosmetic eyelash product containing 0.025% DDDE was investigated for the skin sensitisation and cumulative irritation potential in a HRIPT using 51 human volunteers. In this study, the formulation

was applied to the skin on the back (intrascapular area) of 51 human subjects (ages 18 – 59 years) for 24 hours, 3 times/week for 3 consecutive weeks (total of 9, 24-hour exposures). Prior to each reapplication, the test sites were evaluated by trained laboratory personnel. Fourteen days after removal of the last induction patches, a challenge and re-challenge patch was applied to each of the study participants. After 48 and 96 hours of application, the test sites were evaluated. Each evaluation was scored as given in the following table:

Table 15. Scoring for the evaluation of skin reactions

Score	Reaction
0	No
1	Erythema throughout at least ¾ of the patch area
2	erythema and induration throughout at least 2/4 of patch area
3	Erythema, induration and vesicles
4	Erythema, induration and bullae

Results

Two out of 561 evaluations were scored "1", all other evaluations were scored "0". No adverse reactions of any kind were reported during this study.

Conclusion

Under the conditions of the HRIPT, the formulation containing 0.025% DDDE did not show any signs or symptoms indicative of a skin sensitisation response in any of 51 panellists completing the study.

(Gunt, 2009)

Overall conclusion on skin sensitisation potential

Based on the overall absence of skin sensitisation responses in guideline compliant *in vitro* DPRA and KeratinoSens™ studies with neat DDDE and in two HRIPTs conducted with 7.5% DDDE in phenoxyethanol and with a formulation containing 0.025% DDDE, no skin sensitisation concern is considered for DDDE at its intended use concentration of 0.018%.

3.3.5. Repeated dose toxicity

No repeated dose toxicity studies could be identified for DDDE. Therefore, data available for the analogue tafluprost is used for assessing the systemic toxicity potential of DDDE by means of read across. The scientific justification for the read across is provided in **Section 3.3**. with more details provided in **Annex I**.

The database for the assessment of repeated dose toxicity with the analogue tafluprost includes 4 intravenous (2 subacute, 2 subchronic), 2 subcutaneous (subchronic) and 4 ocular studies (1 subacute, 2 subchronic and 1 chronic). The overview of the repeated dose studies with the analogue tafluprost has been summarised in **Table 16**.

Table 16. Overview of repeated dose toxicity studies with the analogue tafluprost

Study type, Species	Doses	Key findings	NOAEL/LOAEL	Reference
Intravenous route				
28 days, intravenous study, rats (strain not specified) (12/sex/dose)	0, 10, 30 and 100 µg/kg bw/day	No significant effects at any dose level.	NOAEL = 100 µg/kg bw/day	(CDER, 2011)
28 days, intravenous study, dogs (strain not specified) (4/sex/dose)	0, 0.1, 1 and 10 µg/kg bw/day	At 10 µg/kg bw/day, salivation, vomiting, miosis, increased respiratory rate, increased heart rate, and	NOAEL = 1 µg/kg bw/day	(CDER, 2011)

Study type, Species	Doses	Key findings	NOAEL/LOAEL	Reference
		prolonged QTc interval in both sexes, increased diastolic pressure only in males, and reduced urinary chloride concentration only in females		
26 weeks, intravenous study, rats (Crl:CD®(SD)IGSBR) (15/sex/dose)	0, 10, 30 and 100 µg/kg bw/day	At all dose levels, histopathology findings in bone marrow in the femoral and sternum bone, spleen, liver, and kidney.	LOAEL = 10 µg/kg bw/day *	(CDER, 2011)
39 weeks, intravenous study, dogs (strain not specified) (4/sex/dose)	0, 0.1, 1 and 10 µg/kg bw/day	At 10 µg/kg bw/day, transient clinical signs of nausea, transient miosis, transient slight elevations in heart rate, blood pressure, and respiratory rate, minor adrenal cortical eosinophilia in 3 out of 4 dogs and acinar cell hypertrophy in salivary glands in all dogs	NOAEL = 1 µg/kg bw/day	(CDER, 2011)
Subcutaneous route (SC)				
13-week, subcutaneous study, rats (Crl:CD®(SD) IGSBR) (10/sex/dose) Range finding studies for carcinogenicity study.	0, 3, 10, and 30 µg/kg bw/day	At 30 µg/kg bw/day, minor histopathological changes in spleen and kidney	NOAEL = 30 µg/kg bw/day*	(CDER, 2011)
13-week, subcutaneous study, mice (Crl:CD-1(ICR)BR) (12/sex/dose) Range finding studies for carcinogenicity study.	0, 3, 10, 30 and 100 µg/kg bw/day	No significant adverse effects any dose level	NOAEL = 100 µg/kg bw/day*	(CDER, 2011)
24-month, subcutaneous study, rats (Crj:CD(SD) IGSBR) (60/sex/dose)	0, 3, 9 and 30 µg/kg bw/day	<u>Systemic</u> At 3 µg/kg bw/day, significant reduction in body weight in both sexes. <u>Non-neoplastic</u> Histopathology changes including hyperostosis of the sternum and femur in some animals and increased incidence of extramedullary haematopoiesis in the spleen in males at all dose levels	Systemic LOAEL = 3 µg/kg bw/day* Carcinogenicity NOAEL = 30 µg/kg bw/day	(CDER, 2011)

Study type, Species	Doses	Key findings	NOAEL/LOAEL	Reference
		No carcinogenicity at any dose level		
78 weeks, subcutaneous study, mice (Crl:CD-1(ICR)BR) (51/sex/dose)	0, 10, 30 and 100 µg/kg bw/day	No significant adverse effects at any dose level	Systemic and carcinogenicity NOAEL = 100 µg/kg bw/day*	(CDER, 2011)
Topical ocular[7]				
28 days, ocular study, monkeys (strain not specified) (3/sex/dose)	0.0005, 0.005% and 0.05% of the ophthalmic solution (i.e., equivalent to 0.067–0.1, 0.67–1 and 6.7–10 µg/kg bw/day)	Systemic and local No significant toxicity at any dose level	Systemic NOAEL = 6.7–10 µg/kg bw/day	(CDER, 2011; TGA, 2012)
13-week, ocular study, monkeys (strain not specified) (4/sex/dose)	0.0005, 0.005% and 0.05% of the ophthalmic solution (i.e., equivalent to 0.075–0.1, 0.75–1 and 7.5–10 µg/kg bw/day)	Systemic No systemic toxicity at any dose level Local At all dose levels, irreversible darkening in iris colour in left treated eye[8].	Systemic NOAEL = 7.5- 10 µg/kg bw/day	(CDER, 2011; TGA, 2012)
13-week, ocular study, monkeys (strain not specified) (3/sex/dose)[9]	0.0045% of the ophthalmic solution (i.e., equivalent to 0.75 – 0.9 µg/kg bw/day)	Systemic No systemic toxicity at any dose level Local At all dose levels, changes in iris colour in 2 animals[10]	Systemic NOAEL = 0.75 – 0.9 µg/kg bw/day	(CDER, 2011; TGA, 2012)
52-weeks, ocular study, monkeys (strain not specified) (4/sex/dose)	0.0005, 0.005% and 0.05% of the ophthalmic solution (i.e., equivalent to 0.075–0.1, 0.75–1 and 7.5–10 µg/kg bw/day)	Systemic No systemic toxicity at any dose level Local At all dose levels, sunken, dark iris colour, blue-grey discolouration in 3/4 animals of both the genders throughout study period[11]	Systemic NOAEL = 7.5- 10 µg/kg bw/day	(CDER, 2011; TGA, 2012)

** The NOAEL/LOAELs were not established in the source documents but was concluded based on the available details on the adverse effects in the underlying studies.*

[7]Doses in µg/kg bw/day are reported in the Australian public assessment report (TGA, 2012).

[8] These effects are considered to be mainly cosmetic, not associated with loss of function, and not toxicologically significant (CDER, 2011).

[9] The study was conducted in multiple test groups, including a combination of tafluprost with timolol. Only the test group with standalone administration of tafluprost was considered for the assessment (CDER, 2011).

[10,14] These effects are considered to be mainly cosmetic, not associated with loss of function, and not toxicologically significant (CDER, 2011).

3.3.5.1. Intravenous route

3.3.5.1.1 Subacute toxicity

1st study: Subacute toxicity study in rats via IV route

Guideline:	Not specified
Species/strain:	Rats/ Not specified
Number of animals:	12/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Vehicle:	Not specified
Batch:	Not specified
Purity:	Not specified
Route:	Intravenous
Dose levels:	0, 10, 30 and 100 µg/kg bw/day
Duration:	28 days
Recovery	14 days
GLP:	Yes
Study period/year:	Not specified (pre-2013)

The subacute repeated dose toxicity of the analogue tafluprost (purity not specified) was investigated in rats following intravenous administration of 0, 10, 30 and 100 µg/kg bw/day daily for 28 days followed by a 14-day recovery period. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Haematological parameters, clinical chemistry parameters, urinalysis, ophthalmic and electrocardiographic parameters were examined. At the termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, clinical signs, or changes in body weight, water consumption, clinical chemistry, urinalysis parameters, ophthalmic parameters, electrocardiograph traces, organ weights, gross pathology or histopathology were noted. Slightly lower haemoglobin concentration, erythrocyte numbers, and packed cell volume occurred in males at 100 µg/kg bw/day and erythrocyte numbers were marginally reduced in males at 30 and 10 µg/kg bw/day. Also, platelet numbers were marginally low in all treated females and males at 30 and 100 µg/kg bw/day. However, significant differences did not occur, and all of these parameters returned to normal levels during the recovery period.

Conclusion

Under the conditions of the study, the NOAEL for the test substance tafluprost was established at 100 µg/kg bw/day (highest tested dose) in rats.

(CDER, 2011)

2nd study: Subacute toxicity study in dogs via IV route

Guideline:	Not specified
Species/strain:	Dogs/ Not specified
Number of animals:	4/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Vehicle:	Not specified
Batch:	Not specified

Purity:	Not specified
Route:	Intravenous
Dose levels:	0, 0.1, 1 and 10 µg/kg bw/day
Duration:	28 days
Recovery	14 days
GLP:	Yes
Study period/year:	Not specified (pre-2013)

The subacute repeated dose toxicity of the analogue tafluprost (purity not specified) was investigated in dogs following intravenous administration of 0, 0.1, 1 and 10 µg/kg bw/day daily for 28 days followed by a 14- day recovery period. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Haematological parameters, clinical chemistry parameters, urinalysis, ophthalmic parameters, and electrocardiograph were assessed. At the termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, clinical signs, or changes in body weight, food consumption, haematology, body temperature, ophthalmology, gross pathology, organ weights or histopathology were noted. Slight miosis, sporadic salivation and vomiting were observed in both sexes at 1 µg/kg bw/day. Salivation, vomiting, miosis, increased respiratory rate, increased heart rate, and prolonged QTc interval were observed in both sexes at 10 µg/kg bw/day. Increased alanine aminotransferase (ALT) activity, increased urine volume and decreased urinary potassium concentration were observed in both sexes during the dosing period, but these effects disappeared after the recovery period. Increased diastolic pressure was found only in males and reduced urinary chloride concentration occurred only in females.

Conclusion

Under the conditions of the study, the NOAEL for the test substance tafluprost was established at 1 µg/kg bw/day (mid dose) in dogs.

(CDER, 2011)

3.3.5.1.2 Subchronic toxicity

1st Study: Subchronic toxicity study in rats via IV route

Guideline:	EMA note for guidance on repeated dose toxicity (CPMP/SWP/1042/99)
Species/strain:	Rats/Crl:CD®(SD)IGSBR
Number of animals:	15/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	FP-0002
Purity:	98.6%
Route:	Intravenous
Dose levels:	0, 10, 30 and 100 µg/kg bw/day
Dose volume:	10 ml/kg
Vehicle:	Sterile saline (0.9% NaCl)
Satellite group	8/sex/group for toxicokinetic analysis
Duration:	26-weeks
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the outcome or integrity of the study.

Study year: 2001

The subchronic toxicity of the analogue tafluprost (98.6% purity) was investigated for repeated dose toxicity according to the EMA note for guidance on repeated dose toxicity (CPMP/SWP/1042/99) (TGA, 2012). Crl:CD®(SD)IGSBR rats (15/sex/group) were administered intravenously daily at doses of 0, 10, 30 and 100 µg/kg bw/day for 26-weeks. The stability of the dosing solution was confirmed by analysis. During the treatment period, animals were observed for clinical signs, mortality, body weight, food consumption and water consumption at defined intervals. Haematological, biochemical, ophthalmological examination and urinalysis were performed. Electrocardiogram (ECG) measurements were performed pre-treatment and in Week 26 before dosing. At the termination of treatment, all animals were sacrificed after overnight fasting and macroscopically examined, organs were weighed, and comprehensive histopathology was performed. The study report noted multiple minor deviations from the study protocol. However, these deviations were not considered to have altered the outcome or integrity of the study.

Results

Fifteen animals (eleven males and four females) died or were sacrificed during the treatment period. The apparent cause of death of one male in the low dose group and two males in the intermediate dose group was due to glomerulonephropathy, but the cause of death for the other animals (all intermediate and high dose) was not apparent.

No treatment-related clinical signs in surviving animals, changes in body weight, water consumption, clinical chemistry, or ophthalmic parameters were noted. Few ECG findings were of uncertain relationship to the test substance administration. In haematological examinations, some effects were evident in males and females. In Week 26, haemoglobin concentration and erythrocyte numbers were slightly decreased in females in all dose groups. Reticulocyte numbers, mean cell volume, mean cell haemoglobin, and red cell distribution widths were slightly higher in females at 30 and 100 µg/kg bw/day. Similar patterns were observed in males at 100 µg/kg bw/day but generally to a lesser extent for most parameters. Platelet numbers and platelet crit were slightly low at all dose levels in males, and the mean platelet volume and platelet distribution width were slightly high in males at 0.03 and 100 µg/kg bw/day. For these parameters, similar patterns were observed in females at 100 µg/kg bw/day but generally, to a lesser extent for most parameters. Neutrophils were also slightly increased in high-dose males, but not in females.

In urinalysis, slightly smaller volumes of urine with slightly greater specific gravity were noted in males. The total output of sodium, potassium, and calcium was similar to controls. These changes were considered to be toxicological insignificant. No changes were observed in females.

Analysis of bone marrow smears revealed reduced numbers of early erythropoietic and myelopoietic cell types and increased late erythropoietic and myelopoietic cell types at 100 µg/kg bw/day. A slight but significant increase in mean spleen weights was recorded in males at 100 µg/kg bw/day and in females at 30 µg/kg bw/day and 100 µg/kg bw/day.

Histopathology findings were noted in bone marrow in femoral and sternum bone, spleen, liver, and kidney in all dose groups. The findings included dose-related hyperostosis and myelofibrosis in the femoral and sternum bone marrow. Also increased haematopoiesis was noted in the spleen, liver, and male femoral bone marrow. Generally, minor liver haematopoiesis occurred as foci in liver parenchyma in all dose groups without a clear dose-dependent trend for severity. A dose-dependent increase in the incidence and severity of femoral bone marrow haematopoiesis was only apparent in males. A dose-dependent increase in the incidence and severity of corticomedullary mineralization was observed only in females. The histopathology was consistent with the known pharmacological activity of prostaglandin F_{2a} analogues.

Conclusion

Under the conditions of the study, the LOAEL for the test substance was considered to be 10 µg/kg bw/day in rats based on histopathology findings in bone marrow in femoral and sternum bone, spleen, liver, and kidney in all dose groups. A NOAEL value could not be established.

(CDER, 2011; TGA, 2012)

2nd study: Subchronic toxicity study in dogs via IV route

Guideline:	EMA note for guidance on repeated dose toxicity (CPMP/SWP/1042/99)
Species/strain:	Beagle dogs
Number of animals:	4/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	FP-0002
Purity:	98.6%
Route:	Intravenous
Dose levels:	0, 0.1, 1, and 10 µg/kg bw/day
Dose volume:	1 mL/kg
Vehicle:	0.9% sodium chloride
Satellite group	None
Duration:	39 weeks
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the outcome or integrity of the study.
Study year:	2000

The subchronic toxicity of the analogue tafluprost (98.6% purity) was investigated for repeated dose toxicity according to the EMA note for guidance on repeated dose toxicity (CPMP/SWP/1042/99) (TGA, 2012). Beagle dogs (4/sex/group) were dosed daily via the intravenous route at doses of 0, 0.1, 1, and 10 µg/kg bw/day for 39 weeks. The stability of dosing solution was confirmed by analysis. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Ophthalmological examinations were performed in all animals' pre-treatment and in Weeks 13, 26, and 39. An indirect binocular ophthalmoscope was used to examine the eyelids, optic disc, tapedal and non-tapedal fundus, and retinal blood vessels. In addition, a pupillary light response examined in weeks 14, 27, 38/39 of the study for all animals. Electrocardiograms were performed on all animals. In addition, blood pressure, body temperature, respiratory rate measurements were made pre-treatment and before dosing and 5, 30 and 120 minutes after dosing in weeks 4, 8, 13, 26, and 39. Blood samples for haematological and clinical-chemical examination were collected from all animals before treatment, and in weeks 13, 26, and 39. For urinalysis, urine samples were collected overnight from all animals pre-treatment and in weeks 12, 26, and 38. At the termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

One high-dose male displaying multiple indications of hepatic failure was killed *in extremis* during Week 19 of treatment of the experiment. Several clinical signs were observed on an almost daily basis including salivation, emesis/retching, and pacing at 10 µg/kg bw/day. Emesis/retching and loose faeces were less frequently observed at 1 µg/kg bw/day. Other less frequent clinical signs included panting, subdued mood, vocalization, aggression, loose faeces, poor mobility, and vasodilation were observed at 10 µg/kg bw/day. Several clinical signs were observed in high-dose animals on an almost daily basis including salivation, emesis/retching, and pacing. Emesis/retching and loose faeces were

less frequently observed in dogs receiving 1 µg/kg/day. Clinical signs in single high-dose male killed *in extremis* included, inappetence, progressive body weight loss, thin appearance, sluggishness, and yellow coloration of the whole body.

No treatment-related changes in body weight, food consumption, body temperature, or urine parameters were noted.

No ocular changes were considered to be treatment-related other than dose-dependent changes in pupillary light response. Marked miosis was observed shortly after dosing during weeks 14, 27, and 39 at 10 µg/kg bw/day. Miosis was slight to moderate two hours after dosing in weeks 27 and 39 for most high-dose animals. Slight to moderate miosis occurred at 1 µg/kg bw/day after dosing in Weeks 14, 27, and 39. No changes were observed at a low dose level of 0.1 µg/kg bw/day.

In ECG examination, increased heart rate was observed in a dose-dependent manner at 5 and/or 30 minutes after dosing. As a consequence of increased heart rate, RR-intervals were slightly shortened. The QT values corrected for heart rate (QTc) were normal.

Slight but significant increased mean arterial blood pressure (MAP) was observed 30 minutes after dosing during weeks 4, 26, and 39 in females at 10 µg/kg bw/day. Also, MAP was significantly increased at a single time-point, 5 minutes after dosing in Week 26 in males at 1 and 10 µg/kg bw/day.

Respiratory rates were slightly but significantly elevated in animals at 30 minutes after dosing at 10 µg/kg bw/day. However, the rates were less elevated or similar to control, two hours after dosing indicating a transient effect.

No treatment-related changes in haematology and clinical chemistry parameters were observed in any of the animals except the high-dose male killed in extremis. For this animal, prolonged prothrombin, activated partial thromboplastin times and increased aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase and alkaline phosphatase activities and total bilirubin concentration were noted.

The primary gross pathology finding was reddening at injection site but not in a dose-dependent manner. No other treatment-related findings were observed except the high-dose male killed in extremis. For this animal, a small, firm, mottled liver, a small thymus, a slightly enlarged kidney and a discoloured intestinal tract were observed. The relative salivary gland weights were increased by 28 and 37% in males and females respectively compared to controls. No other treatment-related changes were observed.

In histopathology examination, minor adrenal cortical eosinophilia and acinar cell hypertrophy in salivary glands were observed primarily in males and females at 10 µg/kg bw/day. For the affected animals, the salivary gland acini tended to be larger due to an increased amount of normal appearing cytoplasm. No other treatment-related findings were observed except the high-dose male killed in extremis. For this animal, extensive histopathology occurred in the liver. The centrilobular cords were atrophic and consisted mainly of collapsed sinusoids with pigmented cells and few normal hepatocytes. The periportal region consisted of proliferating bile ducts variably surrounded by basophilic hypertrophic hepatocytes interspersed with necrotic cells and mitosis.

Conclusion

Under the conditions of the study, the NOAEL for the test substance tafluprost was established at 1 µg/kg bw/day in dogs based transient clinical signs of nausea, transient miosis, transient slight elevations in heart rate, blood pressure, and respiratory rate, enlarged salivary glands, and adrenal and salivary gland histopathology at 10 µg/kg bw/day.

(CDER, 2011; TGA, 2012)

Overall conclusion for repeated dose toxicity potential via the IV route

Subacute and subchronic toxicity studies in rats and dogs available for the close structural analogue tafluprost via the IV route revealed increased respiratory rate, altered cardiac parameters, hyperostosis and myelofibrosis in femoral and sternum bone marrow and haematopoiesis in spleen, liver and male femoral bone marrow, and increased corticomedullary mineralization of the kidney of females at a dose ≥10 µg/kg bw/day. The NOAELs ranged from 1 to 100 µg/kg bw/day. Due to the chemical and presumed toxicological similarity of DDDE to tafluprost, similar NOAELs can be established for DDDE.

3.3.5.2. Subcutaneous route

3.3.5.2.1 Subchronic toxicity

1st study: Sub-chronic dose range finding study for carcinogenicity study in mouse via SC route

Guideline:	Not specified
Species/strain:	Mice/Crl:CD-1(ICR)BR
Number of animals:	12/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Vehicle:	Not specified
Batch/Lot:	F01X010
Purity:	99.5%
Route:	Subcutaneous
Dose levels:	0, 3, 10, 30 and 100 µg/kg bw/day
Duration:	90 days
GLP:	Yes
Study year:	2003

The sub-chronic repeated dose toxicity of the analogue tafluprost (99.5% purity) was investigated in Crl:CD-1(ICR)BR mice following subcutaneous administration of 0, 3, 10, 30 and 100 µg/kg bw/day daily for 90 days. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Haematological and clinical chemistry parameters were examined. At the termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, clinical signs, or changes in body weight, food consumption, haematology parameters, clinical chemistry parameters, gross pathology, organ weight or histopathology were observed at any dose levels.

Conclusion

Under the conditions of the study, the NOAEL for the test substance tafluprost can be set at 100 µg/kg bw/day in mice.

(CDER, 2011; TGA, 2012)

2nd study: Sub-chronic dose range finding study for carcinogenicity study in rats via SC route

Guideline:	Not specified
Species/strain:	Rats/Crl:CD®(SD)IGSBR
Number of animals:	10/sex/group
Test substance:	Tafluprost
Product name:	AFP-168

Vehicle:	Not specified
Batch/Lot:	F01X010
Purity:	99.5%
Route:	Subcutaneous
Dose levels:	0, 3, 10, and 30 µg/kg bw/day
Duration:	90 days
GLP:	Yes
Study year:	2003

The sub-chronic repeated dose toxicity of the analogue tafluprost (99.5% purity) was investigated in Crl:CD®(SD)IGSBR rats following subcutaneous administration of 0, 3, 10, and 30 µg/kg bw/day daily for 90 days. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Haematological parameters, and clinical chemistry were assessed. At the termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

Only minor and/or dose-independent changes were noted for mortality. No treatment related effects on clinical signs, gross pathology, or changes in body weight, food consumption, haematology, clotting parameters, clinical chemistry, or organ weights were observed.

Histopathology in the spleen and kidney was considered to be treatment related. In the spleen, a minor increase in the incidence and severity of haematopoiesis in high-dose males and females compared to vehicle control animals was noted. In the kidney, a minor increase in the incidence and severity of corticomedullary mineralization in high-dose females was noted. However, the histopathological changes in spleen and kidney were not considered to be statistically significant.

Conclusion

Under the conditions of the study, the NOAEL for the test substance tafluprost was considered to be 30 µg/kg bw/day in rats.

(CDER, 2011; TGA, 2012)

See Section **3.3.8.3** for the details on the chronic/carcinogenicity studies via SC routes in rats and mice.

Overall conclusion for repeated dose toxicity potential via SC route

Following repeated **subcutaneous** administration of analogue tafluprost for 13 weeks in rats, produced only minor non-significant histopathological changes in spleen and kidney at 30 µg/kg bw/day. However, following chronic administration for 24 months in the carcinogenicity study, it revealed reduced body weight and histopathology changes including hyperostosis of the sternum and femur in some animals, and increased incidence of extramedullary haematopoiesis in the spleen at ≥3 µg/kg bw/day. No significant adverse effects were observed at any dose level in the dose range finder as well as chronic carcinogenicity studies in mice at doses up to 100 µg/kg bw/day study in mice (see **Section 3.3.8.3** for the details on the chronic/carcinogenicity studies). Based on the chronic studies, a NOAEL of <3 µg/kg bw/day is derived in rats and 100 µg/kg bw/day in mice. Due to the chemical and presumed toxicological similarity of DDDE to tafluprost, similar NOAELs can be established for DDDE.

3.3.5.3. Ocular route

3.3.5.3.1 Subacute toxicity

1st study: Subacute toxicity study in monkeys via the ocular route

Guideline:	Not specified
Species/strain:	Monkey/Not specified
Number of animals:	3/sex/group
Test substance:	Ophthalmic solution containing 0.0005-0.05% tafluprost
Product name:	AFP-168
Test concentrations:	0, 0.0005%, 0.005%, 0.05%
Batch:	Not specified
Purity:	Not specified
Route:	Topical ocular
Dose levels:	0, 0.15, 1.5 and 15 µg/left eye/time, twice daily (i.e., 0, 0.3, 3 and 30 µg/eye/day) Equivalent to 0, 0.067–0.1, 0.67–1 and 6.7–10 µg/kg bw/day[12]
Formulation/Vehicle:	Not specified
Duration:	28 days
GLP:	Yes
Study period/year:	Not specified (pre-2013)

The ocular toxicity of an ophthalmic solution of analogue tafluprost (purity not specified) was investigated in a subacute toxicity study in monkeys. Monkeys (3/sex/group) were exposed to ophthalmic solutions containing 0, 0.0005%, 0.005% and 0.05% tafluprost (i.e., equivalent to 0, 0.067–0.1, 0.67–1, 6.7–10 µg/kg bw/day) via the ocular route for 28 days. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Haematological parameters, clinical chemistry parameters, ECG parameters, blood pressure, electroretinogram parameters, macroscopic ocular findings and intraocular pressure were examined. At termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, clinical signs, changes in body weight, food consumption, macroscopic ocular findings, lens or vitreous body changes, or changes in intraocular pressure, electroretinogram parameters, or eye lash colour, ECG parameters, blood pressure, haematology parameters, clinical chemistry, gross pathology, organ weights or histopathology were noted.

Treatment-related changes were restricted to local changes in iris colour in two animals at 3 µg/eye/day (i.e., 0.67–1 µg/kg bw/day). In addition, transient corneal precipitates, anterior chamber cells, superficial corneal opacity and erosion, positive epithelial topical ocular fluorescein staining, and conjunctiva redness were occasionally observed at the two highest tested doses. The erosion, positive epithelial topical ocular fluorescein staining, and conjunctiva redness were also observed in vehicle control animals and untreated eyes and may have been related to corneal surface drying due to the anaesthesia.

Conclusion

Under the conditions of the study, the NOAEL for systemic toxicity was established at 30 µg/eye/day (i.e., 6.7–10 µg/kg bw/day) due to the lack of any systemic effects of tafluprost in monkeys.

[12] Reported in the Australian public assessment report (TGA, 2012)

(CDER, 2011; TGA, 2012)

3.3.5.3.2 Subchronic toxicity

1st study: Subchronic toxicity study in monkeys via the ocular route

Guideline:	Not specified
Species/strain:	Cynomolgus monkey
Number of animals:	4/sex/group
Test formulation:	Ophthalmic solution containing 0.0005-0.05% tafluprost
Test substance:	AFP-168
Test concentrations:	0, 0.0005%, 0.005%, 0.05%
Batch/Lot:	Not specified
Purity:	Not specified
Route:	Topical ocular
Dose levels:	0, 0.15, 1.5, 15 µg/left eye/time, twice daily (i.e., 0, 0.3, 3 and 30 µg/eye/day) Equivalent to 0, 0.075–0.1, 0.75–1 and 7.5–10 µg/kg bw/day[13]
Dose volume:	Not specified
Formulation/Vehicle:	The vehicle for the 0.05, 0.005% and 0.0005% AFP-168 ophthalmic solutions contained Tween 80, NaH_2PO_4 X H_2O, EDTA x 2Na, glycerine, 0.01 % benzalkonium chloride, and NaCl
Duration:	13 weeks
Recovery:	28 days
GLP:	Yes
Study period/year:	Not specified (pre-2013)

The ocular toxicity of an ophthalmic solution of analogue tafluprost (purity not specified) was investigated in a subchronic toxicity study in monkeys following dosing for 13 weeks. Monkeys (4/sex/group) were exposed to ophthalmic solutions containing 0, 0.0005%, 0.005% and 0.05% tafluprost (i.e., equivalent to 0, 0.075–0.1, 0.75–1 and 7.5–10 µg/kg bw/day) via ocular route for 13 weeks followed by 4-week recovery. The right eyes remained untreated. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Macroscopic ocular examination as well as fundus examination, slit lamp examination and fluorescein staining of the cornea, and IOP measurements were performed. Similarly, iridial colour, eyelash colour examinations, electroretinography (ERG), electrocardiograms (ECG) and blood pressure measurement were performed. Blood samples for haematological and clinical-chemical examination were collected from all animals. For urinalysis, urine samples were collected overnight from all animals. At termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, changes in body weight, food consumption, gross pathology, or histopathology, or changes in ECG, blood pressure, haematology parameters, clinical chemistry parameters, urinalysis, or organ weights were observed. No treatment-related effects in fundus and slit lamp examination or no changes in electroretinogram (ERG) parameters, or eyelash colour and ocular histopathology were observed.

Treatment-related findings in the treated eye included a reversible finding of sunken eyelids, slight punctuate fluorescein staining of the cornea, a tendency to reduce intraocular pressure, and irreversible iris colour darkening were observed at all dose levels. Changes in iris colour in animals

[13] Reported in the Australian public assessment report (TGA, 2012)

were not reversed following a 4-week treatment-free period. The ocular effects were considered to be mainly cosmetic, not associated with loss of function, and not toxicologically significant. These effects were however noted in the warnings and precautions of the product labels.

Conclusion

Under the conditions of the study, the local (ocular) and systemic NOAEL for the test substance tafluprost was established at 30 µg/eye/day (7.5–10 µg/kg bw/day).

(CDER, 2011; TGA, 2012)

2nd study: Subchronic toxicity study in monkeys via ocular route

Guideline:	Not specified
Species/strain:	Monkey/not specified
Number of animals:	3/sex/group
Test substance:	Ophthalmic solution containing 0.0045% tafluprost
Test concentrations:	0, 0.0045%
Batch/Lot:	100002-04
Purity:	102.4%
Route:	Ocular
Dose levels:	0, 1.35 µg/left eye/time, twice daily (i.e., 0, 2.7 µg/eye/day) Equivalent to 0, 0.75–0.9 µg/kg bw/day
Dose volume:	30 µL/eye/dose
Formulation/Vehicle:	Vehicle for DE-111 ophthalmic solution contained benzalkonium chloride with a pH of approximately 7.0, and an osmolar ratio of approximately 1)
Satellite group	None
Duration:	13 weeks
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the results or the validity and integrity of the study
Study year:	2010

The ocular toxicity of an ophthalmic solution of analogue tafluprost (purity: 102.4% purity) was investigated in a subchronic toxicity study in monkeys. Monkeys (3/sex/group) were exposed to ophthalmic solutions containing 0, 0.0045% tafluprost (i.e., equivalent to 0, 0.67–0.9 µg/kg bw/day) via the ocular route for 13 weeks. The right eyes remained untreated. The stability of the dosing solutions was analytically confirmed. During the treatment period, animals were observed for clinical signs, mortality and body weight at defined intervals. Macroscopic ocular examination as well as fundus examination, slit lamp examination and fluorescein staining of the cornea, and IOP measurements were performed once before dosing, and 2 to 6 hours after dosing in Weeks 4, 8 and 13. Similarly, iridial and eyelash colour examinations were performed before dosing, and 2 hours 26 minutes to 5 hours 13 minutes after dosing in weeks 4, 8, and 13. Electroretinography (ERG) was performed before dosing and during Week 13. Scotopic ERG, oscillatory potentials, 30 Hz flicker, and photopic ERG were measured. At the termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, changes in body weight, macroscopic ocular findings, fundus, or slit lamp examinations or changes in electroretinogram parameters, gross pathology, organ weights or histopathology were noted. Treatment-related changes in eye colour were observed in animals.

A slight tendency for decreased IOP was observed compared to the untreated eyes; however, the reduced IOP in the treated eyes fell within the normal variations. The iridial colour was found to be darkened relative to pre-dose colours in two male animals. The ocular effects were considered to be mainly cosmetic, not associated with loss of function, and not toxicologically significant. These effects were however noted in the warnings and precautions of the product labels.

Conclusion

Under the conditions of the study, the local (ocular) or systemic NOAEL for the test substance tafluprost was established at 2.7 µg/eye/day (0.67–0.9 µg/kg bw/day).

(CDER, 2011)

3.3.5.3.3 Chronic toxicity

1st study: Chronic toxicity study in monkeys via ocular route

Guideline:	EMA note for guidance on repeated dose toxicity (CPMP/SWP/1042/99)
Species/strain:	Monkey/not specified
Number of animals:	4/sex/group
Test substance:	Ophthalmic solution containing 0.0005-0.05% tafluprost
Product name:	AFP-168
Test concentrations:	0, 0.0005, 0.005 and 0.05%
Batch/Lot:	D01114, D01115, D01116
Purity:	98, 100, 102% respectively
Route:	Topical ocular
Dose levels:	0, 0.15, 1.5, 15 µg/left eye/time, twice daily (i.e., 0, 0.3, 3 and 30 µg/eye/day) Equivalent to 0, 0.075–0.1, 0.75–1 and 7.5–10 µg/kg bw/day[14]
Dose volume:	30 µL/eye/dose
Formulation/Vehicle:	The vehicle for the 0.05 (30 µg/eye/day), 0.005% (3 µg/eye/day) and 0.0005% (0.3 µg/eye/day) AFP-168 ophthalmic solutions contained Tween 80, NaH_2PO_4 X H_2O, EDTA x 2Na, glycerine, 0.01 % benzalkonium chloride, and NaCl.
Satellite group	None
Duration:	52 weeks
GLP:	Yes
Study deviation	Yes, study deviations were not considered to have altered the results and integrity of the study
Study year:	2001

The ocular toxicity of an ophthalmic solution of the analogue tafluprost (98-102% purity) was investigated in a chronic toxicity study in monkeys, according to the EMA note for guidance on repeated dose toxicity (CPMP/SWP/1042/99) (TGA, 2012). Monkeys (4/sex/group) were exposed ophthalmic solutions containing 0, 0.0005%, 0.005% and 0.05% tafluprost (i.e., equivalent to 0, 0.075–0.1, 0.75–1 and 7.5–10 µg/kg bw/day) via the ocular route for 52 weeks. The right eyes remained untreated. The stability of dosing solutions was analysed but no stability information was provided. During the treatment period, animals were observed for clinical signs, mortality body weight and food consumption at defined intervals. Macroscopic ocular examination as well as fundus examination, slit lamp examination and fluorescein staining of the cornea, and IOP measurements were performed once before dosing, and 2 to 6 hours after first dosing in weeks 13, 26, 39 and 52. Similarly, iridial and

[14] Reported in the Australian public assessment report (TGA, 2012)

eyelash colour examinations were performed once before dosing, and once after dosing in Weeks 13, 26, 39 and 52. ERG was performed once before dosing and during weeks 25 and 26, 29 or 30 , 51 or 52. Scotopic ERG, oscillatory potentials, 30 Hz flicker, and photopic ERG were measured. Electrocardiograms (EKG) were performed on all animals (unanaesthetised, temporarily restrained) once before treatment and in weeks 13, 26 and 52 (prior to and 15 minutes after the first dosing). Heart rates (beats/minute), ECG intervals, RR, P, PR, QRS, and QT intervals (QTc and Qt dispersion), as well as voltage measurement of P, R, S, and T were measured. Blood samples for haematological and clinical-chemical examination were collected from all animals once before treatment, and once in Weeks 13, 26, and 52. For urinalysis, urine samples were collected overnight from all animals once pre-treatment and once in Weeks 13, 26, and 52. At termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, changes in body weight, food consumption, fundus examination, pupil size, irritation score, corneal examination or changes in electroretinography, blood pressure, haematology, clinical chemistry, or urine parameters, were noted.

Only eye-related clinical signs were considered to be treatment-related. A sunken eye was noted at the two highest doses and darker iris colour was noted in all doses in at least 3 of 4 animals of each gender in the beginning as early as Day 23, and this change was permanent throughout the experiment. Four animals (three males and one female) demonstrated sunken eyes on single occasions. Many of the same animals demonstrated blue-grey discolouration of the lower eyelid. A statistically significant decrease in IOP occurred in males and females in the high-dose group during Week 26. The IOP of the treated eye remained lower compared to the untreated eye for the rest of the study, but not significant. Slight decreases in IOP also occurred in low and intermediate-dose groups during Week 26. The lower eyelid darkening was observed in the intermediate and high dose group.

In gross pathology, no treatment-related changes were noted other than iris darkening, sunken eye. No treatment-related changes in organ weights were noted. Significant increase in the group mean thymus/brain weight ratios relative to control animals were noted in two males of intermediate and high dose groups. However, for all other animals in these groups, non-statistical increases were noted in similar thymus/brain weight ratios and absolute thymus and thymus weight/body weight ratios.

No systemic organ histopathology was considered to be related to the treatment. Most of the animals in all dose groups demonstrated increased melanocyte pigment in the iris stroma of the left eye which is in line with the observed iris darkening. In addition, the treated eyes showed increased pigment around the hair follicles and minimal focal inflammation in the epithelium predominantly in high-dose males and females.

All the ocular effects were considered to be mainly cosmetic, not associated with loss of function, and not toxicologically significant.

Conclusion

Under the conditions of the study, the Local (ocular) and systemic NOAEL for the test substance tafluprost was established at 30 µg/eye/day (7.5–10 µg/kg bw/day).

(CDER, 2011; TGA, 2012)

Overall conclusion for repeated dose toxicity potential via the ocular route

Subacute, subchronic and chronic ocular dose toxicity studies in monkeys available for the ophthalmic solutions containing 0.0005-0.05% tafluprost (i.e., equivalent to 0.067 to 10 µg/kg bw/day) did not produce systemic toxicity up to the highest concentrations. As per the FDA report, *"these data strongly suggest that clinical administration of tafluprost by the topical ocular route is unlikely to cause*

systemic toxicity." (CDER, 2011; TGA, 2012). Due to the chemical and presumed toxicological similarity of DDDE to tafluprost, similar NOAELs can be established for DDDE (i.e., 10 µg/kg bw/day).

3.3.6. Reproductive and developmental toxicity

No reproductive and developmental toxicity studies could be identified for DDDE itself. Therefore, data available on the close structural analogue tafluprost is used for assessing this endpoint. The database for assessing reproductive and developmental toxicity comprises studies conducted in rats and rabbits by the intravenous route.

The overview of the reproductive and developmental toxicity studies with the analogue tafluprost are summarised in **Table 17**.

Table 17. Overview of reproductive and developmental toxicity studies with analogue tafluprost

Study type, Species	Doses	Key findings	NOAEL	Reference
Reproductive toxicity				
Reproductive toxicity, intravenous study, rats (Crl:CD(SD)IGSBR rats) (24/sex/dose)	0, 10, 30 and 100 µg/kg bw/day	No adverse effects at any dose level	NOAEL = 100 µg/kg bw/day	(CDER, 2011)
Developmental toxicity				
Dose range finding studies				
Prenatal developmental toxicity, intravenous study, rats (strain not specified) (7 females/dose)	0, 10, 30 and 100 µg/kg bw/day Gestation day (GD): Not specified	Pale extremities were observed immediately. after dosing in animals at 30 and 100 µg/kg bw/day A dose-dependent increase in the incidence of post-implantation loss in the intermediate (12.1%) and high-dose (49.6%) groups compared to controls (6.0%) with two high-dose animals having litters with no live foetuses.	NOAEL for maternal toxicity = 10 µg/kg bw/day* NOAEL for developmental toxicity = 10 µg/kg bw/day*	(CDER, 2011)
Prenatal and postnatal development toxicity, intravenous study, rats (strain not specified) (7 females/dose)	0, 0.3, 1, 3 and 10 µg/kg bw/day GD: Not specified.	Decreased maternal body weight gains at ≥3 µg/kg bw/day. The increased number of dead foetuses and reduced birth index and viability index on Day 4 of lactation in the animals at 3 and 10 µg/kg bw/day	NOAEL for maternal toxicity = 1 µg/kg bw/day* NOAEL for developmental toxicity = 1 µg/kg bw/day*	(CDER, 2011)
Prenatal developmental toxicity, intravenous study, rabbits (strain not specified) (7 females/dose)	0, 1, 3 and 10 µg/kg bw/day GD: Not specified.	Clinical signs include tremors, splayed legs and excessive licking immediately after dosing in animals at 10 µg/kg bw/day. Intrauterine deaths with no live foetuses in 2 females at 1 µg/kg bw/day, and no live	LOAEL for maternal toxicity = 1 µg/kg bw/day* LOAEL for developmental toxicity = 1 µg/kg bw/day*	(CDER, 2011)

Study type, Species	Doses	Key findings	NOAEL	Reference
		foetuses occurred in all the females at 3 and 10 µg/kg bw/day		
Main studies				
Prenatal developmental toxicity, intravenous study, rats (Crl:CD(SD)IGSBR) (24 females/dose)	0, 3, 10, and 30 µg/kg bw/day GD: 6-17	Increased number of intrauterine deaths reduced foetal maturity, and an incidence of defects of the vertebral column at 10 and 30 µg/kg bw/day.	NOAEL for maternal toxicity = 30 µg/kg bw/day NOAEL for developmental toxicity = 3 µg/kg bw/day	(CDER, 2011)
Prenatal and postnatal development toxicity, intravenous study, rats (Crl:CD(SD)IGSBR) (22 females/dose)	0, 0.3, 1, 3, and 10 µg/kg bw/day GD: 6 to lactation Day 20	Poor nursing behaviour in some dams at all dose levels. Poor nursing resulting in decreased F1 offspring viability at ≥ 1 µg/kg bw/day and delayed pinna unfolding, increased F1 newborn mortality and decreased body weight at 10 µg/kg bw/day	NOAEL for maternal toxicity = 10 µg/kg bw/day NOAEL for developmental toxicity (F1 generation) = 0.3 µg/kg bw/day **#Selected as the PoD study**	(CDER, 2011)
Prenatal developmental toxicity, intravenous study, rabbits (Crl.NZW/Kbl BR rabbits) (24 females/dose)	0, 0.03, 0.1 and 3 µg/kg bw/day GD: 1-19	Abortions in eleven and three animals at 0.1 and 3 µg/kg bw/day respectively compared to one control female. Significant increased early post-implantation loss at 0.1 and 3 µg/kg bw/day. No live foetuses in animals at 3 µg/kg bw/day and only two litters contained live foetuses at 0.1 µg/kg bw/day At 0.03 µg/kg bw/day, majority of litters viable but three foetuses in separate litters had abdominal wall malformations, and six foetuses in five litters had cranial and/or spinal malformations.	No NOAELs were established (see the below summary for details)	(CDER, 2011)
Prenatal developmental toxicity, intravenous study, rabbits (Crl.NZW/Kbl BR rabbits) (24 females/dose)	0, 0.001, 0.003 and 0.01 µg/kg bw/day GD: 7-19	No adverse effects were observed at any dose level	Maternal and developmental NOAEL = 0.01 µg/kg bw/day	(CDER, 2011)

** The NOAEL/LOAELs were not established in the source documents but was concluded based on the available details on the adverse effects in the underlying studies.*

*3.3.6.1 **Reproductive toxicity via the IV route***

Guideline:	Not specified
Species/strain:	Rats/Crl:CD(SD)IGSBR rats
Number of animals:	24/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	FP-0002
Purity:	98.6%
Route:	Intravenous
Dose levels:	0, 10, 30 and 100 µg/kg bw/day
Dose volume:	10 mL/kg
Formulation/Vehicle:	0.9% sodium chloride
Satellite groups:	No
Duration:	Two weeks before mating, throughout the mating period, and until Day 6 of gestation for the females or until necropsy in Week 9 of the treatment period for the males.
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the results or integrity of the study
Study year:	2001

The reproductive toxicity of the analogue tafluprost (98.6% purity) was investigated in rats via the IV route. Crl:CD(SD)IGSBR rats (24/sex/group) were dosed daily at 0, 10, 30 and 100 µg/kg bw/day via the intravenous route for two weeks before mating, throughout the mating period, and until Day 6 of gestation for the females or until necropsy in Week 9 of the treatment period for the males. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals.

The stage of oestrous was recorded for each female from 15 days prior to treatment until mating confirmation. The stages of oestrous were assessed via daily vaginal washings. Rats were paired on a 1:1 basis within each treatment group during the mating period. A vaginal copulatory plug or the presence of sperm in a vaginal washing confirmed positive evidence of mating. The day on which mating was confirmed was designated Day 0 of gestation. On Gestation Day (GD) 13, mated females were sacrificed and examined macroscopically. The ovaries and uteri were removed and examined. The pregnancy status, number of corpora lutea, number and intrauterine position of implantations with recording of live embryos, early intrauterine deaths, and late intrauterine deaths were recorded.

Rats were sacrificed and examined macroscopically for structural or pathological changes. The tissues included ovaries, uterus, cervix, vagina, pituitary, testes, epididymides, seminal vesicles, prostate, coagulation gland, and lesions from all adult animals were retained. The histopathology of reproductive organs from all control and high-dose animals were examined.

Results

No treatment-related changes in body weight, food consumption, fertility or histopathology were noted.

Mortality was found in 2 animals on Day 10 and 15 respectively at 100 µg/kg bw/day. These animals were found dead within one hour of dosing. The animals appeared unremarkable at necropsy. Treatment-related clinical signs including pale extremities were observed in all dose groups immediately after treatment and appeared to be of approximately equal severity in all groups. This effect was observed daily from the second day of treatment in the 100 µg/kg bw/day and from the third day of treatment for the 10 and 30 µg/kg bw/day groups. The effect was transient and disappeared after one hour.

No treatment-related effects were observed on the oestrous cycle except one control pairing, all males mated within the initial four days of mating. No treatment-related effects were observed on fertility parameters (Mating/Fertility Index, Corpora Lutea, Preimplantation Loss, etc.). The mating index was found to be 100% for all treatment and control groups. The fertility and fecundity indexes were 100, 91.7, 91.7, and 100% for both males and females in the control group, low, intermediate and high dose group respectively. On GD 13, no effects were observed on the outcome of the pregnancy and all pregnant females had live embryos. The mean number of corpora lutea and the mean number of implantations per female were similar to the controls. Similar or fewer losses in the mean pre-and post-implantation loss were observed in the treated animals when compared to the control animals.

In histopathology examination, no treatment-related changes in reproductive organs were observed. No treatment-related abnormalities were observed in any of the cell types present within the different stages of the spermatogenic cycle in testis staging.

Conclusion:

Under the conditions of the study, the NOAEL for reproduction/fertility of the test substance tafluprost was established at 100 µg/kg bw/day in rats based on the absence of toxicity.

(CDER, 2011; TGA, 2012)

Overall conclusion for reproductive toxicity potential

The available reproductive toxicity study with the analogue tafluprost via the intravenous route does not indicate any significant treatment-related effects on fertility or reproductive parameters up to a dose of 100 µg/kg bw/day in rats. Based on read across, similar absence of effects on reproduction is expected for DDDE up to a dose of 100 µg/kg bw/day.

3.3.6.2 Developmental toxicity

The key developmental toxicity studies in rats and rabbits available for the analogue tafluprost hare summarised below:

1st Study: Prenatal developmental toxicity study in rats via the IV route

Guideline:	Not specified
Species/strain:	Rats/Crl:CD(SD)IGSBR
Number of animals:	24 females/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	FP-0002
Purity:	98.6%
Route:	Intravenous
Dos/kg levels:	0, 3, 10 and 30 µg/kg bw/day
Dose volume:	10 mL/kg
Formulation/Vehicle:	0.9% sodium chloride
Basis of dose selection	Range-finding study
Satellite groups:	No
Duration:	12 days (GD: 6 to 17)
Termination:	GD 20
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the results or integrity of the study
Study year:	2001

The prenatal developmental toxicity of the analogue tafluprost (98.6% purity) was investigated in rats. Based on the findings in a preceding dose range-finding study, Crl:CD(SD)IGSBR rats (24 females/group) were dosed daily via the intravenous route at doses of 0, 3, 10 and 30 µg/kg bw/day during GD 6 to 17. The stability of dosing solutions was analysed and proven to be stable. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. At sacrifice (GD 20), the ovaries and uterus of each female were removed, and the parameters included pregnancy status, gravid uterus weight, and the number of corpora lutea were assessed. Also, the status of each implantation site was recorded (live, dead, early resorption or late resorption). Fully formed foetuses that appeared to have died shortly before necropsy were classified as dead foetuses. Each live foetus was removed, weighed, sexed, and examined for gross abnormalities. Approximately half of the foetuses in each litter were subjected to a dissection and examined for developmental abnormalities in internal organs and skeletal abnormalities.

Results

No treatment-related mortality, clinical signs, change in body weight and food consumption were noted in dams.

No maternal toxicity was observed at any dose level. The 20% mean post-implantation loss was greater in the high-dose group compared to the 8.6% loss in the vehicle control group. The mean number of corpora lutea and the mean number of implantations was similar to those of the controls in all dose groups. Total litter loss in two rats was noted at 30 µg/kg bw/day and one in the vehicle control group mainly due to late intrauterine deaths for all three females. An increased number of early and late intrauterine deaths increased post implantation loss and decreased mean numbers of foetuses were observed at 30 µg/kg bw/day. However, no statistically significant differences were observed except for the number of late intrauterine deaths.

Foetal weights were significantly decreased at 10 and 30 µg/kg bw/day compared to controls with mean foetal weights of 3.70, 3.59 and 3.89 g in 10, 30 µg/kg bw/day and control group respectively. Slight, but non-significant increased mean placental weight was noted. Visceral malformations included but were not limited to the additional structure in the eye, severe renal pelvic cavitation, severely distended left ureter, umbilical hernia, absent kidneys and ureters, and abnormal lung lobulation in all the dose groups. However, specific visceral malformations occurred in only one litter per dose group, different visceral malformations occurred with the different doses and the least number of malformations occurred in the high-dose group. The visceral malformations were not affected in terms of the number of foetuses or litters affected. These factors indicate that the visceral malformations were not treatment related.

Skeletal malformations occurred at 10 and 30 µg /kg bw/day. A greater number of variations of the lumbar centra, thoracic arches, and thoracic centra were observed at 30 µg/kg bw/day. Also, a dose-related significant increase in the numbers of litters with unossified 5th sternebrae was observed at 10 and 30 µg/kg bw/day. No effects occurred at the low dose (3 µg/kg bw/day).

Conclusion

Under the conditions of this study, the maternal NOAEL was established at 30 µg/kg bw/day based on the absence of significant toxicity. The developmental NOAEL was established at 3 µg/kg bw/day based on increased number of intrauterine deaths, reduced foetal maturity, and an incidence of defects of the vertebral column at 10 and 30 µg/kg bw/day.

(CDER, 2011; TGA, 2012)

2nd Study: Pre- and post-natal developmental toxicity study in rats via the IV route

Guideline:	Not specified
Species/strain:	Rats/Crj:CD(SD)IGS
Number of animals:	22 females/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	037010
Purity:	101.5%
Route:	Intravenous
Dose levels:	0, 0.3, 1, 3, and 10 µg/kg bw/day
Dose volume:	3 mL/kg
Formulation/Vehicle:	0.9% sodium chloride
Basis of dose selection	Range-finding study
Satellite groups:	No
Duration:	35 days (GD 6 to lactation Day (LD) 20)
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the results or integrity of the study
Study year:	2004

The pre and postnatal developmental toxicity of the analogue tafluprost (101.5% purity) was investigated in rats. Based on the findings in a preceding dose range finding study, Crj:CD(SD)IGS rats (22 females/group) were dosed daily via intravenous route at 0, 0.3, 1, 3, and 10 µg/kg bw/day during GD 6 to LD 20. The stability of dosing solutions was analysed and proven to be stable.

In F0 dams, mortality, clinical signs toxicity, body weights and body weight changes, food consumption, uterine content, necropsy findings were assessed. Delivery, gestation and nursing parameters were also assessed.

In F1 generation, mortality, clinical signs toxicity, change in body weights and body weight changes, food consumption, physical development, gross pathology, or necropsy findings were assessed. Neurological parameters and reproduction parameters were also evaluated.

In F2 generation, mortality, number of corpora lutea, implantations, live F2 embryos, or preimplantation loss were evaluated. However, body weights, male/female ratio were not assessed, and F2 foetuses were not externally evaluated for malformations.

Minimal deviations from the study protocol were noted. These deviations were not considered to have altered the study results or compromised the integrity of the study.

Results

In F0 dams, no treatment-related mortality, clinical signs, changes in body weight and food consumption were noted. No significant difference was observed in the number of implantation sites, total number of newborns, delivery index, or proportion of male live newborns compared to control. No abnormal gross pathology findings were observed at necropsy. Delayed delivery was noted in one female at 3 µg/kg bw/day. However, no abnormal delivery was noted in any dam. No significant differences were observed in the duration of gestation, gestation index, or delivery index between any of the treatment groups. Poor nursing behaviour was observed in one dam in 0.3, 3 and 10 µg/kg bw/day dose group and in 3 dams in 1 µg/kg bw/day dose group. Poor nursing behaviour resulted in mortality within 2 days after birth in offspring from 2 dams in each of the 1, 3 and 10 µg/kg bw/day dose groups.

In F1 generation, at birth, the number of dead newborns, and a lower birth index were not significantly increased in 10 µg/kg bw/day dose group compared to the control group. No treatment-related effects

were observed in the number of implantation sites, total number of newborns, delivery index, or male proportion of live newborns. During Lactation, the viability index on LD 4 was lower in the 1, 3 and 10 µg/kg bw/day dose groups compared to the control group with a significant reduction for the high-dose group. No treatment-related effects were observed in the weaning index.

The absence of milk in the stomach was observed in newborns at 1, 3 and 10 µg/kg bw/day. All newborns from 2 dams in each of these groups died within 2 days of birth. During the lactation period, mortality was observed in a few offspring among 2 to 3 dams in control and 0.3, 1, 3 µg/kg bw/day and in some of the offspring from 10 dams in the 10 µg/kg bw/day. After weaning, no abnormal clinical signs were evident in any offspring.

The body weight was significantly decreased in male and female newborns at 10 µg/kg bw/day. However, similar body weights were observed in the offspring in all groups after ≥ 4 days of age. A significant reduction in body weight was observed in 28 days old female offspring at 10 µg/kg bw/day. However, this effect was transient and therefore was not considered to be treatment related.

Growth retardation was observed in the high-dose group. The incidence of pinna unfolding at 3 days of age was statistically lower in the high-dose group (56.5%) compared to the control group (95.5%). This effect was associated with the low birth weights in the high-dose group. Approximately 100% incidence of pinna unfolding was observed at ≥ 4 days of age in all of the groups.

No significant difference was observed for the incidence of back righting or negative geotaxis before weaning compared to the control group. At post-weaning, all F1 offspring exhibited positive visual placing responses, pupillary reflexes, Preyer's reflexes and pain responses. No treatment-related gross pathology findings were noted in any male or female offspring culled at four days of age or in offspring at 21 days of age. A few no dose-related incidences including dilation of the renal pelvis were noted at both ages. Gross pathology was not evident at 10 weeks of age.

For reproduction parameters, no treatment-related changes were observed in preputial separation or vaginal opening. Also, for mating or the fertility index, no significant differences were observed between any dose groups and the control group.

In F2 generation, no treatment-related effects were observed for embryonic mortality, a number of corpora lutea, implantations, live F2 embryos, or preimplantation loss.

Conclusion

Under the conditions of this study, the NOAEL for maternal toxicity was established at 10 µg/kg bw/day based on the absence of treatment related effects in dams. The NOAEL for developmental toxicity was established at 0.3 µg/kg bw/day, based on decreased F1 offspring viability at ≥ 1 µg/kg bw/day and delayed pinna unfolding, increased F1 newborn mortality and decreased body weight at 10 µg/kg bw/day.

(CDER, 2011; TGA, 2012)

Note: The intravenous developmental NOAEL of 0.3 µg/kg bw/day from this study was considered in the present assessment as the PoD for MoS calculations.

3rd Study: Prenatal developmental toxicity study in rabbits via the IV route

Guideline:	Not specified
Species/strain:	Rabbits/Crl.NZW/Kbl BR
Number of animals:	24 females/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	FP-0002
Purity:	98.6%

Route:	Intravenous
Dose levels:	0, 0.03, 0.1 and 3 µg/kg bw/day
Dose volume:	1 mL/kg
Formulation/Vehicle:	0.9% sodium chloride
Basis of dose selection	Range-finding study
Satellite groups:	No
Duration:	19 days (GD 1 to 19)
Termination:	GD 29
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the results or integrity of the study
Study year:	2001

The prenatal developmental toxicity of the analogue tafluprost (98.6% purity) was investigated in rabbits. Based on the findings in a preceding dose range-finding study, Crl.NZW/Kbl BR rabbits (24 females/group) were dosed at 0, 0.03, 0.1 and 3 µg/kg bw/day daily via intravenous rout, during GD 1 to 19. The stability of dosing solutions was analysed and proven to be stable. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Surviving females were sacrificed on Day 29 after mating and examined for gross pathology. At sacrifice, the ovaries and uteri of each female were removed, and the parameters included pregnancy status, gravid uterus weight, number of corpora lutea, the number and intrauterine position of implantations subdivided into live foetuses, early intrauterine deaths, late intrauterine deaths, and dead foetuses were assessed. Live foetuses were sacrificed, and individual foetal and placental weights were recorded. Each live foetus was sexed and examined for gross abnormalities. The heads and hearts of approximately one-half of the foetuses in each litter were fixed for later examination. All foetuses were subjected to dissection and examined for developmental abnormalities in internal organs and skeletal abnormalities.

Results

No treatment-related changes in food consumption were noted. Animals were not found dead during the experiment. However, single animals in the vehicle control and the low dose groups, and eleven and three mothers in 0.1 and 3 µg/kg bw/day groups aborted their pregnancies between GD 17 and GD 23 and were euthanized prior to GD 29. The only clinical sign observed was red tissue and fluid under the cages of the mothers that aborted their pregnancies were observed.

A significant weight loss was observed between GD 12 and GD 15 only at 0.1 µg/kg bw/day. A slight weight loss occurred in the intermediate and high-dose animals between GD 7 and GD 10 and between GD 10 and GD 15 relative to the control animals. The mean gravid uterus weight was significantly decreased in the two intermediate-dose females with viable foetuses.

Treatment-related effects on pregnancy outcome parameters occurred in all dose groups. The mean number of implantations were significantly decreased in 10 and 13 females of the intermediate and high dose group respectively that had evidence of pregnancy on GD 29. The mean implantation values of 10.5, 7.9, and 7.8 were observed in the control, 0.1 and 3 µg/kg bw/day group respectively. In addition, in 8/10 intermediate-dose, and 13/13 high-dose females, all implantations died early in gestation without live foetuses. The mean incidences of post-implantation loss were significantly higher in the intermediate (91.6%) and high dose group (100%) compared to control females (13.1%). Also, the mean number of corpora lutea in the intermediate (3.6) and high dose (3.6) groups was significantly lower compared to control animals (11.5). The mean post-implantation loss in the low-dose group was 29.2% compared to 13.1% in control females with three low-dose females and one control female having total intrauterine death with no live foetuses. In low-dose females with live foetuses, the percentage of post-implantation loss (18.0%) was slightly higher than that of control females (8.7%) with the majority of intrauterine deaths occurring late in gestation.

No live foetuses were observed in the 3 µg/kg bw/day group. The mean foetal weight in the two litters of the intermediate-dose group was similar to controls. A slight but not significant increase in placental weight was observed. Also, the mean litter weight was reduced compared to controls due to the reduced number of live births and one litter contained only females and one litter was approximately equal for each gender.

The mean foetal, and placental weights, and the litter gender proportionality in the low dose group were similar to those of the controls. The reduced number of live foetuses was reflected by the mean litter weight. Foetal malformations were observed in nine foetuses from six mothers. Defects of the skull, brain, and/or spine and three had abdominal wall defects were observed in six affected foetuses. Overall, in the low-dose group, the number of foetal variations was similar to that of the controls, but the skeletal variations were more varied than in the control group and often associated with abnormalities. Visceral and/or skeletal variations were similar in incidence and type compared to the control litters in the two intermediate-dose litters with surviving foetuses.

Conclusion

Under the conditions of this study, abortions, reduced body weight and/or uterus weight were observed in dams at ≥0.1 µg/kg bw/day. However, due to high post implantation loss observed at mid and high doses and the lack of details on statistical significance, the abdominal wall malformations and cranial and/or spinal malformations observed at the low dose (0.03 µg/kg bw/day) could not be assessed further (see the below note).

(CDER, 2011; TGA, 2012)

Note: The study is considered to be of questionable reliability due to the following reasons:

- Dosing started from GD1, which is not as per the standard guidelines from EMA or US-CDER/CBER, and is very likely the reason for the high implantation loss observed in the study[15];
- High implantation loss, led to low number of foetuses, which did not allow any further evaluation of the developmental parameters or establishment of dose response relationship;
- Absence of reporting of statistical significance of the effects observed at the lowest tested dose;
- Absence of reporting of historical control data.

Due to these limitations, the above study was considered to be of questionable reliability and was therefore not considered further for risk assessment purposes.

4th Study: Prenatal developmental toxicity study in rabbits via the IV route

Guideline:	Not specified
Species/strain:	Rabbits/Crl.NZW/Kbl BR
Number of animals:	24 females/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	FP-0002
Purity:	98.6%
Route:	Intravenous
Dose levels:	0, 0.001, 0.003 and 0.01 µg/kg bw/day
Dose volume:	Control: 0.1 mL/kg Low dose: 0.01 mL/kg Intermediate dose: 0.03 mL/kg High dose: 0.1 mL/kg

[15] Dosing during the initial days post fertilisation is to be avoided as this corresponds to a very sensitive stage involving the transport of the zygote and implantation of the embryo in the endometrium of the uterus. Any disturbance by handling, dose application or toxic effects by substances during this period may lead to a very high rate of pre-implantation loss up to total loss.

Formulation/Vehicle:	0.9% sodium chloride
Basis of dose selection	Range-finding study
Satellite groups:	3 pregnant females/group for low, intermediate, and high dose group
Duration:	13 days (GD: 7 to 19)
Termination:	GD 29
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the results or integrity of the study
Study year:	2002

A second intravenous prenatal developmental toxicity study with the analogue tafluprost (98.6% purity) was conducted in rabbits. Based on the findings from the first rabbit study, 24 females/group were dosed at 0, 0.001, 0.003 and 0.01 µg/kg bw/day via intravenous route, during GD 7 to 19. The stability of dosing solutions was analysed and proven to be stable. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Surviving females were sacrificed on Day 29 after mating and examined for gross pathology. At sacrifice, the ovaries and uteri of each female were removed, and the parameters included pregnancy status, gravid uterus weight, number of corpora lutea, the number and intrauterine position of implantations, early intrauterine deaths, late intrauterine deaths, and dead foetuses were assessed. Live foetuses were sacrificed, and individual foetal and placental weights were recorded. Each live foetus was sexed and examined for gross abnormalities. The heads and hearts of approximately one-half of the foetuses in each litter were fixed for later examination. All foetuses were subjected to dissection and examined for developmental abnormalities in internal organs and skeletal abnormalities.

Multiple deviations from the study protocol were noted but the deviations were not considered to have altered the study results or compromised the integrity of the study.

Results

No treatment-related mortality, changes in body weight and mean gravid uterus weight were noted.

Clinical signs included distended urinary bladders noted upon euthanasia and necropsy in all of the animals that were euthanized due to very low food consumption appeared thin, and of these animals one control female and three intermediate-dose females. In the surviving animals, only one high-dose female appeared thin between GD 25 and GD 29 attributable to weight loss and reduced food intake during this period. Also, the pale and mottled liver was observed at the necropsy of this animal. The food intake was found to be very low from GD 4 in three control and four intermediate-dose animals and these animals were euthanized. However, this effect was not considered related to be treatment related.

No treatment-related effects were observed in a mean number of foetuses, and the group mean pre- and post-implantation loss, mean foetal weight, litter weight and placental weights. Individual malformation was not significantly increased in any of the dose groups compared to the control group. Generally, a similar number of foetuses with external, visceral, and skeletal variations were observed in all dose groups compared to the control group. The statistically non-significant increased number of foetuses with abnormally pale contents in the gall bladder and/or non-eruption of the incisors were observed in all dose groups.

Conclusion

Under the conditions of this study, the maternal and developmental NOAEL was established at 0.01 µg/kg bw/day in rabbits.

(CDER, 2011; TGA, 2012)

Overall conclusion for developmental toxicity potential

The available developmental toxicity studies with the analogue tafluprost in rats and rabbits via the intravenous route, showed effects on development (such as intrauterine death and decreased viability) and teratogenicity (such as defects in vertebral column and delayed pinna unfolding), in the absence of maternal toxicity. The maternal NOAELs based on the studies in rats ranged from 10 to 30 µg/kg bw/day, while the developmental NOAELs ranged from 0.3 to 3 µg/kg bw/day. Out of the two studies in rabbits, one was considered to be of limited quality, while in the other study, tafluprost was tested at very low doses leading to a no-observed-effect level (NOEL) of 0.01 µg/kg bw/day for maternal and developmental and teratogenic effects. Due to the chemical and presumed toxicological similarity of DDDE to tafluprost, similar NOAELs can be established for DDDE.

3.3.7. Mutagenicity/Genotoxicity

One guideline compliant *in vitro* Ames test as well as an *in vitro* micronucleus test (MNT) is available for DDDE.

3.3.7.1 Mutagenicity / Genotoxicity *in vitro*

1st study: *In vitro* Bacterial Reverse Mutation Test (Ames)

Guideline:	OECD Guideline 471
Test system:	*Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, *Escherichia coli* WP2 uvrA (pKM101)
Replicates:	Triplicate
Test substance:	DDDE (neat oil)
Vehicle:	DMSO
Batch:	TAF-10-1122-01
Purity:	99.78%
Test concentrations:	**Experiment I:** 3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate (TA100) 31.6, 100, 316, 1000, 2500 and 5000 µg/plate (TA98, TA1535, TA1537, E. coli WP2 uvrA (pKM101)) **Experiment II:** 3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate (TA100, TA1535 [without S9-mix]) 31.6, 100, 316, 1000, 2500 and 5000 µg/plate (TA98, TA1535 [with S9-mix], TA1537, E. coli WP2 uvrA (pKM101))
Negative control:	Purified water
Positive control:	4-nitro-o-phenylene-diamine, methylmethanesulfonate sodium azide (without S9-mix); 2-aminoanthracene (with S9-mix)
GLP:	Yes
Study period:	2023

The mutagenicity potential was evaluated for the test substance DDDE in an *in vitro* bacterial reverse mutation assay, according to OECD Guideline 471 in compliance with GLP. In this assay, the test substance DDDE was tested according to the plate incorporation method (experiment I) and the pre-incubation method (experiment II) using Salmonella typhimurium strains TA98, TA100, TA1535, TA1537 and tester strain E. coli WP2 uvrA (pKM101). The test concentrations ranged from 3.16 to 5000 µg/plate in both experiment I and II.

Results

No precipitation of the test substance was observed in any tester strain used in experiment I and II (with and without S9-mix).

In experiment I, toxic effects of the test substance were observed in tester strains TA98, TA100 and TA1535 (with and without metabolic activation) and in tester strain E. coli WP2 uvrA (pKM101) (without metabolic activation) at concentrations of 2500 µg/plate and higher. In experiment II, toxic effects of the test substance were noted at concentrations of ≥316 µg/plate (with and without S9-mix), depending on the particular tester strain (i.e., at ≥1000 µg/plate in the strain TA 98; ≥316 µg/plate in the strain TA 100 and TA1535; ≥2500 µg/plate in the strains TA 1537 and E. coli WP2 uvrA)

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with the substance at any concentration level, neither in the presence nor absence of metabolic activation in experiments I and II.

Overall, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Conclusion

Under the conditions of the study, DDDE was not mutagenic in the bacterial reverse mutation assay (Ames test), neither in the presence nor absence of metabolic activation.

(Klock, 2023b)

2st study: *In vitro* Micronucleus Test (MNT)

Guideline:	OECD Guideline 487
Test system:	Human lymphocytes
Replicates:	Duplicate
Test substance:	DDDE (neat oil)
Vehicle:	DMSO
Batch:	TAF-10-1122-01
Purity:	99.78%
Test concentrations:	**Pre-Experiment:** 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 1500, 2000 µg/mL (with and without S9-mix) **Experiment I:** 100, 250, 300, 325, 350, 375, 400, 425, 450 and 500 µg/mL (with and without S9-mix) **Experiment II:** 25, 50, 100, 150, 200, 250, 300 and 350 µg/mL (without S9-mix)
Main experiment (microscopic analysis)	Experiment I (short-term exposure- 4 hours): 250, 325 and 350 µg/mL (without S9-mix) 100, 250 and 300 µg/mL (with S9-mix) Experiment II (long-term exposure- 44 hours): 25, 50 and 100 µg/mL (without S9-mix)
Negative control:	Cell culture medium
Positive control:	Methylmethanesulfonate (MMS) and colchicine (without S9-mix) Cyclophosphamide (CPA) (with S9-mix)
GLP:	Yes
Study period:	2023

The genotoxicity of the test substance DDDE was evaluated in an OECD Guideline 487 and GLP-compliant *in vitro* MNT using human lymphocytes. In this assay, the test substance, dissolved in DMSO, was tested in the presence and absence of metabolic activation (i.e., phenobarbital/β-naphthoflavone-induced rats' liver S9-mix). The selection of the concentrations was based on data from the pre-experiment. In the main experiment, the test concentrations in experiment I with 4 h short-term exposure were 250, 325 and 350 µg/mL, without S9 mix and 100, 250 and 300 µg/mL, with S9 mix. In experiment II, with 44 h long-term exposure, the test concentrations were 25, 50 and 100 µg/mL, without S9 mix.

Methylmethanesulfonate (50 and 65 µg/mL) and cyclophosphamide (15 µg/mL) were used as clastogenic controls. Colchicine (0.02 and 0.4 µg/mL) was used as aneugenic control. A solvent control (DMSO) was also included in the test.

Results

No precipitation of the test substance was observed up to the highest concentration used in experiments I (with and without S9-mix) and II (without S9-mix).

In experiment I (without S9-mix), no increase of the cytostasis above 30% was noted up to 250 µg/mL. At 325 and 350 µg/mL, a cytostasis of 37 and 55%, respectively, was observed. In experiment I (with S9-mix), no increase of the cytostasis above 30% was noted up to 100 µg/mL. At 250 and 300 µg/mL, a cytostasis of 44 and 59%, respectively, was observed.

In experiment II (without S9-mix), no increase of the cytostasis above 30% was noted up to 50 µg/mL. At 100 µg/mL, a cytostasis of 66% was observed.

The numbers of micronucleated cells were within the historical control limits of the solvent control, and they did not show a biologically relevant increase compared to the concurrent solvent control in both experiments I and II.

The micronucleated cell frequency of the negative and solvent control was also within the historical control limits. Clastogenic (methylmethanesulfonate, cyclophosphamide) and aneugenic positive controls (colchicine) induced distinct and statistically significant increases in the micronucleus frequency, demonstrating the validity of the assay.

Overall, treatment with test substance DDDE resulted did not result in a statistically significant increase in the number of cells with micronuclei compared to concurrent solvent controls in experiments I and II, both in the presence and absence of an S9-mix.

Conclusion

Under the conditions of the study, the test substance did not induce structural and/or numerical chromosomal damage in human lymphocytes.

(Klock, 2023c)

Overall conclusion on the genotoxicity potential of DDDE

Based on the absence of mutagenic response in the Ames test and clastogenic response in the *in vitro* MNT assays conducted with neat DDDE, no genotoxic concern is considered for DDDE. This is further supported by the absence of genotoxicity observed for the analogue tafluprost in an Ames test, *in vitro* chromosomal aberration assay and *in vivo* MNT assay in mice (CDER, 2011).

3.3.8. Carcinogenicity

No carcinogenicity studies could be identified for DDDE. Therefore, the endpoint was assessed based on (Q)SAR analysis and data available on the structural analogue tafluprost.

Two complementary (Q)SAR models, an expert rule-based and a statistical-based model, were used to evaluated DDDE's carcinogenicity potential.

3.3.8.1 SAR analysis

The SAR analysis was conducted using the OECD QSAR Toolbox v.4.6 (OECD, 2023) and the expert rule-based tool, Derek Nexus tool from Lhasa (Barber *et al.*, 2015) v.6.2.1. As summarized in **Table 18**, DDDE does not present any structural alert indicating a potential for carcinogenicity.

Table 18. SAR analysis of DDDE

SAR tool	Profiler/Endpoint	Results
OECD QSAR Toolbox v.4.6	Carcinogenicity (genotox and non-genotox) alerts by ISS (v.2.7)	No alert found
	Oncologic Primary Classification (v.4.3)	Not classified
Derek Nexus v.6.2.1	Bladder urothelial hyperplasia	No alert
	Carcinogenicity	No alert
	Photocarcinogenicity	No alert

3.3.8.2. QSAR analysis

Statistically-based QSAR analysis was conducted using the carcinogenicity models of the VEGA platform (Benfenati *et al.*, 2013) v. 1.2 and the 'liver specific cancer (rat/mouse *in vivo*)' of the Danish (Q)SAR models platform (Danish (Q)SAR Database, 2023).

As shown in **Table 19**, the predictions by the VEGA v.1.2 and the Danish (Q)SAR models are not considered reliable as all predictions are outside the applicability domain for most of the criteria set by each model, and the expert analysis of the nearest neighbours of the training set does not indicate an adequate similarity to DDDE.

Table 19. QSAR analysis of DDDE

QSAR platform	Model	Results
VEGA v.1.2	Carcinogenicity model CAESAR v.2.1.10	Positive - Outside the applicability domain
	Carcinogenicity model (ISS) 1.0.3	Negative - Outside the applicability domain
	Carcinogenicity model (IRFMN-ISSCAN-CGX) 1.0.1	Positive - Outside the applicability domain
	Carcinogenicity model (IRFMN-Antares) 1.0.1	Positive - Outside the applicability domain*
	Carcinogenicity oral classification model (IRFMN) 1.0.1	Negative - Outside the applicability domain*
	Carcinogenicity inhalation classification model (IRFMN) 1.0.1	Negative - Outside the applicability domain*
Danish (Q)SAR models	Liver specific cancer (rat/mouse *in vivo*)	Inconclusive - Outside the applicability domain

* Based on expert judgement

Based on the experience acquired so far, the current QSAR models for carcinogenicity are not powered enough to provide reliable results.

Therefore, the carcinogenicity data available on the analogue tafluprost was used additionally for assessing this endpoint.

3.3.8.3. Carcinogenicity data for the analogue tafluprost

Two guideline-compliant carcinogenicity studies via the SC route in mice and rats are available for the carcinogenicity assessment of analogue tafluprost.

1st study: 18-month carcinogenicity study in mouse via the SC route

Guideline:	EMA note for guidance on carcinogenic potential (CPMP/SWP/2877/00) (TGA, 2012)
Species/strain:	Mice/Crl:CD-1(ICR)BR
Number of animals:	51/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	F01X010
Purity:	100.7%
Route:	Subcutaneous
Dose levels:	0, 10, 30 and 100 µg/kg bw/day
Dose volume:	10 mL/kg
Formulation/Vehicle:	The stock AFP-168 solution was 0.0015% AFP-168 dissolved in the vehicle (0.9% saline)
Basis of dose selection	13-week range-finding study
Satellite groups:	Yes; 18 animals/sex/group for low dose group and 26 animals/sex/group for intermediate and high dose group respectively for toxicokinetic analysis:
Dual control:	Yes, two saline groups
Interim sacrifice:	No
Duration:	18 months
GLP:	Yes
Study deviation:	Yes, study deviations were not considered to have altered the results. or integrity of the study
Study year:	2004

The carcinogenicity study of tafluprost (100.7% purity) was investigated in mice according to the EMA note for guidance on carcinogenic potential (CPMP/SWP/2877/00) (TGA, 2012). Based on the findings in a 13-week dose range finding study, Crl:CD-1(ICR)BR mice (51/sex/group) were dosed daily via the subcutaneous route at 0, 10, 30 and 100 µg/kg bw/day for 18 months. The dual control groups were used. The stability of dosing solutions was analytically confirmed. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. The moribund animals were sacrificed, and full necropsy were performed. Blood samples for haematological examination were collected from unfasted animals at the terminal sacrifice. At termination of the treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

No treatment-related mortality, clinical signs, haematology parameters, gross pathology or non-neoplastic histopathology were noted. In neoplastic histopathological evaluation, a non-significant increased incidence rates for neoplastic lesions were observed in the high-dose group when compared to the vehicle control groups.

Conclusion

Under the study conditions, the NOAEL for systemic toxicity and carcinogenicity of the test substance was set at the highest tested dose of 100 µg/kg bw/day.

(CDER, 2011; TGA, 2012)

2nd study: 2-years carcinogenicity study in rats

Guideline:	EMA note for guidance on carcinogenic potential (CPMP/SWP/2877/00)
Species/strain:	Rats/Crj:CD(SD)IGSBR
Number of animals:	60/sex/group
Test substance:	Tafluprost
Product name:	AFP-168
Batch/Lot:	037010
Purity:	101.5%
Route:	Subcutaneous
Dose levels:	0, 3, 9 and 30 µg/kg bw/day
Dose volume:	3 mL/kg
Formulation/Vehicle:	The AFP 168 stock solution was 0.0015% AFP-168 dissolved in the vehicle (isotonic sodium chloride solution).
Basis of dose selection	13-week range-finding study
Satellite group	Yes; 12 animals/sex/group for toxicokinetic analysis:
Dual control	Yes, two saline groups
Interim sacrifice	No
Duration:	2 years
GLP:	Yes
Study deviation	Yes, study deviations were not considered to have altered the results or integrity of the study
Study year:	2003

The carcinogenicity of the analogue tafluprost (101.5% purity) was investigated in a study according to the EMA note for guidance on carcinogenic potential (CPMP/SWP/2877/00) (TGA, 2012). On the basis of the findings in a 13-week dose range finding study, Crj:CD(SD)IGSBR rats (60/sex/group) were dosed daily via subcutaneous route at 0, 3, 9 and 30 µg/kg bw/day for 2 years. The dual control groups were used. The stability of dosing solutions was analysed and proven to be stable. During the treatment period, animals were observed for clinical signs, mortality, body weight and food consumption at defined intervals. Animals that died during the study were necropsied and examined for gross pathology as soon as possible. Blood samples were obtained, when possible, from moribund animals, before the animals were euthanized. For both the animals that died and the moribund animals, following necropsy the organs were weighed and histopathology sections were prepared. Blood samples for haematological examination were collected from animals one day after the termination of dosing. At termination of treatment, all animals were sacrificed and macroscopically examined, organs were weighed, and comprehensive histopathology was performed.

Results

Only minor and/or dose-independent changes in mortality, clinical signs, and food consumption were noted. Significant decreases in body weight were noted in male and female animals at 30 µg/kg bw/day. Treatment-related significant changes in haematology parameters included decreased red blood cells and increased white blood cells in male animals at 30 µg/kg bw/day. However, the change in RBCs was considered to be related to hyperostosis and an associated decrease in bone marrow cavity volume. Increased white blood cell counts were noted in males in the 30 µg/kg bw/day group. This changed was driven by extremely high values in two males suffering from leukaemia. However, overall, the incidence of leukaemia determined through histopathology was not increased in the 30 µg/kg group compared to the vehicle control groups.

Gross pathology did not show presence of any statistically significant or treatment related lesions in any organs.

Absolute and relative spleen weights were increased significantly in males at 30 µg/kg bw/day. The increased spleen weights were influenced due to leukaemia in one male and more severe extramedullary haematopoiesis in males at 30 µg/kg bw/day. Also, absolute, and relative adrenal weights were increased significantly in males at 30 µg/kg bw/day. However, no correlation was observed with histopathological evidence of adrenal tumours in any dose groups.

The relative but not absolute weights of kidney liver, lungs, heart, and brain were significantly increased in males and/or females at 30 µg/kg bw/day. However, these relative organ weight changes were considered attributable to decreased body weight in the high-dose group and not considered to be a direct treatment-related effect. Other weight changes included decreased absolute brain weight and increased relative brain weight high-dose group animals were also considered to be attributed to decreased body weight.

In histopathological evaluation, none of the neoplastic lesions in any of the dose groups was considered to be treatment related. Some significant differences were noted for the incidence of adenocarcinoma and adenoma in the pars distalis of the pituitary between male animals in one or both of the vehicle control groups and one or more of the dose groups. Also, a significant increase in the specific mammary gland and uterine tumours in female rats in one or more dose groups compared to one of the vehicle control groups were noted. In general, no dose-related pattern was observed in tumour incidence changes and in some instances, lower tumour incidences were observed in the treatment groups compared to the controls. These comparisons suggest that the pituitary gland, mammary gland, or uterine tumour incidence changes were toxicologically insignificant. Overall, no treatment-related biologically significant changes in the incidence of neoplastic lesions were observed.

Treatment-related non-neoplastic histopathological changes were observed which included hyperostosis of the sternum and femur in some animals and increased incidence of extramedullary haematopoiesis in the spleen in males of all dose groups. This change was attributable to a decreased volume of the bone marrow cavity due to hyperostosis.

Conclusion

Under the study conditions, the NOAEL for carcinogenicity was set at 30 µg/kg bw/day. Taking into consideration the non-neoplastic histopathology changes in sternum and femur in some animals and increased incidence of extramedullary haematopoiesis in the spleen in males at all dose levels, the LOAEL for systemic toxicity of the test substance can be considered at 3 µg/kg bw/day.

(CDER, 2011; TGA, 2012)

Overall conclusion on carcinogenicity potential of DDDE

The absence of genotoxicity and structural alerts for carcinogenicity using *in silico* tools together with the absence of significant treatment-related tumorigenic potential of the analogue tafluprost in two chronic carcinogenicity assays in mice and rats, indicates that there are no carcinogenicity concerns for DDDE at its intended use concentration of 0.018%.

3.3.9. Photoinduced toxicity

No publicly available photoinduced toxicity studies on DDDE could be identified. However, a recently conducted OECD Guideline 101 UV/VIS study with DDDE (neat oil) revealed an absorption band in the range 210 – 240 nm with maximum absorption at 226 nm and an absorption band in the range 250 – 285 nm with three maxima at 265 nm, 258 nm and 276 nm. The molar extinction coefficients (MCEs) were in the range 1046.2 to 1306.1 L*Mol-1cm-1 for the three maxima.

As the MCE results are above the cut-off of >1000 L/mol-1cm-1, a photo-reactivity potential cannot be entirely ruled out. However, considering the maximum absorbance wavelength cut-off, which is below 313 nm, additional *in vitro* phototoxicity testing is not required as per the SCCS NoG (SCCS, 2023).

(Johannes, 2023)

3.3.10. Human data

Clinical studies

The skin irritation and sensitisation potential of DDDE was evaluated in clinical studies. Under the test conditions, the eyelash cosmetic products containing up to 0.025% DDDE, were neither irritating nor sensitising to skin.

The eye irritation potential of DDDE was evaluated in clinical studies. Under the test conditions, eyelash cosmetic products containing up to 0.025% DDDE were not irritating to eyes.

For the study details, refer to **Sections 3.3.3 and 3.3.4.**

Clinical trials data on tafluprost – Ophthalmic solution

The safety of test substance tafluprost (0.001%-0.005% ophthalmic solution) was assessed in phases I, II and III clinical trials, in compliance with GCP and regulatory requirements. The phase II and phase III clinical trials performed in a masked way are the most important studies for assessing safety. The subjects received at least one dose of tafluprost eyedrop at a concentration of 0.0015% concentration, directly to the surface of the eye. The studies assessed the preservative-containing and/or preservative-free formulation. Long-term data is derived from Study 15-003 over 12 months and Study 74458 over 24 months (see **Table 20**).

Subjects were observed for adverse events (AE), laboratory evaluations (blood chemistry, haematology, and urinalysis) and vital signs assessment. In addition, specific ocular safety assessments were conducted. The status of the cornea, conjunctiva, iris, lens, vitreous and the retina were investigated routinely by recording visual acuity, visual fields, slit-lamp microscopy and ophthalmoscopy. Conjunctival hyperaemia was examined using standard photos as reference. Iris, eyelash and eyelid changes were assessed from photos by an independent, masked assessor. Microscopy in selected centres was used to assess corneal endothelial cells in Study 74458 and 15-003. Aqueous flare was measured in selected centres in Study 74458 (see **Table 20**).

Results

In Phase I clinical trials (Study 15005 and 77551), the most prevalent adverse events (AEs) were ocular redness and ocular hyperaemia. There were neither serious adverse events nor premature

discontinuations due to adverse events. No unexpected findings were detected in the ocular safety variables or systemic safety variables. Formulations were well tolerated and safe (see **Table 21**).

In Phase II/III trials (15002, 74458, 15003, 74460 and 77550), AEs occurred in 484/724 (66.9%) patients treated with tafluprost and ocular AEs in 343/724 (47.4%) subjects. The most common AE in patients treated with 0.0015% tafluprost was ocular/conjunctival hyperaemia, reported in 16% of subjects, followed by eye pruritus (7.5%), eye irritation (6.8%), eye pain (5.9%), growth of eye lashes (4.1%), visual field defects (4.1%), dry eye (3.9%) and blurred vision (3.3%). Most ocular AEs (77%) were considered by the investigators to be related to study medication (see **Table 20**).

Systemic adverse events included headache was the most commonly reported at 6.9%, followed by nasopharyngitis (6.6%), cough (4.0%), and hypertension (4.0%). No marked effects were observed on blood pressure, heart rate or laboratory examinations (see **Table 20**).

Conclusion

The results of the clinical trials support the clinical safety and efficacy of tafluprost eyedrops for the treatment of elevated intraocular pressure in patients with open-angle glaucoma or ocular hypertension.

In addition, the data from the clinical trials did not reveal any critical adverse reactions with regards to safety of the product.

Overall, the administration of tafluprost eyedrops directly onto the surface of the eye, appeared to be well tolerated despite the high incidence of ocular AEs. The study subject's discontinuation due to an AE and an ocular AE was low (i.e., 3% and 2% of subjects respectively).

(CDER, 2012; TGA, 2012)

Table 20. Clinical safety studies with tafluprost

Clinical trial phase	Dose / duration / No. of volunteers	Study details	Adverse events (AEs)	Reference
Phase II	**Dose:** 0.15 mL of 0.001, 0.0025, 0.005% (i.e., 0.025, 0.0625, 0.125 µg/kg bw/day) **Duration:** 28 days **No. of volunteers:** 152 patients	Study 15-001 was a prospective, multi-center, double-masked, parallel group, randomized, dose ranging trial designed to investigate the dose-response relationship of preservative-containing (PC) tafluprost in patients with open-angle glaucoma or ocular hypertension and to compare the safety and efficacy of three concentrations of PC tafluprost (0.001, 0.0025, 0.005%) with placebo (vehicle) and latanoprost 0.005%. A total of **152** patients were enrolled and 142 completed the study. Patients received masked study medication for **28 days**. Intra ocular pressure (IOP) was measured at defined intervals.	**Ocular effects:** The incidence of ocular adverse events (AEs) was 40.0% with 0.001% tafluprost, 50% with 0.0025%, and 43.0% with 0.005% compared to 16.7% with placebo and 40.0% with latanoprost. **Systemic effects:** No systemic effects were reported.	(CDER, 2012; TGA, 2012)
Phase II	**Dose:** 0.0003, 0.0015, 0.0025% (i.e., 0.0075, 0.0625, 0.125 µg/kg bw/day) **Duration:** 28 days **No. of volunteers:** 144 patients	Study 15-002 was a prospective, multi-center, double-masked, parallel group, randomized, dose-ranging trial designed to investigate the dose-response relationship of PC tafluprost in patients with open-angle glaucoma or ocular hypertension and to compare the safety and efficacy of three concentrations of PC tafluprost (0.0003, 0.0015, 0.0025%) with timolol 0.05% and latanoprost 0.005%. A total of **144 patients** were enrolled and 139 completed the study. Patients received masked study medication for **28 days**.	**Ocular effects:** There was no major difference in the incidence of ocular AEs between the doses (39.3%, 36.7%, 37.9% for 0.0003%, 0.0015%, and 0.0025% tafluprost respectively, compared to 41.4% for timolol and 32.1% for latanoprost). **Conjunctival hyperaemia** was the most common AE and increased with increasing concentration of tafluprost (10.7, 20.0, 24.1% for 0.0003, 0.0015, and 0.0025% tafluprost respectively, compared to 13.8% timolol and 14.3% latanoprost). **Systemic effects:** No systemic effects were reported	(CDER, 2012; TGA, 2012)

Clinical trial phase	Dose / duration / No. of volunteers	Study details	Adverse events (AEs)	Reference
Phase III	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 28 days **No. of volunteers:** 43 patients	Study 77550 was a randomized, investigator-masked, multicenter, cross-over phase III study on two formulations (preserved and unpreserved) of tafluprost 0.0015% eye drops in patients with open-angle glaucoma or ocular hypertension. The study consisted of two treatment periods: preserved followed by unpreserved formulation or unpreserved followed by preserved formulation of study medication tafluprost 0.0015% once daily. The duration of both treatment periods was **four weeks**, separated by a washout period of at least four weeks. A **total of 43 patients** were randomized in the study. IOP was measured at defined intervals.	AEs were reported in 11/43 (25.6%) of the preservative free group compared to 7/43 (16.7%) of preservative-containing tafluprost group. **Ocular effects:** Ocular AEs were more frequent in the preservative free group (20 AEs in 11 subjects, 26%) compared to the preserved formulation group (seven ocular AEs in six subjects, 14%), the most common of which was conjunctival hyperaemia occurred in eight compared to two subjects respectively. Ocular safety was similar between groups. **Systemic effects:** Musculoskeletal and connective tissue disorder (Pain in extremity) in the preservative free group and nausea, Osteoporosis and Tendonitis in the preservative-containing group. There were neither serious adverse events nor withdrawals due to adverse events in this study.	(CDER, 2012; Hamacher *et al.*, 2008; NIH, 2022; TGA, 2012)
Phase III	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 24 months **No. of volunteers:** 269 patients	Study 74458 was a randomized, double-masked, active-controlled, parallel-group, **24- month**, multinational, and multicenter trial comparing efficacy and safety of PC tafluprost 0.0015% comparing with PC latanoprost 0.005%. A total of 533 patients were randomized. At the start of the study **269 patients** were randomized to tafluprost treatment, out of which 246 completed the first 6 months of treatment, 229 completed 12 months of treatment, and 185 completed 24 months of	The overall incidence of AEs was relatively greater with tafluprost than with latanoprost (176/269, 65.4% versus 166/264, 62.9%, respectively) after 24 months of treatment. **Ocular effects:** The tafluprost-treated patients reported more eye disorders (46.5% versus 43.9%), in particular **conjunctival hyperaemia** (9.3% versus 5.7%), **eye pain** (7.1% versus 2.7%), **eye pruritus** (3.7% versus 1.1%), growth of eyelashes (6.3% versus 4.2%),	(CDER, 2011; 2012; TGA, 2012; Uusitalo *et al.*, 2010)

Clinical trial phase	Dose / duration / No. of volunteers	Study details	Adverse events (AEs)	Reference
		treatment. Of the 264 patients randomized to latanoprost, 252 completing the first 6 months, 247 completing 12 months, and 217 completed 24 months of treatment. IOP was measured at defined intervals.	**blurred vision** (2.6% versus 1.1%), and **visual field defect** (6.7% versus 4.9%). Ocular AEs were noted to continue to occur even after months of treatment, as demonstrated by the rise in cumulative AE incidence at 12 and 24 months. At 6 months, 71/269 (26.4%) tafluprost subjects had 130 ocular AEs, at 12 months the cumulative incidence was 102 (37.9%) subjects with 256 ocular AEs. At 24 months there were a total of 127 (47.2%) subjects with 400 ocular AEs. For latanoprost, the incidence at 6 months was 61/264 (23.1%) with 106 ocular AEs. At 12 months there were 91 (34.5%) subjects with 173 ocular AEs and at 24 months there were 117 (44.3%) subjects with 286 ocular AEs. **Systemic effects:** There were no clinically significant changes in blood pressure or heart rate during the 24- month study period or laboratory parameters up to 12 months.	
Phase III	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 12 months **No. of volunteers:** 267 patients	Study 15-003 was randomized, double-masked, parallel group, multicenter, **12-month** trial comparing the efficacy and safety of PC tafluprost 0.0015% with PC timolol 0.5%. A total of 458 patients were randomized. At the start of the study, **267** were randomized to tafluprost, out of which 250 completed the first 6 months of treatment, and 240 completed 12 months of treatment. Of the 191 patients randomized to timolol, 168 completed the first 6 months, and 162 completed 12 months of treatment. IOP was measured at defined intervals.	The incidence of AEs was greater in tafluprost-treated than in timolol-treated subjects (218/267, 81.6% versus 137/191, 71.7%) after 12 months of treatment. **Ocular effects:** Tafluprost-treated subjects had more eye disorders (50.9% versus 44.0%) including **conjunctival hyperaemia** (18.0% versus 6.3%), **eye pruritus** (9.0% versus 2.6%), **dry eyes** (5.6% versus 3.7%), and **foreign body sensation in the eyes** (3.7% versus 2.1%). **Systemic effects:**	(CDER, 2012; TGA, 2012)

Clinical trial phase	Dose / duration / No. of volunteers	Study details	Adverse events (AEs)	Reference
			Systemic events that occurred more in tafluprost subjects compared to timolol were **headache** (13.5% versus 6.8%), **nausea** (3.7% versus 1.0%), **hypercholesterolaemia** (7.1% versus 3.7%) and **cough** (7.9% versus 4.2%).	
Phase III	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 12 weeks **No. of volunteers:** 96 patients	Study 74460 was randomised, double-masked, placebo-controlled, parallel-group, multinational and multicentre **Phase III Study** 74460 evaluated the efficacy and safety of tafluprost 0.0015% eye drops as adjunctive therapy with timolol 0.5% eye drops with open-angle glaucoma or ocular hypertension who are only partially controlled with timolol treatment. The duration of treatment was **12 weeks** (6 weeks treatment period (timolol + tafluprost or timolol + vehicle) followed by 6-week extension period (Vehicle switched to tafluprost) Total 185 patients in a ratio of 1:1 (**96 in the tafluprost group** and 89 in the vehicle group.	There were more AEs (44.8% versus 34.8%) and more mild ocular AEs (41.7% versus 29.2%) in subjects treated with tafluprost+ timolol compared to those treated with vehicle+ timolol. **Ocular effects:** The incidence of **conjunctival hyperaemia** and **eye pruritus** in the tafluprost+ timolol group was 18.8% and 14.6%, respectively, compared to 13.5% and 0% in the vehicle+ timolol group. **Systemic effects:** No clinically significant findings were observed	(CDER, 2012; Egorov *et al.*, 2009; TGA, 2012)
Phase III	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 12 weeks **No. of volunteers:** 320 patients	Study 001 was a randomized, multicenter, active comparator-controlled, **12-week**, double-masked clinical trial to compare the efficacy and safety of preservative-free (PF) tafluprost (0.0015%) and PF timolol 0.5%. A total of 643 patients were randomized, among which **320 patients were randomized to tafluprost** treatment and 306 completed the study. Of 323 patients randomized to timolol, 312 completed the study. IOP was measured at defined intervals.	**Ocular effects:** The adverse events of conjunctival and ocular hyperaemia (2.8 and 1.6%, respectively) were reported more frequently in the PF tafluprost group than in the PF timolol group in which no conjunctival hyperaemia and 0.6% ocular hyperaemia were reported. Photophobia was reported with an incidence of 1.3% in the PF tafluprost group compared with the PF timolol group, which had none. Eye pruritus was reported in 6 (1.9%) patients and 3 (0.9%) patients in the tafluprost and timolol group, respectively. **Systemic effects:**	(CDER, 2012; Chabi *et al.*, 2016; NIH, 2022; TGA, 2012)

Clinical trial phase	Dose / duration / No. of volunteers	Study details	Adverse events (AEs)	Reference
			Headache (1.6%) Serious adverse events occurred in 2 patients (0.6%) treated with PF tafluprost (atrial fibrillation and myocardial infarction) were not thought to be treatment related.	
Phase IIIb	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 12 weeks **No. of volunteers:** 158 patients	An open-label Phase **IIIb** Study 77552 assessed changes in ocular symptoms and signs as well as conjunctival inflammatory markers when **158 patients** with POAG or OHT were switched from preserved latanoprost 0.005% eye drops to tafluprost 0.0015% preservative-free eye drops. The mean IOP at baseline was 16.77mmHg and this was maintained after switching to tafluprost; a mean IOP of 16.36mmHg at Week 6 and 16.44mmHg at **Week 12**. At Week 12, the difference was only marginally statistically significant (p=0.049). While this study predominantly assessed change in symptoms and signs when switching to the preservative free tafluprost formulation, results showed a maintenance of IOP control over 12 weeks of treatment following switch from latanoprost to tafluprost.	**Ocular effects:** There were 11 subjects with 18 ocular AEs (7.0%) and 52 non-ocular AEs in 36 subjects (22.8%). There were four SAEs (2.5% of subjects) all of which were non-ocular. There was **a reduction in the proportion of subjects with ocular symptoms (irritation, foreign body sensation, tearing, itching, dry eye sensation) after 12 weeks of treatment**. An overall score (0 to 20) on five ocular symptoms was found to reduce significantly from a mean at baseline of 7.9 to 4.3 at Week 12 ($p<0.001$) with improvement starting by Week 2 of treatment. **Ocular signs (tear break up time, corneal fluorescein staining, blepharitis, conjunctival redness and tear secretion) were also found to significantly improve by 12 weeks ($p=0.003$ for tear secretion and $p<0.001$ for other signs).** Discomfort on drop instillation was reported to decrease from 60% at baseline to 20% at Week 12.	(TGA, 2012)
Phase III	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 12 weeks **No. of volunteers:** 95 patients	Study 002-01 was randomized, active comparator-controlled, **four-week**, double-masked clinical trial to compare the efficacy and safety of preservative-free MK-2452 (0.0015%) and preservative-free timolol maleate (0.5%) in **190 patients** with open-angle glaucoma or ocular hypertension in India.	**Ocular effects:** The incidence of **conjunctival hyperaemia** (6.45%) and **eye pruritus** (7.53%), eye irritation (6.45%) and Conjunctivitis (9.68%). **Systemic effects:**	(Chabi *et al.*, 2016; NIH, 2022)

Clinical trial phase	Dose / duration / No. of volunteers	Study details	Adverse events (AEs)	Reference
		A total of 190 **patients (PF tafluprost = 95,** PF timolol = 95).	No mortality and other adverse effects were observed	
Phase III	**Dose:** 0.15 mL of 0.0015% (i.e., 0.0375 µg/kg bw/day) **Duration:** 4 weeks **No. of volunteers:** 489 patients	A double-masked study of DE-111 ophthalmic solution versus tafluprost ophthalmic solution 0.0015% alone and concomitant use of tafluprost ophthalmic solution 0.0015% plus timolol ophthalmic solution 0.5% in patients with primary open-angle glaucoma or ocular hypertension -phase 3, confirmatory study. **No. of total patients = 489**	No ocular and systemic effects were recorded.	(NIH, 2022)

Post-marketing experience:

In post-marketing experience, the most common adverse effects were ocular hyperaemia and eye redness. The events were consistent with Phase I-III clinical trials. No marked systemic effects were observed. The spontaneous post-marketing data of tafluprost formulation were consistent with the safety profile from the clinical studies and provides reassurance that no new safety issues have emerged with prolonged use (CDER, 2012; Kuwayama *et al.*, 2017; Kuwayama and Nomura, 2014; Sun *et al.*, 2022; Tumbocon and Macasaet, 2019).

3.3.11. Special investigations

3.3.11.1 Assessment of intraocular pressure and ocular effects in humans

The eye irritation potential of a cosmetic eyelash product containing 0.025% of DDDE was investigated in 19 human volunteers. The test substance was applied daily in accordance with the directions in the package insert for 28 days. The study was conducted under the supervision of an ophthalmologist. The IOP and ocular effects were evaluated. The details of the study are already described in **Section 3.3.3** of the dossier.

The within-eye differences in IOP from the beginning to the end of the study were not statistically significant ($t > 0.05$). No ocular adverse effects were noted by the study ophthalmologist. Only minor ocular effects were self-reported by 4 of 19 volunteers including slight dryness sensation around the eye areas (5%), slight itching sensation (21%), slight stinging sensation (16%), slight eye watering and redness (11%) and moderate to high burning sensation (5%). Overall, the test substance was moderately tolerated by the majority of the volunteers.

Under the conditions of the study, the test substance did not show a significant effect on IOP, but minor ocular effects were observed in the human volunteers (Sebesten, 2010). These data show there is no pharmacological effect on eyes with normal use of the cosmetic eyelash product containing DDDE (Bailey, 2023).

3.3.11.2 Assessment of endocrine disrupting (ED) potential of DDDE

3.3.11.2.1 The ED assessment strategy

The World Health Organization (WHO) defines an endocrine disruptor as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations (WHO/IPCS, 2002).

The strategy to identify the potential endocrine disrupting properties of DDDE included the gathering and collation of ED relevant information, the assessment of all evidence, a mode of action analysis if deemed necessary, and subsequently a safety evaluation (**Chapter 3.4**).

3.3.11.2.3. Gathering and assessment of the evidence

In silico data for DDDE and *in vivo* studies for the analogue tafluprost were available for the assessment of the potential ED properties.

3.3.11.2.3.1. Existing data and non-test information (OECD Conceptual Framework Level 1)

The endocrine activity potential of DDDE was evaluated using the *in silico* tools (see **Table 21**, below)

Table 21. *In silico* predictions for DDDE

QSAR model		Results
Oestrogen	OECD QSAR Toolbox v.4.6	Strong binder, OH group
	Derek Nexus version 6.2.1	No alerts
	Danish QSAR models	ER_α binding: Positive, within the applicability domain (ID) ER_α activation: Negative, out of the applicability domain (OD) ER activation: Negative, ID
	VEGA v.1.2.3	ER relative binding affinity: Inactive, OD ER mediated effects: Inactive, ID
	Endocrine disruptome	Medium binding to ERα as agonist/antagonist

QSAR model		Results
Androgen	Derek Nexus version 6.2.1	No alerts
	Danish QSAR models	AR binding: Negative, OD AR activation: Negative, ID AR inhibition: Negative, ID
	VEGA v.1.2.3	AR binding activity: Active, OD
	Endocrine disruptome	Potential binding to AR as antagonist
Thyroid modalities	Derek Nexus version 6.2.1	No alerts
	Danish QSAR models	Thyroid peroxidase (TPO) inhibition: Negative, ID Sodium/iodide symporter (NIS): Negative, ID
	VEGA v.1.2.3	TR_α and TR_β binding: Inactive, ID
	Endocrine disruptome	Medium probability to bind TR_α
Steroidogenesis	Derek Nexus version 6.2.1	No alerts
	Endocrine disruptome	Medium probability to bind to glucocorticoid receptor (GR) as agonist

Overall, the (Q)SAR results indicate that DDDE may have some ED activity. However, this will need to be evaluated further based on the *in vivo* studies available for the analogue tafluprost to determine if there is a plausible link between the potential ED activity and the ED-mediated adversity (if any).

3.3.11.2.3.2. *In vitro* assays providing data about selected ED mechanisms / pathways (OECD Conceptual Framework Level 2)

No ED relevant *in vitro* assays as defined in the OECD Conceptual Framework Level 2 could be identified for DDDE or its analogue tafluprost.

3.3.11.2.3.3. *In vivo* assays providing data about selected ED mechanisms/ pathways (OECD Conceptual Framework Level 3)

No *in vivo* assays investing ED selective mechanisms or mode of actions (e.g., Uterotrophic assay - OECD TG 440, Hershberger assay - OECD TG 441) could be identified for DDDE or its analogue tafluprost. However, safety pharmacology studies for the common hydrolytic metabolite tafluprost acid, showed an increase in the force of uterine contractions in uteri of non-pregnant rats and rabbits at ≥ 10^{-9} g/mL (CDER, 2011).

3.3.11.2.3.4. *In vivo* assays providing data on adverse effects on ED related endpoints (OECD Conceptual Framework Level 4)

The potential for endocrine adversity of DDDE can be assessed based on the endocrine, androgen, thyroid and steroidogenesis-mediated ('EATS-mediated') and 'Sensitive to, but not diagnostic of EATS' (EATS-sensitive) parameters (see the below list) evaluated in repeated dose, reproductive, and developmental toxicity studies.

As per the ECHA EFSA guidance document for the identification of endocrine disruptors (EFSA/ECHA, 2018), the EATS-mediated and sensitive parameters typically investigated in these studies include:

- EATS-mediated: Weights and histopathology of thyroid, ovaries, uterus, cervix, vagina, testes, epididymis, prostate and seminal vesicles and sperm parameters (morphology, motility, and count), Oestrus cyclicity.
- EATS-sensitive: Weights and histopathology of adrenals, reproductive and developmental parameters such as fertility, gestation length, litter size and weight, litter viability, and mortality.

The available repeated dose, reproductive and developmental toxicity studies for the analogue tafluprost do not show any toxicologically significant effects on EATS-mediated parameters (see **Sections 3.3.5 and 3.3.6**). The effects on the EATS-sensitive, but not diagnostic parameters such as litter viability, littler size and weight was taken into consideration while establishing the PoD (see **Section 3.4.1**).

3.3.11.2.3.5. *In vivo* assays providing more comprehensive data on adverse effects on ED related endpoints over more extensive parts of the life cycle of the organism (OECD Conceptual Framework Level 5)

No OECD Level 5 *in vivo* assays defined in the OECD Conceptual Framework Level 5 could be identified for DDDE or its analogue tafluprost.

Overall conclusion on endocrine disrupting properties of DDDE

Based on the available information from *in silico* modelling for DDDE paired with the evidence from *in vivo* studies for the analogue tafluprost, DDDE may have some potential for ED activity. Available data from *in vivo* testing of the analogue tafluprost (i.e., repeated dose toxicity; developmental and reproductive toxicity) did not reveal any evidence for EATS-mediated adversity but showed adverse effects on 'EATS-sensitive' parameters. Due to lack of specificity of these parameters, no conclusive evidence of adverse effects due to an endocrine-related mechanism is available. Furthermore, the proposed point of departure (PoD) for risk assessment is based on a NOAEL derived from a developmental toxicity study via the intravenous route and is therefore considered to also adequately cover for any potential EATS-sensitive effects.

3.4. SAFETY EVALUATION (INCLUDING CALCULATION OF THE MOS)

3.4.1 Selection of the PoD

In the absence of repeated dose, reproductive and developmental toxicity studies for DDDE, its systemic toxicity can be assessed based on toxicity data available for the close structural analogue tafluprost by means of read across. The scientific justification for the read across is provided in **section 3.3**. with more details provided in **Annex I**.

A range of repeated dose toxicity and developmental toxicity studies are available on the analogue tafluprost to establish a critical NOAEL suitable as PoD for the risk assessment of DDDE used in cosmetic applications.

Considering all available information and in line with the approach chosen by the German BfR for the risk assessment of DDDE in cosmetic eye product formulations (BfR, 2017), the **NOAEL of 0.3 µg/kg bw/day** derived from the prenatal developmental toxicity study in rats following intravenous administration is considered as the most appropriate PoD. As the NOAEL is based on critical effects i.e., developmental/teratogenic effects following intravenous dosing, it represents a very conservative and worst-case PoD when compared with the exposure route (i.e., dermal) for the intended application of DDDE present in cosmetic eye product formulations. Further, the study is considered to be of good quality with appropriate dose spacing and it corresponds to the lowest NOAEL established in the available set of studies for tafluprost.

As discussed in **Section 3.3.1.2**, no bioavailability correction is required for deriving a systemic dose **(PoD_{sys})** as the PoD has been derived from an intravenous study, resulting in a systemic PoD (**PoD_{sys}**) of **0.3 µg/kg bw/day or 0.0003 mg/kg bw/day**.

3.4.2 Exposure assessment

As described in **Section 3.2**, DDDE is used in the cosmetic eyelash product at 0.018% concentration. It is intended to be used as an eyelash conditioner that "helps to strengthen eyelashes while protecting against breakage and brittleness" while "improving flexibility, moisture and shine for bold, beautiful, more dramatic looking lashes" ([redacted] 2020). The concentration of DDDE in [redacted] is 0.018%.

3.4.2.1 Instructions for use of the eyelash cosmetic formulation containing DDDE

The cosmetic eyelash product should be applied with a multi-use fine brush applicator as a thin line directly to eyelashes (primarily the upper eyelashes) above the lash line. It is formulated with a thickener (cellulose gum) to ensure that the product stays on the eyelashes where it is applied and does not migrate to contact the fluid or membranes surrounding the eye. Therefore, the cosmetic eyelash product is designed to affect the appearance of eyelashes and not to come in contact with eyes.

Further, the below 'Directions for Use' and 'Caution Statements' are provided in the packaging for cosmetic eyelash product in Europe.

Directions For Use	Caution Statements
Once a day, apply a thin line of the cosmetic eyelash product directly to eyelashes, above the lash line. Let dry completely before applying additional beauty products.	Do not get in eye. Rinse immediately with water if eye contact occurs. If irritation develops, reduce frequency of use until irritation resolves. If irritation persists or is excessive, discontinue use and consult a physician. Some users have reported a faint darkening of the eyelash base (primarily with excessive use); if this is of concern, do not use. Keep out of reach of children.

3.4.2.2 Concentration of DDDE in the cosmetic eyelash product

The concentration of DDDE in the cosmetic eyelash product, is 0.018%.

3.4.2.3 Amount of product applied per application

A very small amount of DDDE is applied per application to the eyelashes. The amount of the cosmetic eyelash product applied per brushstroke to the upper eyelashes was determined by weighing the brush applicator that is part of the cosmetic eyelash product container after the applicator was removed from the tube container (pre-application) and again immediately after it was used to apply the cosmetic eyelash product to the upper eyelashes, above the lash line (i.e., before and after a single brushstroke to the eyelashes). Ten different applicator brushes were used in the study with the same person applying the cosmetic eyelash product. The range of the cosmetic eyelash product applied per brush stroke was 1 – 4 mg. On **average, 2.4 mg** of cosmetic eyelash product was applied to the upper eyelashes with each brush stroke ([redacted] 2013) (see **Annex V** for details).

In another recently conducted in-house test, the amount of cosmetic eyelash product per brush stroke was determined by applying it to commercially available mink hair samples. The results are presented in **Annex V.** The tests used different combinations of tube containers and applicators. In Procedure 1, the same tube container was used with 10 different applicators. In Procedure 2, the same applicator was used with different tube containers. In both procedures, two different methods of inserting the applicator into the tube were used. In Insertion Method A, the applicator tip was pushed to the bottom of the tube. In Insertion Method B, the applicator tip was inserted just to the neck of the tube. The amount of cosmetic eyelash product applied per brushstroke was calculated as the difference between the amount on the applicator brush before and after application of the product across the mink hair samples. There were 10 replications of each procedure/insertion method. The range of the cosmetic eyelash product applied per brush stroke was 0.64 – 3.72 mg. The highest '**average amount'** from both the procedures (**2.42 mg**) is almost identical to the average amount of cosmetic eyelash product applied (2.4 mg) per brushstroke to human upper eyelashes. However, the highest **'maximum amount'** is lower (i.e., **3.72 mg)** than the maximum amount of cosmetic eyelash product applied (4 mg) per brushstroke to human upper eyelashes. Calculations of the average and maximum amounts of DDDE used per application to the upper eyelashes from both the methods are presented in **Annex V**.

Based on the above measurements and as a conservative approach, both, the 'average amount' and the 'maximum amount' per brush stroke, were taken into consideration for the purpose of risk assessment. Therefore, considering once daily application to both eyes, the 'average amount' of **4.8 mg/day** and the 'maximum amount' of **8 mg/day** were used for the MoS calculations.

3.4.2.4 Systemic exposure dose calculations (SED)

DDDE is present at concentrations up to **0.018%** in the cosmetic eyelash product which has to be applied as a thin line to the eyelashes, above the lash line. Although not completely identical to the product types defined in the SCCS NoG, in application terms, it is close to the eyeliner and mascara cosmetic products for which the dermal, and to some minor extent, the ocular routes are the potential routes of exposure. However, unlike eyeliners, the cosmetic eyelash product is applied directly to eyelashes (similar to mascara), which decreases the risk of dermal exposure. Further, the risk of its ocular exposure is also low, as a relatively small amount (see **Section 3.4.2.3**) of the cosmetic eyelash product is applied as a fine line across the eyelashes above the lash line. This is in contrast to mascara where a significantly large amount (i.e., 25 mg as per the SCCS NoG) is applied along the full length of eyelashes.

In addition, unlike the other cosmetic products used near the eyes, the cosmetic eyelash product includes cellulose gum which acts as a 'viscosity increasing agent 'or 'thickener' that will minimise the migration of the product to the eye lid skin or eye. Therefore, the quantity of DDDE that is likely to migrate to the eyelid and be available for skin penetration is negligible (Bailey, 2023). Nevertheless, for a worst-case calculation, 50% of the applied amount (i.e., an average amount of 2.4 mg and maximum amount of 4 mg) was used for the MoS calculations.

Therefore, when compared to other cosmetics applied near eyes, there is less potential for dermal or ocular exposure with the cosmetic eyelash product. Accidental unintended exposure to eyes may occur but is not expected to present a significant risk. DDDE was found to be non-irritating to the eyes, when tested neat in an EpiOcular^{TM} RhCE test (see **Section 3.3.3.2**) and did not reduce IOP following exposure to 0.025% DDDE in a cosmetic eyelash product formulation in female volunteers for 28-days (see **Section 3.3.11**). Therefore, 0.018% concentration of DDDE in the cosmetic product is not considered to alter the overall eye irritation profile or to have a physiological effect on the eye. This is further supported by data on consumer experience with the cosmetic eyelash product, which shows that the rate of adverse event reports is very low (0.154%, April 2022 – April 2023) (Bailey, 2023). This is further supported by the absence of systemic effects following subchronic/chronic repeated administration of ophthalmic formulations containing the analogue tafluprost in monkeys (CDER, 2011).

Taking all the above information into consideration, only a potential **systemic exposure dose (SED)** via the dermal route was estimated in the present assessment.

The estimated **'SED'** from the dermal route can be calculated as follows:

$$\mathbf{SED = E_{product} \times C/100 \times DAp/100}$$

With
- **SED** = Systemic Exposure Dosage from dermal route (mg/kg bw/day)
- **Eproduct**= Estimated daily exposure to a cosmetic product per kg bw
- **C** = Concentration of the ingredient under study in the finished cosmetic product (%)
- **DAp** = Dermal Absorption expressed as a percentage of the test dose assumed to be applied in real life conditions (%)

The dermal SED calculation is based on:

- $\mathbf{E_{product}}$ – Considering 50% of the measured 'average amount' or 'maximum amount' of the cosmetic product per kg bw is 0.04-0.067 mg/kg bw/day for a 60 kg adult (i.e., 50% x 4.8 mg/day ÷ 60 kg = 0.04 mg/kg bw/day; 50% x 8 mg/day ÷ 60 kg bw = 0.067 mg/kg bw/day) (see **Section 3.4.2.3**)
- **C** – The concentration of the substance in the cosmetic product (C) (i.e., **0.018%**; see **Section 3.4.2.2)**

- **DAp** – dermal absorption = **8.67%** (see **Section 3.3.1.1**)

The resulting estimated SEDs from daily application of the cosmetic eyelash product containing 0.018% of DDDE are calculated to be:

- SED (based on average amount of 4.8 mg the cosmetic eyelash product) = **6.24E-07 mg/kg bw/day** [i.e., (0.04 mg/kg bw x 0.018% x 8.67%)].
- SED (based on maximum amount of 8 mg the cosmetic eyelash product) = **1.04E-06 mg/kg bw/day** [i.e., (0.0667 mg/kg bw x 0.018% x 8.67%)].

It is important to note that the SED calculations are based on conservative worst-case assumptions. The underlying assumption a maximum of 50% of the cosmetic eyelash product[16] could migrate to the eyelid and be available for dermal exposure is made even though the product is applied as a thin line to the eyelashes above the lash line and an added viscosity agent further minimises product migration to the eyelid.

3.4.3 Margin of Safety calculation (MoS)

The MoS, which represents the ratio between the systemic PoD (PoD_{sys}) and the estimated SED, was calculated according to the following algorithm. The results are presented in **Table 23**:

$$\mathbf{MoS = PoD_{sys}/SED}$$

With:
- PoD_{sys} = Systemic Point of Departure (mg/kg bw/day)
- SED = Systemic Exposure Dosage (mg/kg bw/day)

In accordance with SCCS NoG, to consider a substance to be safe for use or an acceptable risk assessment, the MoS for systemic toxicity should be ≥100.

Table 23. SED and MoS calculations

Product daily amount (mg)	SED (mg/kg bw/d)	PoD_{sys} (mg/kg bw/d)	MoS
Based on average daily amount of 4.8 mg the cosmetic eyelash product	6.24E-07	0.0003	481
Based on maximum daily amount of 8 mg the cosmetic eyelash product	1.04E-06	0.0003	288

3.4.4 Conclusion

Based on the available data and the conservative SED calculation, the present safety assessment reveals a calculated MoS greater than 100 and thereby supports the safe use of DDDE at a concentration up to 0.018% in cosmetic eyelash products under the conditions presented in this evaluation.

[16] 50% of the average amount of 4.8 mg = 2.4 mg and 50% pf the maximum amount of 8 mg = 4 mg

3.5. DISCUSSION

Physicochemical properties

DDDE is a colourless to pale yellow solution. It has a high boiling point (503.76 °C) and a low vapour pressure (1.2E-13 Pa at 25°C), indicating DDDE not to be volatile. In experimental studies, DDDE was found to have a moderate water solubility (1.05 g/L at 20°C) and high lipophilicity (log Kow = 5.03). Its maximum UV absorbance ranges between 226 and 276 nm. The physicochemical properties of DDDE are presented in **Section 3.1.**

Function and uses

DDDE is used at a concentration of 0.018% in a cosmetic eyelash product formulation. It is intended to be used as an eyelash conditioner that "helps to strengthen eyelashes while protecting against breakage and brittleness" while "improving flexibility, moisture and shine for bold, beautiful, more dramatic looking lashes". The cosmetic eyelash product is not marketed to grow eyelashes.

Toxicological evaluation

Toxicological information is available for DDDE on dermal absorption, skin irritation, eye irritation, skin sensitisation and genotoxicity. Toxicological data gaps were identified for acute toxicity, repeated dose toxicity, carcinogenicity, developmental and reproductive toxicity endpoints. These endpoints were addressed by means of read across to data available for the analogue, tafluprost. A justification for the read across approach is provided in **Section 3.3** as well as in **Annex III**.

Toxicokinetics

No toxicokinetic studies with DDDE could be identified. Considering its physicochemical properties, the oral absorption of DDDE is expected to be moderate.

Dermal absorption

The dermal absorption of DDDE has been assessed based on *in vitro* percutaneous absorption study with 0.018% DDDE in a representative eyelash cosmetic formulation. In this study, the dermal penetration of DDDE was determined to be 6.51±2.16% of the applied dose.

Acute toxicity

Based on the data available for the analogue tafluprost in rats, DDDE is not expected to show mortality at doses up to 100 mg/kg bw/day via the oral route or 3 mg/kg bw/day via the intravenous route.

Irritation and corrosivity

The skin irritation potential of DDDE has been assessed based on *in vitro* RhE studies with neat DDDE and an HRIPT with a cosmetic eyelash product formulation containing 0.025% DDDE in 51 panellists. Based on the outcome of these studies, it can be concluded that DDDE is not irritating to skin at the intended use concentration of 0.018%.

The eye irritation potential of DDDE has been evaluated in an *in vitro* RhCE study with neat DDDE, as well as an *in vitro* HET-CAM study and a 28-day human volunteers' study, conducted with a cosmetic eyelash product formulation containing 0.025% DDDE. Based on the results from these studies, no eye irritation concern is considered for DDDE at the intended use concentration of 0.018%.

Skin sensitisation

The skin sensitisation potential of DDDE has been assessed based on multiple studies. These included two *in vitro* DPRA, two *in vitro* KeratinoSens™ studies using neat DDDE as well as two HRIPTs using 7.5% DDDE in phenoxyethanol and a formulation containing 0.025% DDDE.

The results of the two DPRA revealed mean peptide depletion values well below the threshold of 13.89%, suggesting 'no or minimal reactivity' in the cysteine1:10 prediction model. Regarding the two

KeratinoSens™ assays, one assay was considered inconclusive as it revealed a ≥1.5-fold increase in luciferase induction only at the highest non-cytotoxic concentration (250 µM) in the absence of a clear dose response. However, the second assay showed a clear negative result, as the luciferase induction values did not exceed the 1.5-fold threshold at any of the tested concentrations. Further, the two HRIPTs did not show any signs of skin sensitisation response. Based on the results from these studies, DDDE is not assessed to cause skin sensitisation at its intended use concentration of 0.018%.

Repeated dose toxicity

No repeated dose toxicity studies could be identified for DDDE. Therefore, the data available for the analogue tafluprost has been used for assessing the repeated dose toxicity of DDDE by means of read-across. The database for the assessment of repeated dose toxicity with the analogue tafluprost includes intravenous (2 subacute, 2 subchronic), subcutaneous (2 subchronic) and ocular (1 subacute, 2 subchronic and 1 chronic) studies.

The repeated **intravenous** administration of tafluprost for up to 26 weeks in rats and up to 39 weeks in dogs produced a species-specific pattern of systemic toxicity. In rats, the major findings were limited to effects at all dose levels on bone, bone marrow/haemopoiesis and red blood cell indices. As a result, the LOAEL for the test substance was considered to be 10 µg/kg bw/day in rats. In dogs, similar effects in bone marrow or haematological parameters were observed. In addition, transient clinical signs, slight elevations in heart rate, blood pressure, and respiratory rate, prolonged QTc interval, enlarged salivary glands, and adrenal and salivary gland histopathology were observed at 10 µg/kg bw/day. As a result, the NOAEL was established at the dose of 1 µg/kg bw/day in dogs.

Repeated **subcutaneous** administration of the analogue tafluprost for 13 weeks in rats produced only minor histopathological changes in spleen and kidney at 30 µg/kg bw/day. However, chronic administration for 24 months in the carcinogenicity study induced reduced body weight and histopathology changes including hyperostosis of the sternum and femur in some animals, and increased incidence of extramedullary haematopoiesis in the spleen at ≥3 µg/kg bw/day. No significant adverse effects were observed at any dose levels in the 13-week dose range finder as well as in a 78-weeks chronic carcinogenicity studies in mice at doses up to 100 µg/kg bw/day study. Based on these studies, a NOAEL of <3 µg/kg bw/day was established in rats and a NOAEL of 100 µg/kg bw/day in mice.

Topical ocular administration of ophthalmic formulations containing 0.0005-0.05% tafluprost (i.e., equivalent to 0.067-10 µg/kg bw/day) in subacute, subchronic and chronic toxicity studies in monkeys did not produce systemic toxicity up to the highest tested concentrations. However, these studies showed ocular changes including iridial darkening, sunken eyelids, and blue-grey discoloration of the lower eyelid, which were not associated with loss of function and considered to be of cosmetic nature. Other, more serious ocular toxicities including pronounced inflammation or alterations in electroretinography were not observed with topical ocular administration of tafluprost at any of the administered doses. Therefore, the ocular effects were not considered to be toxicologically significant. Further, the FDA pharmacology review report on tafluprost concluded that *"these data strongly suggest that clinical administration of tafluprost by the topical ocular route is unlikely to cause systemic toxicity'.* It is of critical note that unlike the analogue, which is applied as an eyedrop directly to the eye, the cosmetic eyelash product is applied to eyelashes with a viscosity agent that minimises migration of the product away from the eyelashes. Further, the amount of DDDE (i.e., 0.432 µg) applied per application is orders of magnitude smaller than the amount of analogue (2.25 µg) applied per application (Bailey, 2023).

Reproductive and developmental toxicity

In the absence of reproductive and developmental toxicity studies with DDDE, these endpoints were addressed based on data available for the analogue tafluprost. A reproductive toxicity study with the analogue tafluprost via the intravenous route did not indicate any significant treatment-related effects on fertility or reproductive parameters up to a dose of 100 µg/kg bw/day in rats.

Four studies, two in rats and two in rabbits, were available for the assessment of the developmental toxicity endpoint. Out of the two pre-natal developmental toxicity studies in rabbits, one was of questionable reliability due to the dosing during the initial days of post fertilisation, when typically, any disturbance or handling should be avoided. In the second study, tafluprost was tested at very low doses, leading to extremely low blood concentrations[17] and a NOEL of 0.01 µg/kg bw/day was established due to the absence of any adverse effects.

In rats, a prenatal developmental toxicity study showed in the absence of maternal toxicity an increased number of intrauterine deaths, decreased foetal weights, and skeletal malformations in the vertebral column and a greater number of lumbar and thoracic vertebrae at doses ≥10 µg/kg bw/day. Therefore, the NOAEL for foetal toxicity was established at 3 µg/kg bw/day, while the NOAEL for maternal toxicity was established at the highest tested dose of 30 µg/kg bw/day. Further, in a pre-post-natal developmental toxicity study in rats, poor nursing behaviour for some F0 females resulted in decreased F1 offspring viability at doses ≥1 µg/kg bw/day. In addition, delayed pinna unfolding at 3 days of age for F1 offspring as well as decreased body weights and increased F1 newborn mortality were observed at 10 µg/kg bw/day. As a result, the NOAEL for nursing and development of the F1 generation was established at 0.3 µg/kg bw/day in rats. In the F2 generation, no significant differences in embryonic mortality or the number of corpora lutea, implantations, live F2 embryos, or pre-implantation loss were noted for any of the tafluprost treatment groups. However, F2 generation body weights, male/female ratio were not assessed, and F2 foetuses were not externally evaluated for malformations.

Mutagenicity/ genotoxicity

The genotoxicity of neat DDDE has been assessed in an Ames test as well as in an *in vitro* MNT using human lymphocytes. No mutagenic or clastogenic response was observed in these recent OECD guideline-compliant assays. This is further supported by the absence of genotoxicity observed for the analogue tafluprost in an Ames test, *in vitro* chromosomal aberration assay and *in vivo* MNT in mice. Based on this information, DDDE is not assessed to be genotoxic.

Carcinogenicity

No carcinogenicity studies could be identified for DDDE. However, the absence of genotoxicity and structural alerts for carcinogenicity using *in silico* tools together with the absence of significant treatment-related tumorigenic potential of the analogue tafluprost in two chronic carcinogenicity assays in mice and rats via subcutaneous routes at doses up to 100 µg/kg bw/day, indicate that there is only a low carcinogenicity concern for DDDE at the intended use concentration of 0.018%.

Photo-induced toxicity

The UV/Visible spectrum showed one absorption band in the range 210 – 240 nm with maximum absorption at 226 nm and another absorption band in the range 250 – 285 nm, with three maxima at 265 nm, 258 nm and 276 nm. Considering that the maximum absorbance wavelengths are below the cut-off of 313 nm, DDDE is not likely to have phototoxic concern and no additional *in vitro* phototoxicity testing is required as per the SCCS NoG (SCCS, 2023).

[17] The blood concentrations were below the limit of detection (20 pg/mL) of the analytical method.

Human data

As described above, the clinical studies with eyelash cosmetic formulations containing 0.025% DDDE did not show skin irritation, eye irritation, and skin sensitisation responses at the tested concentration. Also, there was no statistically significant reduction in IOP over the 28-day study in humans. The clinical trials with the 0.0015% ophthalmic solutions containing the analogue tafluprost, did not reveal any critical adverse reactions with regards to safety of the product, although they showed an impact on IOP.

Special investigations

Evaluation of effect on IOP

A clinical study conducted in 19 human volunteers, did not show statistically significant reduction in IOP following exposure to a cosmetic eyelash product containing 0.025% DDDE for 28 days. Except for minor ocular effects, which were self-reported by 4 of 19 volunteers, no ocular adverse effects were noted by the study ophthalmologist. This data shows that there is no pharmacological effect on eyes with normal use of the cosmetic eyelash product containing DDDE.

Evaluation of ED potential DDDE

Based on the available information from *in silico* modelling for DDDE paired with the evidence from *in vivo* studies for the analogue tafluprost, DDDE may have some ED activity. Available data from *in vivo* testing of the analogue tafluprost (i.e., repeated dose toxicity; developmental and reproductive toxicity) did not reveal any evidence for EATS-mediated adversity but showed adverse effects on 'EATS-sensitive' parameters. Due to lack of specificity of 'EATS-sensitive' parameters, no conclusive evidence of adverse effects due to an endocrine-related mechanism is available. Furthermore, the proposed PoD for risk assessment is based on a NOAEL derived from a developmental toxicity study via the intravenous route and is therefore considered to also cover for any potential EATS-sensitive effects adequately.

Point of departure

As discussed above, due to the absence of data on DDDE, repeated dose toxicity and developmental toxicity studies available for the analogue tafluprost were evaluated to establish a critical NOAEL suitable as PoD for the risk assessment of DDDE used in cosmetic applications.

Considering all available information and in line with the approach chosen by the German BfR for the risk assessment of DDDE in cosmetic eye product formulations (BfR, 2017), the **NOAEL of 0.3 µg/kg bw/day** (or 0.0003 mg/kg bw/day), derived from the pre-post-natal developmental toxicity study in rats following intravenous administration, is considered to be the most appropriate PoD. The underlying study is of good quality with appropriate dose spacing and it corresponds to the lowest NOAEL established in the available set of studies for tafluprost. Further, as the NOAEL is based on critical effects i.e., developmental/teratogenic effects following intravenous dosing, it represents a worst case when compared with the exposure route (i.e., dermal) for the intended application of DDDE present in cosmetic eye product formulations. In addition, due to the intravenous dosing, no bioavailability correction is required for deriving the PoD_{sys}.

Exposure and risk assessment

Taking into consideration the use conditions of the cosmetic eyelash product containing DDDE at 0.018% concentrations, the exposure is expected to be mostly via the dermal route, similar to eyeliners and mascara. However, unlike eyeliners the cosmetic eyelash product containing DDDE is applied directly to the eyelashes, similar to mascara, and unlike mascara, a relatively small amount of the product is applied as fine line across the eyelashes above the lash line. Further, the inclusion of a thickener cellulose gum, in the cosmetic eyelash product, minimises the migration of the product to the eye lid skin or into the eye.

Under the conservative worst case assumption that a maximum of 50% of the amount of cosmetic eyelash product applied to the eyelash could reach the eyelid and thus be available for dermal exposure with a dermal penetration value of 8.7% for DDDE, the maximum systemic exposure dose (SED) is calculated to be **1.04E-06 mg/kg bw/day**. The ratio of the PoD_{sys} value of 0.0003 mg/kg bw/day and the maximum SED, leads to a **MoS >100**, thereby supporting the safe use of DDDE at a concentration up to 0.018% in cosmetic eyelash products under the conditions presented in this evaluation.

Accidental unintended exposure to eyes may occur but is not expected to present a significant risk. DDDE was found to be non-irritating to the eyes, when tested neat in an EpiOcular™ RhCE test and did not reduce IOP following exposure to 0.025% DDDE in a cosmetic eyelash product formulation in female volunteers for 28-days. Therefore, 0.018% of DDDE in the cosmetic product is not considered to alter the overall eye irritation profile of the product formulation or to have a physiological effect on the eye. This is further supported by the absence of systemic effects following repeated administration of ophthalmic formulations containing the analogue tafluprost in monkeys.

4. CONCLUSION

This dossier has been prepared in accordance with the 12th revision of the SCCS Notes of Guidance (2023) to demonstrate the safety of DDDE when used at concentrations up to **0.018% in cosmetic eyelash products**.

The assessment was conducted using an MoS approach, according to which a level considered to be safe for human health, expressed as the PoD_{sys}, was compared with the estimated dermal SED.

The selected PoD was based on the NOAEL of **0.3 µg/kg bw/day** derived from a pre- and post-natal developmental toxicity study by intravenous administration in rats conducted on the analogue tafluprost. Based on an intravenous bioavailability of 100%, no correction was required for deriving a systemic dose **(PoDsys)**. Thus, the PoD of **0.3 µg/kg bw/day** was considered as the PoD_{sys} which was taken forward for risk assessment (MoS calculation).

Under the conservative worst case assumption that a maximum of 50% of the amount of cosmetic eyelash product applied to the eyelash could reach the eyelid and thus be available for dermal exposure with a dermal penetration value of 8.7% for DDDE, the maximum systemic exposure dose (SED) is calculated to be **1.04E-06 mg/kg bw/day**.

Despite the conservative assumptions, the ratio of the PoD_{sys} value of 0.0003 mg/kg bw/day and the maximum SED, leads to an **MoS >100,** demonstrating the safe use of DDDE at a concentration up to 0.018% in cosmetic eyelash products. Additionally, apart from the observed effects on 'EATS-sensitive' parameters in the studies conducted with the analogue, there is no direct evidence of adverse effects due to endocrine-related mechanisms. Nevertheless, the risk assessment conducted using the PoD_{sys} is considered to adequately cover any potential EATS-sensitive effects. Thus, the use of DDDE in cosmetic products at the proposed maximum use levels does not present a human safety concern.

The submitter hereby confirms that the information contained in this dossier complies with the provisions on animal testing as laid down in Article 18(1) of the Cosmetic Products Regulation (EC) No 1223/2009.

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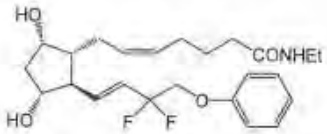
Annex I – Certificate of Analysis

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{33}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications.

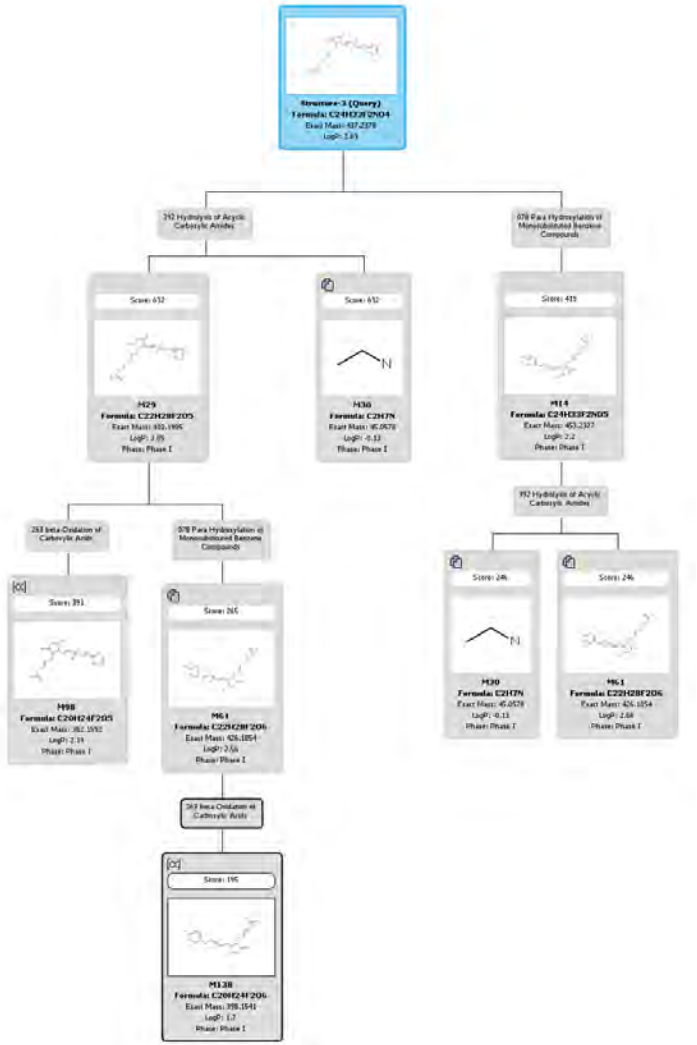
Quality Control: [redacted] *Date:* 23 NOV 2022

Approved: [redacted] *Date:* 23 Nov 2022

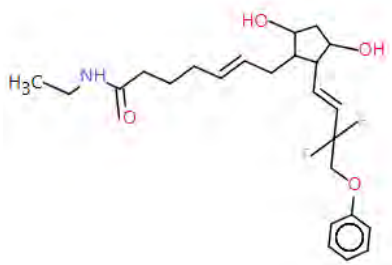
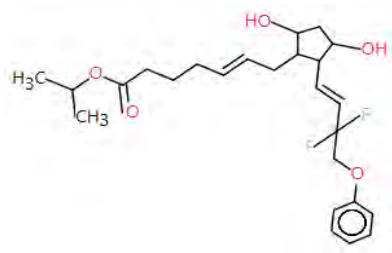
Quality Control Laboratory
[redacted]

Qualitätssicherung
11 Jan. 2023
Kürzel: BAE

Annex II – Prediction of the metabolic pathway of DDDE using Meteor Nexus v.3.1.0



Annex III – Analogue evaluation

	Target substance	Analogue
CAS number	1185851-52-8	209860-87-7
Name	Dechloro dihydroxy difluoro ethylcloprostenolamide (DDDE) or Tafluprost ethyl amide	Tafluprost
2D Structure		
SMILES from PubChem	CCNC(=O)CCCC=CCC1C(O)CC(O)C1C=CC(F)(F)COc1ccccc1	CC(C)OC(=O)CCCC=CCC1C(O)CC(O)C1C=CC(F)(F)COc1ccccc1
Assessment of the analogues on the basis of 4 criteria: 1) structural similarity 2) physico-chemical properties 3) functional gorups and structural alerts 4) first metabolic reaction	/	- With respect to the structural similarity, the analogue has a relatively high DICE index. '- The analogue has physicochemical properties in the same range as compared to the target substance, with a slightly higher log Kow and lower water solubility. These differences in log Kow and water solubility suggest the analogue to be less bioavailable under oral exposure conditions compared to the target substance. '- It shares the key functional group (cycloalkane, ether moiety, alkyl halide and aryl groups); it presents the carboxylic acid ester group instead of the amide group and it contains the isopropyl group. '- It presents the same structural alerts as compared to the target substance. '- With respect to metabolism, the substance is predicted to undergo hydrolysis similar to the target substance.

	Target substance	Analogue
		Conclusion: the analogue is ranked as suitable with interpretation. The watchout is related to the fact that (1) the target gives rise to ethyl amine as a result of hydrolysis, and (2) the source substance seems to be less bioavailable as compared to the target substance.
Analogue ranking		**Suitable with interpretation**
Structural similarity		
Dice index from OECD Tool box v.4.5	-	0.86
Physico-chemical properties		
Molecular weight (Da)	437.53	452.54
Melting Point (deg C)- Estimated (Experimental) Prediction software: MPBPWIN v.1.44	247.58	204.56
Boiling Point (deg C)- Estimated (Experimental) Prediction software: MPBPWIN v.1.44	574.90	509.44
Vapour Pressure (Pa, 25°C)- Estimated (Experimental) Prediction software: MPBPWIN v.1.44	1.2E-013	5.06E-011
Log Kow - Estimated (Experimental) Prediction software: KOWWIN v1.69	5.03	6.51

	Target substance	Analogue
Water solubility (mg/L) - Estimated (Experimental) Prediction software: WSKOW v1.43	0.091	0.0039
Functional groups and profiling		
Organic groups from OECD TB v.4.5	**Organic functional groups** Alcohol Alkene moiety Alkyl halide Allyl Aryl Cycloalkane Dihydroxyl derivatives Ether moiety Organic amide and thioamide	**Organic functional groups** Alcohol Alkane, branched with secondary carbon Alkene moiety Alkyl halide Allyl Aryl Carboxylic acid ester Cycloalkane Dihydroxyl derivatives Ether moiety Isopropyl
Structural alerts from OECD TB v.4.5	**Estrogen Receptor Binding:** Strong binder, OH group **Toxic hazard classification by Cramer:** High (Class III) **Toxic hazard classification by Cramer (extended):** High (Class III) **Oncologic Primary Classification:** Alpha- and beta-Haloether Reactive Functional Groups	**Estrogen Receptor Binding:** Strong binder, OH group **Toxic hazard classification by Cramer:** High (Class III) **Toxic hazard classification by Cramer (extended):** High (Class III) **Oncologic Primary Classification:** Alpha- and beta-Haloether Reactive Functional Groups

	Target substance	Analogue
Metabolism		
Prediction of the firmetabolic pathway using Meteor Nexus v.3.1.0 and literature data	**Meteor:** Hydrolysis of Acyclic Carboxylic Amides with the formation of tafluprost acid and aromatic hydroxylation (see **Annex II**).	***Literature****:* Tafluprost is an ester prodrug which is rapidly hydrolysed by corneal esterases to form its biologically active acid metabolite. Tafluprost acid is further metabolized via fatty acid β-oxidation and phase II conjugation (CDER, 2011).

Annex IV – Data matrix

Comparison of toxicological data for tafluprost and the two uncommon hydrolytic products (isopropanol and ethylamine)

	Tafluprost	**Isopropanol**	**Ethylamine**
	CAS No. 209860-87-7	CAS No. 67-63-0	CAS No. 75-04-7
Acute toxicity: oral	LD_{50} (Rat): 100 mg/kg bw (CDER, 2011)	LD_{50} (Rat): 4710 - 5840 mg/kg bw; LD_{50} (Mouse): 4475 mg/kg bw; LD_{50} (Rabbit): 5030 mg/kg bw; LD_{50} (Dog): 4830 mg/kg bw (OECD SIDS, 1997)	LD_{50} (Rat): 390-400 mg/kg bw (ECHA, 2023)
Acute toxicity: inhalation	-	LC50 (4 h) (Rat): 72.6 mg/L bw. (OECD SIDS, 1997)	LC50 (4 h) (Rat): 12.6 mg/L bw for male/female (similar to OECD 403) (ECHA, 2023; OECD SIDS, 2011) LC50 (4 h) (Rat): 8000 - 16000 ppm (i.e., 14.75- 29.50 mg/L) (similar to OECD 403) (ECHA, 2023)
Acute toxicity: dermal	-	LD_{50} (Rabbit): 12,870 mg/kg bw (OECD SIDS, 1997)	LD_{50} (Rabbit): 265 - 360 mg/kg bw (ECHA, 2023)
Acute toxicity: parenteral	LD_{50} (Rat): >3 mg/kg bw ((CDER, 2011)		
Skin irritation / corrosion (*in vitro*)	-	-	Corrosive in artificial membrane barrier model (49 CFR 173.136 & 173.173 and similar to OECD 435) (ECHA, 2023)
Skin irritation / corrosion (*in vivo*)	-	Not a skin irritant in rabbits and guinea pigs (No guideline followed) (OECD SIDS, 1997)	Corrosive in rabbits (similar to OECD 404) (ECHA, 2023)
Eye irritation (*in vitro*)	-	Eye irritant (OECD 491)	-

	Tafluprost	**Isopropanol**	**Ethylamine**
	CAS No. 209860-87-7	CAS No. 67-63-0	CAS No. 75-04-7
		(OECD SIDS, 1997)	
Eye irritation (*in vivo*)	Slight eye irritant in rabbit (0.005% and 0.05% of an ophthalmic solution containing tafluprost) (CDER, 2011)	Eye irritant in rabbit (Similar to OECD 405) (OECD SIDS, 1997)	Corrosive in rabbits (ECHA, 2023)
Skin sensitisation	Not a skin sensitiser in GPMT and patch tests (0.005% and 0.05% of an ophthalmic solution containing tafluprost) (CDER, 2011)	Not a skin sensitiser in guinea pigs (Buehler test) (OECD 406) (ECHA, 2022)	-
Repeated dose toxicity: oral	-	27-week study repeated dose in rats via oral drinking water, NOAEL: 600-1000 mg/ kg bw/day based decreased body weight at 2300-3900 mg/kg bw/day --- 12-week repeated dose study in male rat via oral drinking water, NOAEL: 870 mg/ kg bw/day (1%) based on increased relative organ weights of liver, kidneys, and adrenals (OECD SIDS, 1997)	**RA to methylamine (CAS No.74-89-5)** Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test in rats by oral gavage, NOAEL (systemic): 500 mg/kg bw/day based on reductions in parental body weights and food consumption at 1000 mg/kg bw/day (according to OECD 422) (OECD SIDS, 2011)
Repeated dose toxicity: inhalation	-	104-week (6 hours/day, 5 days/week) repeated dose study in Fischer 344 rat via inhalation (whole body), NOEC: 500 ppm based on macroscopic changes such as granular kidney in males and females of 2500 and 5000 ppm groups; (OECD 451) --- 13-weeks (6 hours/day, 5 days/week) repeated dose study in Fischer 344 rats	24-week (6 h/day, 5 days/week) repeated dose study in rat via inhalation, NOEC: 100 ppm based on decreased body weights and histopathological changes of the nasal passages at 500 ppm (no guideline followed) (ECHA, 2023)

	Tafluprost	**Isopropanol**	**Ethylamine**
	CAS No. 209860-87-7	CAS No. 67-63-0	CAS No. 75-04-7
		and CD®-1 mice via inhalation (whole body), NOEC (Mice): 500 ppm based on increased body weight gain at ≥1500 ppm; NOEC (Rat): 1500 ppm based on increased relative liver weight at 5000 ppm (OECD 413) (ECHA, 2022)	120 days repeated dose study in rat via inhalation, NOEC: 100 ppm based on moderate to marked amounts of atrophic rhinitis occurred at 500 ppm (no guideline followed) (ECHA, 2023)
Repeated dose toxicity: parenteral	Repeated dose toxicity potential in rats and dogs via the IV route: NOAELs = 1-100 µg/kg bw/day based on increased respiratory rate, altered cardiac parameters, hyperostosis and myelofibrosis in femoral and sternum bone marrow and haematopoiesis in spleen, liver and male femoral bone marrow, and increased corticomedullary mineralization of the kidney of females at a dose of ≥10 µg/kg bw/day --- Repeated dose toxicity potential in rodents via the SC route: NOAELs = <3-100 µg/kg bw/day based on reduced body weight and histopathology changes including hyperostosis of the sternum and femur in some animals, and increased incidence of extramedullary haematopoiesis in the spleen at ≥3 µg/kg bw/day (CDER, 2011)		

	Tafluprost	**Isopropanol**	**Ethylamine**
	CAS No. 209860-87-7	CAS No. 67-63-0	CAS No. 75-04-7
Repeated dose toxicity: ocular	Repeated dose toxicity potential in monkeys via ocular route: NOAELs =0.067 to 10 µg/kg bw/day based on absence of systemic toxicity up to highest tested dose (CDER, 2011)		
Genetic toxicity *in vitro* (bacteria)	Negative in Ames test with and without S9 (similar to OECD 471) (CDER, 2011)	Negative in Ames test with and without S9 (similar to OECD 471) (ECHA, 2022; OECD SIDS, 1997) Negative in modified ames test with and without S9 (similar to OECD 471) (ECHA, 2022)	Negative in Ames test with and without S9 (no guideline followed) (ECHA, 2023)
Genetic toxicity *in vitro* (cytogenicity study in mammalian cells)	Negative in chromosomal aberration assay (CA) with and without S9 (CDER, 2011)	Negative in Sister chromatid exchange assay with and without S9 (OECD SIDS, 1997)	
Genetic toxicity *in vitro* (mutagenicity study in mammalian cells)		Negative in *in vitro* mammalian cell gene mutation test with and without S9 (similar to OECD 476) (OECD SIDS, 1997)	
Genetic toxicity *in vivo* cytogenicity study in mammalian cells	Negative *in vivo* mammalian erythrocyte MNT in mice via intraperitoneal route (CDER, 2011)	Negative in *in vivo* mammalian erythrocyte MNT in ICR mouse by intraperitoneal route (similar to OECD 474) (ECHA, 2022)	Negative in *in vivo* DNA damage and/or repair (no guideline followed) (ECHA, 2023) **RA to methylamine (CAS No.74-89-5)** Negative in *in vivo* mammalian erythrocyte MNT (OECD SIDS, 2011)
Toxicity to reproduction	Fertility study in rats, via intravenous route: NOAEL (reproductive/systemic) = 100 µg/kg bw/day (HD)	Two-generation reproduction toxicity study in rats by oral gavage route, NOAEL (parental): 500 mg/kg bw/day based on increases in absolute and/or relative liver and/or kidney weights	**RA to methylamine (CAS No.74-89-5)** Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test in rats by oral gavage, NOAEL (systemic

	Tafluprost	Isopropanol	Ethylamine
	CAS No. 209860-87-7	CAS No. 67-63-0	CAS No. 75-04-7
		NOAEL (Reproduction): >1000 mg/kg bw/day based on absence of effects; NOAEL (F1 and F2): 100 mg/kg bw/day based on increased mortality and/or, reduced body weights at ≥500 mg/kg bw/ (Similar to OCED 416) (ECHA, 2022) One-generation reproduction toxicity study in rats by oral drinking water, NOEL (Reproduction): 625-825 mg/kg bw/day for males-females based on reduced pup weight gain and decreased survival (similar to OCED 415) (OECD SIDS, 1997)	and reproductive toxicity): 500 mg/kg bw/day. based on reduced corpora lutea and subsequent reductions in implantations and litter size at 1000 mg/kg bw/day (according to OECD 422) 24-week repeated dose inhalation toxicity study, NOAEC: 922 mg/m^3 based the absence of adverse effects on gonads. (OECD SIDS, 2011)
Developmental toxicity	Pre-natal developmental toxicity study in rats, via intravenous route: Maternal NOAELs = 10-30 µg/kg/day and developmental NOAELs = 0.3-3 µg/kg/day based on the increased number of intrauterine deaths, reduced foetal maturity, and a low incidence of defects of the vertebral column at HD and MD Pre-natal developmental toxicity study in rabbits, via intravenous route: NOAEL (maternal and developmental) = 0.01 µg/kg bw/day (HD) (CDER, 2011)	Pre-natal developmental toxicity study in rats, NOAEL (maternal): 400 mg/kg bw/day based on reduced body weight gain and food consumption; NOAEL (developmental): 400 mg/kg bw/day based on reduced foetal body weights/litter at ≥ 800 mg/kg bw/day (similar to OECD 414) Pre-natal developmental toxicity study in rabbits, NOAEL (maternal): 240 mg/kg bw/day based on reduced body weight and clinical signs of toxicity at 480 mg/kg bw/day NOAEL (developmental): 480 mg/kg bw/day based on the absence of adverse effects (similar to OECD 414)	**RA to methylamine (CAS No.74-89-5)** Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test in rats by oral gavage, NOAEL (Developmental): 1000 mg/kg bw/day based on the absence of adverse effects (according to OECD 422) (OECD SIDS, 2011)

	Tafluprost	**Isopropanol**	**Ethylamine**
	CAS No. 209860-87-7	CAS No. 67-63-0	CAS No. 75-04-7
		Pre-natal developmental toxicity study in rat, NOAEL (maternal): 596 mg/kg bw/day based on decreased food consumption, water consumption, and body weight at ≥ 1242 mg/kg bw/day NOAEL (developmental): 596 mg/kg bw/day based on decreased mean foetal body weight at ≥ 1242 Mg/kg bw/day (similar to OECD 414) (ECHA, 2022) Pre-natal developmental toxicity study in rat, NOAEL (maternal and developmental neurotoxicity): 1200 mg/kg bw/day* based on the absence of adverse effects (similar to OECD 414) (OECD SIDS, 1997)	

** The NOAEL/LOAELs were not established in the source documents but was concluded based on the available details on the adverse effects in the underlying studies.*

Annex V – The cosmetic eyelash product application amount in human eyelashes and mink hair

[redacted]

[redacted] – *Eyelash Application Amount*

Purpose: To determine the average amount of [redacted] that is applied to the upper eyelashes per application.

Study Date: January 11, 2013

Study Coordinator: [redacted], QA Manager, [redacted]

Study Assistant: [redacted] Quality Assurance Technician

Study Description: The average amount of [redacted] that is applied per brushstroke to the upper eyelashes was determined by weighing the applicator brush used to apply [redacted] to the eyelashes in 3 conditions: 1) before being inserted into a standard vial of [redacted] (**"Dry"**); 2) after being inserted into a standard vial of [redacted] (**"Wet Pre-Application"**); and 3) after application of [redacted] by the study volunteer to the upper eyelashes in accordance with the package directions for use (**"Wet Post-Application"**).

The average amount of [redacted] that is applied to the applicator ***brush*** = [Wet Pre-Application – Dry].

The average amount of [redacted] that is applied to the ***eyelashes*** = [Wet Pre-Application – Wet Post-Application].

Study Equipment: Mettler Toledo Analytical Balance Model Number MS303S Serial Number B201596128 (**"Mettler Balance"**).

Study Materials: 20 of the following components from inventory:

- Empty (no [redacted] 3.5mL tubes with brush applicator (sku [redacted]).
- Unopened 3.5mL vials filled with [redacted] (sku [redacted]).

Study Volunteers: Five employees at the [redacted] Distribution Center in [redacted].

Study Procedures:

1. Remove the dry applicator brush from the tube and record its weight on the Mettler Balance.
2. Insert the dry applicator brush into the vial filled with [redacted] (= **Dry weight**).
3. Slightly shake the filled vial with inserted brush.
4. Standing near the Mettler Balance, remove the applicator brush from the tube, place on Mettler Balance and record its weight (= **Wet Pre-Application weight**).
5. Remove the wet applicator brush from the Mettler Balance and give to study volunteer, standing near Mettler Balance. Study volunteer used applicator brush to apply [redacted] to upper eyelashes of one eye in accordance with the package directions for use (one continuous brush stroke to upper eyelashes above the skin).
6. Gently take the applicator brush from the study volunteer, place the applicator brush on the Mettler Balance and weigh the applicator brush (= **Wet Post-Application weight**).
7. Discard the applicator brush, tube and vial just used.
8. Repeat steps 1 – 7 with the same study volunteer; apply [redacted] to the opposite eye.
9. Repeat steps 1-8 with a total of 5 study volunteers (total of 10 eyes).

[redacted]

Page 1

[redacted] – *Eyelash Application Amount*

Study Results: On average, **2.4 mg** of [redacted] was applied to the upper eyelashes per normal brushstroke (see Table 1, below).

Table 1. Weights of applicator brush with no [redacted] ("Dry"), with [redacted] before application (Wet Pre-Application") and after application (Wet Post-Application"). Weights measured using MettlerBalance. All weights are in mg.

Sample	Brush Applicator: Dry	Brush Applicator: Wet Pre-Application	Amount of [redacted] on Applicator *Brush*	Brush Applicator: Wet Post-Application	Amount of [redacted] Applied to *Eyelashes*
1	3814	3823	9	3819	4
2	3850	3855	5	3854	1
3	3860	3866	6	3863	3
4	3827	3834	7	3833	1
5	3812	3818	6	3815	3
6	3855	3861	6	3857	4
7	3846	3855	9	3852	3
8	3876	3881	5	3880	1
9	3814	3821	7	3818	3
10	3828	3831	3	3830	1
Average	**3838.2mg**	**3844. 5mg**	**6.3 mg**	**3842.1 mg**	**2.4 mg**

Report Prepared By: [redacted] Date: 1-11-13

Approved By: [redacted] Date: 1/11/2013

[redacted]

Product Testing Mink Lashes Summary Current Component

Three different "swipe" testing protocols were performed on the componentry to determine the amount of product expressed by the current cosmetic eyelash product applicator.

Procedure 1:

Procedure Summary:

This procedure keeps the same tube and the applicator changes. Each applicator is used for 2 applications.

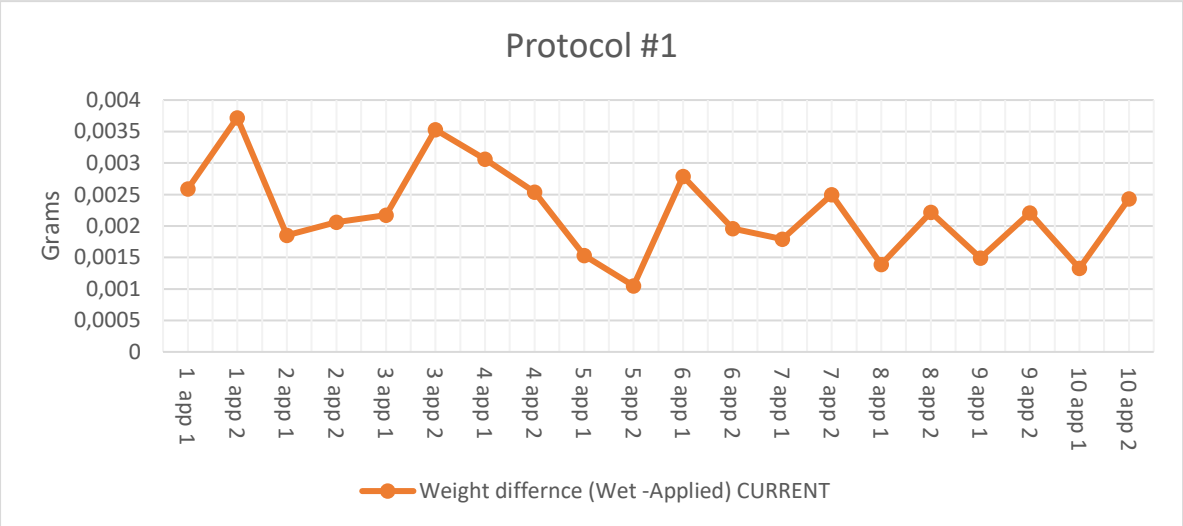
Application 1: Applicator was inserted into the tube, applicator was twisted on all the way, removed, weighed, swiped across measured length of mink eyelash (see photo reference), and then reweighed.

Application 2: Applicator was inserted into the tube, applicator was only pushed down to contact the neck of the tube, applicator was removed, weighed, swiped across a measured length of mink eyelashes, and then reweighed. Then lashes were then discarded, and the applicator set aside. The tube was then set for a rest period. This was done for 10 current applicators.

Current:

Total	0.02410
Average (Total /20)	0.001205
Total App 1	0.01999
Average App 1 (total app 1/10)	0.001999
Total App 2	0.02422
Average App 2 (total app 1/10)	0.002422

Graphical Data:



In terms of molecule consumption per average:

Calculation: $Average_{applied}$ X [DDDE]= $Amount_{DDDE}$ in grams

[DDDE] = 0.00018

1. Average Overall Current: 0.001205 x 0.00018 = 2.169 x 10^{-7} = 0.0000002169 g
2. Average App 1 Current: 0.001999 x 0.00018 = 3.5982 x 10^{-7}= 0.00000035982 g
3. Average App 2 Current: 0.002422 x 0.00018 = 4.3596 x 10^{-7} = 0.00000043596 g

Summary:

All values are less than the 3.4 x 10^{-6} grams of prostaglandin that is mentioned in the [redacted] report. [redacted] stated that [redacted] drop contained .0034 mg of the prostaglandin which converts to 3.4 x 10^{-6} grams.

Procedure 2:

Procedure Summary:

This procedure keeps the same applicator and the tube changes. Each tube is used for 2 applications.

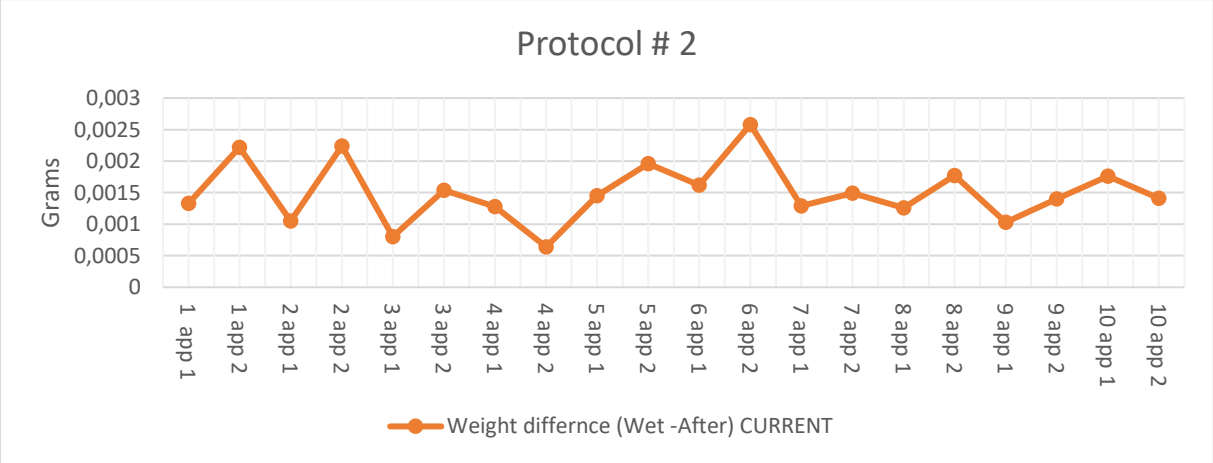
Application 1: Applicator was inserted into the tube, applicator was twisted on all the way, removed, weighed, swiped across measure eyelash (see photo reference), and then reweighed.

Application 2: The applicator was inserted into the tube, applicator was only pushed down to contact the neck of the tube, applicator was removed, weighed, swiped across a measured length of mink eyelashes, and then reweighed. The lashes were then discarded, and the applicator set aside. The applicator was then wiped down with a soft towel and set aside for a drying period. This was done for 10 current tubes.

Current:

Total	0.03012
Average (Total /20)	0.001506
Total App 1	0.01287
Average App 1 (total app 1/10)	0.001287
Total App 2	0.01725
Average App 2 (total app 1/10)	0.001725

Graphical Data:



In terms of molecule consumption per average:

Calculation: $Average_{applied}$ X [DDDE]= $Amount_{DDDE}$ in grams

[DDDE] = 0.00018

1. Average Overall Current: .001506 x .00018 = 2.7108 x 10^{-7} g = 0.00000027108 g
2. Average App 1 Current: .001287 x .00018 = 2.3166 x 10^{-7} g = 0.00000023166 g
3. Average App 2 Current: .001725 x .00018 = 3.105 x 10^{-7} g = 0.0000003105 g

Summary:

All values are less than the 3.4 x 10^{-6} grams of prostaglandin that is mentioned in the [redacted] report. [redacted] stated that [redacted] drop contained .0034 mg of the prostaglandin which converts to 3.4 x 10^{-6} grams.

Original 1 of 1

Study No. CSL-23-0460.04

Study Title

Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide)

Determination of physico-chemical properties
Partition Coefficient (EC A.8. and OECD 117)

Test Guidelines

Regulation EC No. 440/2008 Method A.8.
OECD Test Guideline 117 (2004)

Study Director

Dr. Maike Möller

Study Completion Date

2023-07-05

Sponsor

[redacted]

Test Facility

consilab Gesellschaft für Anlagensicherheit mbH
Industriepark Höchst, G830/G840
65926 Frankfurt am Main
Germany
phone: +49 (0)69 305-16658
fax: +49 (0)69 305-30014

Good Laboratory Practice Compliance Statement

This study was conducted under my direction in compliance with the Principles of Good Laboratory Practice (GLP) as described in

- German Chemicals Act (ChemG), Annex 1, from 2013 in the current version
- OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17, January 21, 1998
- Directive 2004/10/EC, Official Journal of the European Union, L50/44, February 20, 2004

There were no circumstances that might have affected the quality or integrity of the study.

Study Director

2023-07-05
Date

Dr. Maike Möller

Ownership Statement

This report contains the unpublished results of research conducted by consilab Gesellschaft für Anlagensicherheit mbH. These results must not be published, either wholly or in part, or reviewed or quoted in any other publication without the authorization of the sponsor.

Certification of Authenticity

Signatures:

Study Director	2023-07-05	Dr. Maike Möller
	Date	
Test Facility Management	2023-07-05	Dr. Jürgen Franke
	Date	

Quality Assurance Statement

Study Title	Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide) Determination of physico-chemical properties Partition Coefficient (EC A.8. and OECD 117)
Study No.	**CSL-23-0460.04**
Test Guideline	Regulation EC No. 440/2008 Method A.8. OECD Guideline 117 (2004)
Test Facility	consilab Gesellschaft für Anlagensicherheit mbH Industriepark Höchst, G 830 / G 840 65926 Frankfurt am Main, Germany
Study Director	Dr. Maike Möller

This study was periodically inspected. The experimental conduct was inspected on a process-based approach for short-term studies. Properly signed records of these inspections were submitted to the study director and test facility management as listed below. This report has been audited by the quality assurance unit.

Inspection	Phase of Study	Reported
2023-05-03	study plan	2023-05-03
2023-06-21	study plan amendment 1	2023-06-21
2023-07-06	study plan amendment 2	2023-07-06
2023-02-08	process based audit EC A.8.	2023-02-08
2023-07-05	draft report	2023-07-05
2023-07-06	final report	2023-07-06

The reported results accurately reflect the original raw data of the study.

Date: 2023-07-06 Signature:

Tobias Teucke
Quality Assurance Unit (GLP)

DiQualis Deutschland GmbH
Charlottenstraße 7
66119 Saarbrücken, Germany

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1 Study Identification

Sponsor	[redacted]
Study Monitor / Sponsor Representative	Dr. Karsten Schilling IRSC - International Regulatory & Scientific Consulting Paul-Lincke-Str. 36 67304 Eisenberg, Germany
Test Facility	consilab Gesellschaft für Anlagensicherheit mbH Industriepark Höchst, G830/G840 65926 Frankfurt am Main, Germany
Study Director	Dr. Maike Möller
Quality Assurance	Head: Friedrich Kammerer DiQualis GmbH Charlottenstraße 7 66119 Saarbrücken, Germany
Study No.	CSL-23-0460.04
Archives	consilab Gesellschaft für Anlagensicherheit mbH Industriepark Höchst, G830 and G810 65926 Frankfurt am Main, Germany
Involved staff	Diana Thomas
Study starting date	2023-05-03
Experimental starting date	2023-06-05
Experimental completion date	2023-06-05

2 Summary

The partition coefficient at 25 °C of the test item Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide) was determined by means of the HPLC method, according to Regulation EC No. 440/2008 method A.8. and OECD Test Guideline 117 (2004) to be:

$$\log P_{OW} = 2.74 \pm < 0.01$$

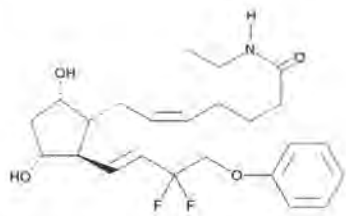
3 Objective

The objective of this study was the determination of the partition coefficient of the test item according to

- Regulation EC No. 440/2008 Part A., Method A.8. Partition Coefficient
- OECD Test Guideline 117 (2004) Partition Coefficient (n-octanol/water) High Performance Liquid Chromatography (HPLC) Method

4 Presentation of the Test Item, Reference Items and Dead Time Marker

4.1 Test Item

Test Item:	Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide)
Chemical name:	N-ethyl-9α,11α-dihydroxy-15,15-difluoro-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide
Batch:	0663687
CAS No.:	1185851-52-8
Structural formula:	
Molecular formula:	$C_{24}H_{33}F_2NO_4$
Molecular weight:	437.5 g/mol
Appearance:	Liquid
Purity:	99.2 % (HPLC)
Certificate of Analysis:	Certificate of Analysis of Tafluprost ethyl amide from [redacted], undated, Product Information Sheet from [redacted], undated and SDS
Storage conditions:	-20 °C
Supplied as:	Clear 10% solution in ethanol
Expiry date:	2023-11-08

4.2 Reference Items

Reference Item 1:	Acetanilide
CAS No.:	103-84-4
Batch No.:	A0402401
Purity:	99.9 % (GC)
Certificate of analysis:	CoA from Acros Organics, ENA23, zone 1, nr 1350, Janssen Pharmaceuticalaan 3a, B-2440 Geel, Belgium, dated 2019-12-09
Storage conditions:	Closed container, dry at room temperature
Expiry date:	2023-11
Reference Item 2:	4-Methyl benzyl alcohol
CAS No.:	589-18-4
Batch No.:	10097989
Purity:	99.9 % (GC)
Certificate of analysis:	CoA from Alfa Aesar
Storage conditions:	Closed container, dry at room temperature
Expiry date:	2024-06-20
Reference Item 3:	Cinnamyl alcohol
CAS No.:	104-54-1
Batch No.:	STBJ9054
Purity:	99.6 % (GC)
Certificate of analysis:	CoA from Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103 USA, dated 2020-08-24
Storage conditions:	Closed container, dry at room temperature
Expiry date:	2026-04-01
Reference Item 4:	Allyl phenyl ether
CAS No.:	1746-13-0
Batch No.:	10233905
Purity:	99.4 % (GC)
Certificate of analysis:	CoA from ThermoFisher Scientific
Storage conditions:	Closed container, dry at room temperature
Expiry date:	2025-03-14

Reference Item 5:	Diphenyl ether
CAS No.:	101-84-8
Batch No.:	10232960
Purity:	99.9 % (GC)
Certificate of analysis:	CoA from ThermoFisher Scientific
Storage conditions:	Closed container, dry at room temperature
Expiry date:	2025-03-14

Reference Item 6:	Fluoranthene
CAS No.:	206-44-0
Batch No.:	LRAD3185
Purity:	98.8 % (mass balance)
Certificate of analysis:	CoA from Supelco, 595 North Harrison Road, Bellefonte, PA 16823-0048 USA, dated 2022-10-18
Storage conditions:	Closed container, dry at room temperature
Expiry date:	2025-09

4.3 Dead Time Marker

Dead time Marker:	Formamide
CAS No.:	75-12-7
Batch No.:	Y22D003
Purity:	99.6 % (GC)
Certificate of analysis:	CoA from Alfa Aesar
Storage conditions:	Closed container, dry at room temperature
Expiry date:	2023-07-07

5 Test Methods

5.1 Test Principle

The partition coefficient (P) is defined as the ratio of the equilibrium concentrations (c_i) of a dissolved substance in a two-phase system consisting of two largely immiscible solvents. In the case of 1-octanol and water:

$$P_{ow} = c_{1\text{-octanol}} / c_{water}$$

The partition coefficient (P) therefore is the quotient of two concentrations and is usually given in the form of its logarithm to base 10 (log P).

For the determination of the partition coefficient three separate procedures can be performed: the shake flask method, the high performance liquid chromatography (HPLC) method and the slow stirring method. The first method is applicable when the log P_{OW} value falls within the range -2 to 4, the second within the range 0 to 6 and the last for highly hydrophobic substances (log P_{OW} values ≥ 5). Before carrying out either of the experimental procedures a preliminary estimate of the partition coefficient should first be obtained.

In this study the HPLC method was used. The principle of the HPLC method is based on a reverse phase HPLC, performed on analytical columns packed with a solid phase containing long hydrocarbon chains (e.g. C8, C18) chemically bound onto silica. The test item injected on such a column partitions between the mobile solvent phase and the hydrocarbon stationary phase as it is transported along the column by the mobile phase. The chemicals are retained in proportion to their hydrocarbon-water partition coefficient, with hydrophilic chemicals eluted first and lipophilic chemicals last. As mobile phase eluents of methanol/water ratios with a minimum water content of 25 % should be used. Alternatively, acetonitrile or 2-propanol can be used instead of methanol. The retention time is described by the capacity factor *k* given by the expression:

$$k = \frac{t_R - t_0}{t_0}$$

where t_R is the retention time of the test item, and t_0 is the dead-time, i.e. the average time a solvent molecule needs to pass the column. As dead time markers, unretained organic substances as thiourea or formamide have to be used. Quantitative analytical methods are not required and only the determination of retention times is necessary.

The octanol/water partition coefficient of the test item can be computed by experimentally determining its capacity factor *k* and then inputting *k* into the following equation:

$$\log P_{OW} = a + b \cdot \log k$$

where a, b = linear regression coefficients.
The equation above can be obtained by linearly regressing the log of octanol/water partition coefficients of reference substances against the log of capacity factors of the reference substances.

5.2 Equipment and materials

- **Laboratory equipment**
  - HPLC System, 1100/1200 Series, Agilent
  - Analytical Balance XS 205 Dual Range, Mettler Toledo
  - 100 and 1000 µl variable pipettes, Eppendorf
- **General laboratory equipment**
- **Materials**
  - Methanol, VWR
  - Buffer solution pH 7:
    - Disodium hydrogen phosphate dihydrate, Merck
    - Potassium dihydrogen phosphate, Merck
    - Sodium hydroxide (1M), Merck
  - Reference items and dead time marker (chapter 4)
  - Double distilled water, Roth

5.3 Test Procedure

The tests were performed according to the following SOPs:

- SOP-LA-241 Determination of the partition coefficient log P_{OW} via HPLC method (EC A.24., OECD 117)
- SOP-PG-020 High Performance Liquid Chromatography (HPLC)

5.4 Test Description

5.4.1 Preliminary Test

Since only a small amount of the test item was available, the preliminary test for the solubility in octanol was not performed.

A pre-test for the HPLC method was carried out, which showed that the HPLC method was suitable for the determination of the partition coefficient. This pre-test is not reported.

5.4.2 Main Test

The main test was performed by isocratic elution with methanol/buffer solution pH 7 in a ratio of 75 : 25 % (pH 6.9). The analytical method used is described in chapter 5.4.3.

For the main test 24.2 mg of the dead time marker formamide were dissolved in 25 mL methanol.

Between 7.4 mg and 33.8 mg of the reference items were dissolved in 25 mL methanol. Acetanilide was diluted again from 1 to 5 mL with methanol and cinnamyl alcohol was diluted again from 1 to 20 mL with methanol.

0.75 mL of the stock solution (100 mg/L) of the test item in ethanol, which was used for the calibration in study CSL-23-0460.03, was diluted to 1 mL with double distilled water.

The dead time marker was injected and measured twice before the reference items and the test item. After that two series of measurements of the reference and test items were performed, wherein the reference substances and the test item were injected separately into the mobile phase.

5.4.3 HPLC Method

The analytical HPLC method used for the determination of the retention times is described in **Table 1**.

Table 1: Analytical HPLC method

Apparatus:	Agilent 1100/1200 System: degasser G1322A, quaternary pump G1311A, autosampler G1329A, ALS thermostat G1330B, COLCOM column oven G1316A, VWDExch. UV-detector G1314A
Stationary phase:	Zorbax Eclipse Plus C18, Agilent Technologies
Column inner diameter:	4.6 mm
Pore size:	5 µm
Column temperature:	25 °C
Injection volume:	10 µL
Solvent flow:	1 mL/min
Wave length:	Dead time marker: 210 nm Reference items: 254 nm Test Item: 205 nm
Mobile phase:	Isocratic: methanol / buffer solution pH 7 75:25 % (v/v) (pH 6.9)

5.4.3.1 Quality criteria

The value of *log* P_{ow} derived from repeated measurements made under identical conditions and using the same set of reference items should fall within a range of ±0.1 *log* units.
Typically, the *correlation coefficient r* for the relationship between *log k* and *log* P_{ow} for a set of test substances is around 0.9, corresponding to an octanol/water partition coefficient of *log* P_{ow} ±0.5 *log* units.

6 Results

6.1 Main Test

After the measurements of the retention times t_R the *log k* values of the reference items were plotted as a function of their *log* P_{OW} values (**Figure 2** and **Figure 3**).

The partition coefficient of the test item was obtained by interpolation of the calculated capacity factor on the calibration graph of the reference items.

Table 2 shows the measurements of the dead time marker.

Table 2: Retention time of the dead time marker

	1st run	2nd run	mean value
t_0 (min)	2.425	2.422	2.424

Table 3 shows the results of the first measurement series of the reference items.

Table 3: Retention times of the reference items (1st measurement)

reference item	t_R	*k*	*log k*	*log* P_{OW}[1]
Acetanilide	3.123	0.289	-0.540	1.0
4-Methyl benzyl alcohol	3.742	0.544	-0.264	1.6
Cinnamyl alcohol	3.812	0.573	-0.242	1.9
Allyl phenyl ether	7.213	1.976	0.296	2.9
Diphenyl ether	12.652	4.221	0.625	4.2
Fluoranthene	26.130	9.782	0.990	5.1

The first measurement and the linear regression of the calibration graph leads to an equation for the *log* P_{OW} of:

$$\log P_{OW} = 2.681 \cdot \log k + 2.396$$

$$correlation\ coefficient\ r = 0.9947$$

Table 4 shows the results of the second measurement series of the reference items.

Table 4: Retention times of the reference items (2nd measurement)

reference item	t_R	*k*	*log k*	*log* P_{OW}[1]
Acetanilide	3.123	0.289	-0.540	1.0
4-Methyl benzyl alcohol	3.742	0.544	-0.264	1.6
Cinnamyl alcohol	3.814	0.574	-0.241	1.9
Allyl phenyl ether	7.219	1.979	0.296	2.9
Diphenyl ether	12.656	4.222	0.626	4.2
Fluoranthene	26.143	9.787	0.991	5.1

The second measurement and the linear regression of the calibration graph leads to an equation for the *log* P_{OW} of:

$$\log P_{OW} = 2.681 \cdot \log k + 2.396$$

$$correlation\ coefficient\ r = 0.9947$$

[1] Literature values of *log* P_{OW} from OECD Test Guideline 117 (2004)

With the determined regression coefficients *a* and *b*, the *log* P_{OW} of the test item can be calculated for both measurements. The HPLC chromatogram of the test item is shown in **Figure 1**.

Table 5 shows the measured retention times of the test item and the calculated *log* P_{OW} values for both measurement series.

Table 5: Retention times of the test item

test item	t_R	*k*	*log k*	*log* P_{OW}	P_{OW}
1st measurement	5.675	1.342	0.128	2.739	548
2nd measurement	5.679	1.343	0.128	2.739	549
			mean value	**2.74**	548
			standard deviation	**< 0.01**	<1

According to Regulation EC No. 440/2008 method A.8. and OECD Test Guideline 117 the test item has a partition coefficient at 25 °C of

$$log\ P_{OW} = 2.74 \pm < 0.01$$

6.2 Final Results

The partition coefficient at 25 °C of the test item Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide) was determined by means of the HPLC method, according to Regulation EC No. 440/2008 method A.8. and OECD Test Guideline 117 (2004) to be:

$$\log P_{OW} = 2.74 \pm < 0.01$$

7 Figures

Data File C:\CHEM32\1\DATA\CSL_23_0460B 2023-06-05 12-16-29\23_0460_0000023.D
Sample Name: CSL-23-0460.04

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Acq. Instrument  : Instrument 1                 Location : Vial 18
Injection Date   : 6/5/2023 12:53:47 PM              Inj :  1
                                              Inj Volume : 10.0 µl
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                   (modified after loading)
Method Info      : CSL-23-0460.04
                   HPLC-System 1
                   Säule 13  Säulen Nr. USUXA47267    Lot-Nr. B22032
                   Zorbax Eclipse Plus   C18

                   Methode für Prüfgegenstand

Additional Info  : Peak(s) manually integrated
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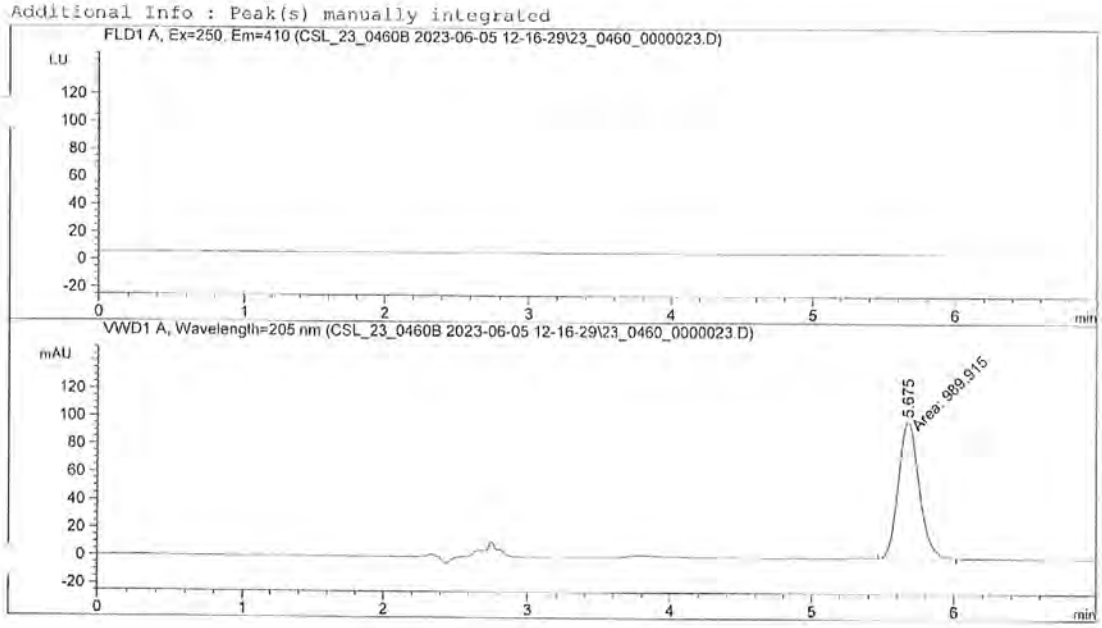


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Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: FLD1 A, Ex=250, Em=410
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Instrument 1 6/5/2023 1:02:59 PM DP

Page 1 of 2

Figure 1: HPLC chromatogram of the test item (Page 1 of 2)

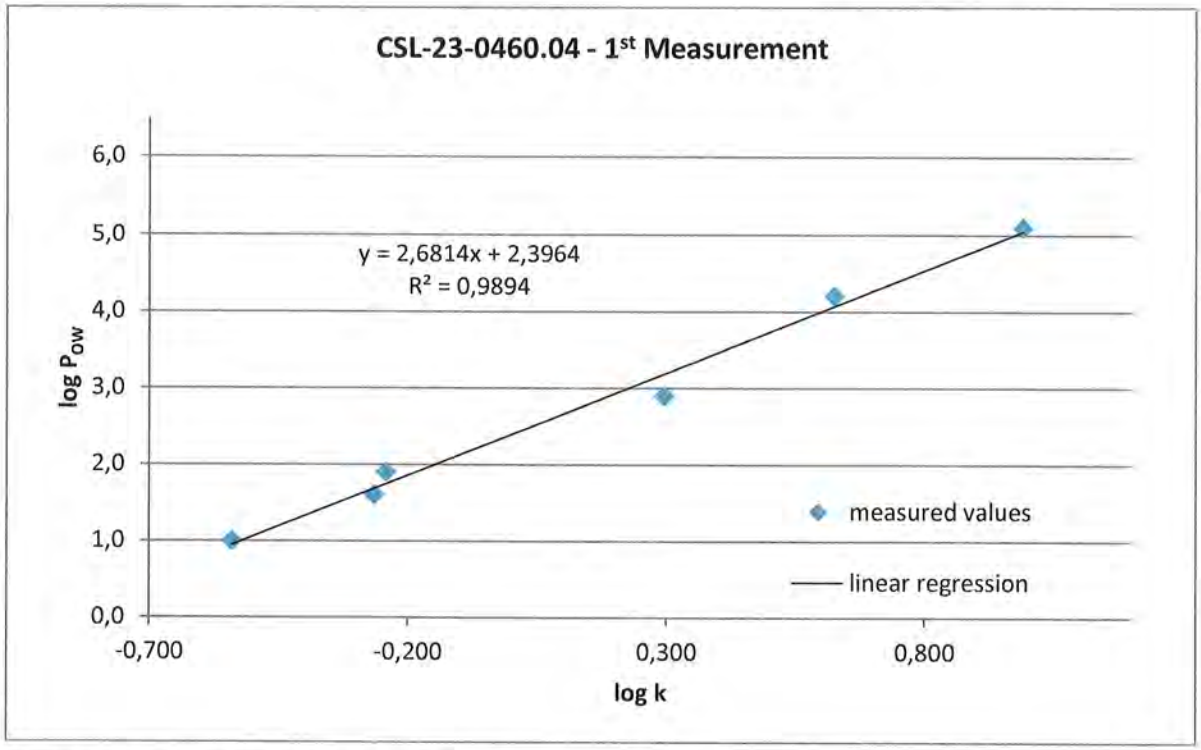


Figure 2: Calibration curve of the first measurement series

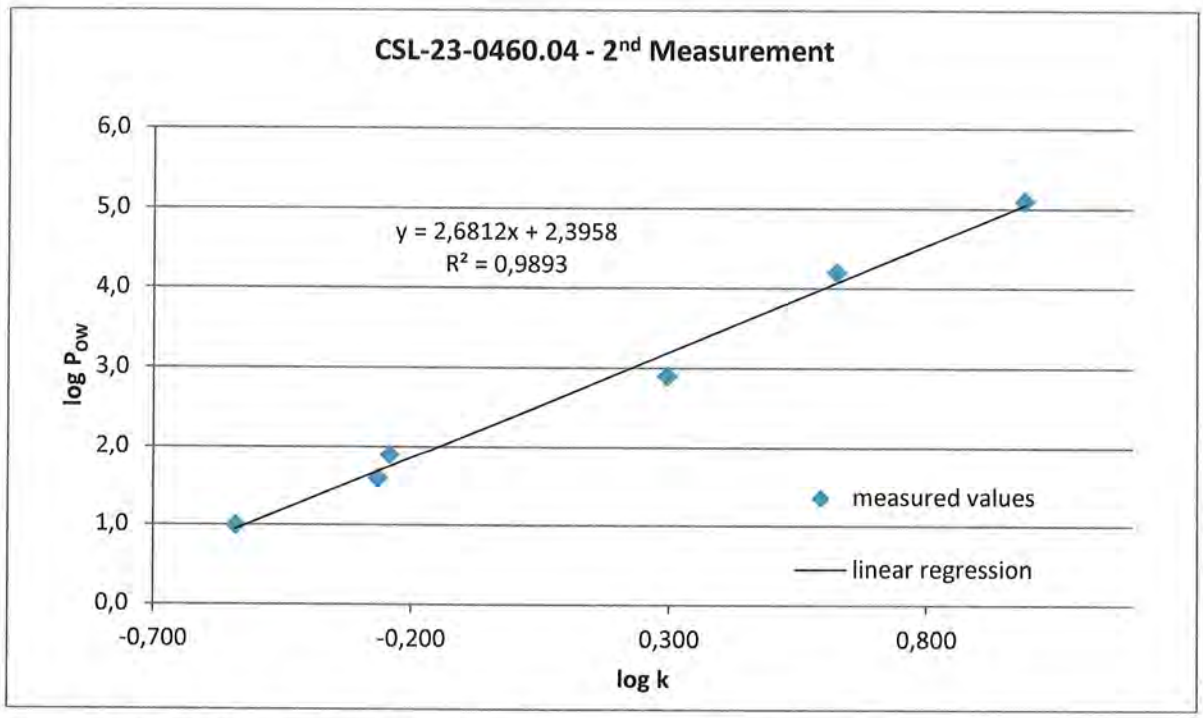


Figure 3: Calibration curve of the second measurement series

8 Appendix

8.1 Certificate of the Test Item

[redacted]

CERTIFICATE of ANALYSIS

Tafluprost ethyl amide
N-ethyl-9α,11α-dihydroxy-15,15-difluoro-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide
Item No. 9000843 • Batch No. 0663687

Purity Specification: ≥98%
Molecular Formula : C24H33F2NO4
CAS Number: 1185851-52-8 Formula Weight: 437.5 Expiry date: 08NOV2023

Overview

Tests	Results
HPLC	Purity: 99.2 %
Mass spec	MH+: 437.9
TLC	Purity: 100 %

Reviewed and approved by: [redacted]

[redacted]

WARNING
THIS PRODUCT IS FOR RESEARCH USE - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE. IT IS THE RESPONSIBILITY OF THE PURCHASER TO DETERMINE SUITABILITY FOR OTHER APPLICATIONS.

SAFETY DATA
This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent via email to your institution.

WARRANTY AND LIMITATION OF REMEDY
Buyer agrees to purchase the material subject to [redacted] Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

[redacted]

8.2 GLP Certificate of the Test Facility

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

consilab Gesellschaft für Anlagensicherheit mbH
Industriepark Höchst, Geb.G 830
65926 Frankfurt am Main

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

1 Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen

1 Physical-chemical testing

5 Prüfungen zum Verhalten im Boden, im Wasser und in der Luft, Prüfungen zur Bioakkumulation und zur Metabolisierung

5 Studies on behaviour in water, soil and air; bioaccumulation

23.11.2022 bis 24.11.2022
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Brandt

Dr. Astrid Brandt, Referentin, Wiesbaden, den 21.März 2023
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

Hess. Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz, Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

Original 1 of 1

Study No. CSL-23-0460.03

Study Title

Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide)

Determination of physico-chemical properties
Water Solubility (EC A.6. and OECD 105)

Test Guidelines

Regulation (EC) No 440/2008 Method A.6.
OECD Test Guideline 105 (1995)

Study Director

Dr. Maike Möller

Study Completion Date

2023-07-26

Sponsor

[redacted]

Test Facility

consilab Gesellschaft für Anlagensicherheit mbH
Industriepark Höchst, G830/G840
65926 Frankfurt am Main
Germany
phone: +49 (0)69 305-16658
fax: +49 (0)69 305-30014

Good Laboratory Practice Compliance Statement

This study was conducted under my direction in compliance with the Principles of Good Laboratory Practice (GLP) as described in

- German Chemicals Act (ChemG), Annex 1, Bundesgesetzblatt 2013, part I No. 55, September 06, 2013
- OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17, January 21, 1998
- Directive 2004/10/EC, Official Journal of the European Union, L50/44, February 20, 2004

There were no circumstances that might have affected the quality or integrity of the study.

Study Director 2023-07-26 Dr. Maike Möller

Date Dr. Maike Möller

Ownership Statement

This report contains the unpublished results of research conducted by consilab Gesellschaft für Anlagensicherheit mbH. These results must not be published, either wholly or in part, or reviewed or quoted in any other publication without the authorization of the sponsor.

Certification of Authenticity

Signatures:

Study Director	2023-07-26	Dr. Maike Möller
	Date	
Test Facility Management	2023-07-26	Dr. Jürgen Franke
	Date	

Quality Assurance Statement

Study Title	Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide) Determination of physico-chemical properties Water Solubility (EC A.6. and OECD 105)
Study No.	**CSL-23-0460.03**
Test Guideline	Regulation (EC) No 440/2008 Method A.6. OECD Test Guideline 105 (1995)
Test Facility	consilab Gesellschaft für Anlagensicherheit mbH Industriepark Höchst, G 830 / G 840 65926 Frankfurt am Main, Germany
Study Director	Dr. Maike Möller

This study was periodically inspected. The experimental conduct was inspected on a process-based approach for short-term studies. Properly signed records of these inspections were submitted to the study director and test facility management as listed below. This report has been audited by the quality assurance unit.

Inspection	Phase of Study	Reported
2023-05-03	study plan	2023-05-03
2023-07-25	study plan amendment 1	2023-07-25
2023-06-29	process based audit EC A.6.	2023-06-29
2023-07-25	draft report	2023-07-25
2023-07-27	final report	2023-07-27

The reported results accurately reflect the original raw data of the study.

Date: 2023-07-27 Signature:

Tobias Teucke
Quality Assurance Unit (GLP)

DiQualis Deutschland GmbH
Charlottenstraße 7
66119 Saarbrücken, Germany

Table of Contents Page

1 Study Identification

Sponsor	[redacted]
Study Monitor	Dr. Karsten Schilling IRSC - International Regulatory & Scientific Consulting Paul-Lincke-Str. 36 67304 Eisenberg, Germany
Test Facility	consilab Gesellschaft für Anlagensicherheit mbH Industriepark Höchst, G830/G840 65926 Frankfurt am Main, Germany
Study Director	Dr. Maike Möller
Quality Assurance	Head: Friedrich Kammerer DiQualis Deutschland GmbH Charlottenstraße 7 66119 Saarbrücken, Germany
Study No.	CSL-23-0460.03
Archives	consilab Gesellschaft für Anlagensicherheit mbH Industriepark Höchst, G830 and G810 65926 Frankfurt am Main, Germany
Involved staff	Diana Thomas, Katharina Gemmel
Study starting date	2023-05-03
Experimental starting date	2023-06-06
Experimental completion date	2023-07-12

2 Summary

According to OECD Test Guideline 105 (1995) and Regulation (EC) No 440/2008 Method A.6. the water solubility at 20 °C of the pure test item Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide) was determined to be:

c_S = 1.05 g/L
RSD = 3.6 %

3 Objective

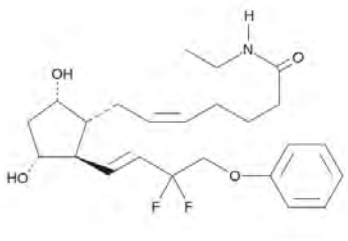
The objective of this study was the determination of the water solubility of the test item according to

- Regulation (EC) No 440/2008, Method A.6. Water Solubility
- OECD Test Guideline 105 (1995) Water Solubility

The following guidelines also apply to the results:

- US EPA OCSPP Test Guideline OPPTS 830.7840 (1998): Water Solubility: Column Elution Method; Shake Flask Method

4 Presentation of the Test Item

Test Item:	Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide)
Chemical name:	N-ethyl-9α,11α-dihydroxy-15,15-difluoro-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide
Batch:	0663687
CAS No.:	1185851-52-8
Structural formula:	
Molecular formula:	$C_{24}H_{33}F_2NO_4$
Molecular weight:	437.5 g/mol
Appearance:	Liquid
Purity:	99.2 % (HPLC)
Certificate of Analysis:	Certificate of Analysis of Tafluprost ethyl amide from [redacted], undated, Product Information Sheet from [redacted], undated and SDS
Storage conditions:	-20 °C
Supplied as:	Clear 10% solution in ethanol
Expiry date:	2023-11-08

5 Test Methods

5.1 Test Principle

The determination of the water solubility can be carried out by two different methods. The flask method is used for test items with an expected solubility in water of > 10 mg/L, and the column elution method is used for test items with an expected solubility in water of < 10 mg/L.

For the estimation of the water solubility, normally, a preliminary test is performed to decide which method is used. In this case, no preliminary test was performed due to the low amount of test item. Although the water solubility should be low, according to the sponsor, the flask method is performed, because a larger amount of test item would be necessary for the performance of the column elution method.

Using the flask method for determination of the water solubility the test item is dissolved in water at a temperature of 30 °C. When saturation is achieved, the mixture is cooled and kept at the test temperature (20 ± 0.5 °C). Subsequently, the mass concentration of the test item in the aqueous solution, which must not contain any undissolved particles, is determined by HPLC.

5.2 Equipment and materials

- **Laboratory equipment**
  - HPLC System, 1100/1200 Series, Agilent
  - Analytical Balance XS 205 Dual Range, Mettler Toledo
  - pH-meter, SevenCompact Duo S213, Mettler Toledo
  - Glass electrode, InLab®Routine Pro, Mettler-Toledo
  - Centrifuge, 2-16PK, Sigma
  - Climate chamber KBF 115, Binder
  - 100, 200, 1000 and 5000 µL variable pipettes, Eppendorf
  - Liquid-in-glass thermometer, THM20
- **General laboratory equipment**
- **Materials**
  - Buffer solutions for the calibration of the pH meter, pH 4.01, 7.00 and 9.21, Mettler-Toledo
  - Methanol, BDH Prolabo
  - Ethanol, Merck
  - Buffer solution pH 7:
    - Disodium hydrogen phosphate dihydrate, Merck
    - Potassium dihydrogen phosphate, Merck
    - Sodium hydroxide (1M), Merck
  - Double distilled water, Roth
  - HCl, Merck

5.3 Test Procedure

The test was performed according to the following SOPs:

- SOP-LA-061 Determination of the water solubility by means of the flask method (EC A.6., OECD 105)
- SOP-PG-020 High-performance liquid chromatography (HPLC)

5.4 Test Description

5.4.1 Preliminary Test

Due to the small amount of test item and the log P_{OW} of 2.74, determined in study no. CSL-23-0460.04, the preliminary test for solubility in water was not performed.

5.4.2 Main Test

Since, the amount of test item was too low for performing the column-elution-method, the flask method was carried out.

The test item was supplied dissolved in ethanol (10%). For the flask method, the ethanol was removed before the test. For this purpose, small amounts of the test item were filled in three glasses (1a, 2a, 3a). Each flask was placed in a glass bottle and purged with a nitrogen flow under reduced pressure (310 - 350 mbar) at approx. 25 °C for 1.5 h to 2 h. The pure test item appeared in the flasks as clear waxy layer.

After reweighing, water was added to the pure test item in the three flasks and the slightly milky mixtures were stirred at 30 °C for different periods of time (24, 48 and 72 h). After the stirring times the mixtures were still milky so that enough test item was in the flasks to ensure the saturation of the solution.

After stirring at 30 °C the flasks were cooled down to 20 °C and stirred at 20 °C for another 125 h. After 125 h stirring time the contents of the flasks were centrifuged for 30 min at 20 °C with 15000 rpm. After centrifugation the water phases were clear and test item could be observed at the walls of the centrifugation tubes.

For the analytical measurement by means of HPLC the centrifuged clear aqueous phases were diluted from 0.02 mL to 1 mL with ethanol.

One additional flask was prepared as blank measurement (4a). Therefore, distilled water was added to the flask, stirred for 72 h at 30 °C and treated like the other samples.

The masses and stirring times for the four flasks are shown in **Table 1**.

Table 1: Flask method for the determination of the water solubility

Flask	**1a**	**2a**	**3a**	**4a**
Mass of ethanolic test item solution (10%) [mg]	406.94	412.98	407.31	--
Pure test item after removing ethanol [mg]	40.77	41.95	41.43	--
Volume of water [mL]	2	2	2	2
Stirring time at 30 °C [h]	72	48	24	72
Stirring time at 20 °C [h]	125	125	125	125
pH value at 28 °C	7.4	7.4	7.4	6.6

5.5 Analytical HPLC Method

The analytical HPLC method used for the determination of the concentrations in the flasks is described in **Table 2**.

Table 2: Analytical HPLC method

Apparatus:	Agilent 1100/1200 System: degasser G1322A, quaternary pump G1311A, autosampler G1329A, ALS thermostat G1330B, COLCOM column oven G1316A, VWDExch. UV-detector G1314A
Stationary phase:	Zorbax Eclipse Plus C18, Agilent Technologies
Column inner diameter:	4.6 mm
Pore size:	5 µm
Column temperature:	25 °C
Injection volume:	10 µL
Solvent flow:	1 mL/min
Wave length:	Test Item: 205 nm
Mobile phase:	Isocratic: methanol / buffer solution pH 7 75:25 % (v/v) (pH 7.0)

5.5.1 Calibration and Validation of the Analytical Method

The analytical method described in section **5.5** was validated based on guideline SANTE/2020/12830 - and found to be valid.

5.5.2 Specificity

The specificity of the analytical method was demonstrated by comparing the chromatograms for the calibration with the test item and the blank experiment (see **Figure 2** and **Figure 4**). No interferences could be observed. The signals between retention times of 2 and 4.5 minutes belong to ethanol.

5.5.3 Linearity

The HPLC apparatus was calibrated using eight different concentrations in the range 1.25 mg/L to 50 mg/L of the test item (external standard method). The test item was diluted with ethanol. Each concentration was injected and measured twice.

The calibration graph is shown in **Figure 1**.

Calibration: $y = mx + b$

$m = 12.76423$

$b = 1.62779$

correlation $r = 0.99898$

The correlation factor **r** for the calibration is **> 0.995**.

5.5.4 Limit of Quantification (LOQ)

The LOQ was defined as the lowest validated concentration with sufficient recovery and precision.

LOQ = 3.5 mg/L

5.5.5 Limit of Detection (LOD)

The LOD was defined as the lowest detectable concentration of the test item, expressed as lowest calibration standard.[1]

LOD = 1.25 mg/L

5.5.6 Accuracy and Precision

The recovery rates of two sets, each of five separate weighed-in samples of the same concentrations, were determined. The accuracy was obtained as mean value of the individual recovery rates. The precision is expressed as the relative standard deviation of the recovery rates (see **Table 3** and **Table 4**)[2].

The acceptance criterion for the accuracy is: 70 – 120 %.

The acceptance criterion for the precision is: ≤ 20 %.

The values represented in the tables are rounded values. The calculation was carried out with the original not rounded values.

Table 3: Accuracy and precision of the analytical method (concentration: 40 mg/L)

References	Nominal concentration [mg/L]	Measured concentration [mg/L]	Recovery rate [%]
Standard 1	40.0	41.3	103.3
Standard 2	40.0	41.7	104.3
Standard 3	40.0	40.0	100.0
Standard 4	40.0	39.5	98.8
Standard 5	40.0	40.0	100.0
Mean recovery rate = Accuracy [%]			**101.3**
RSD = Precision [%]			**2.3**

Table 4: Accuracy and precision of the analytical method (concentration: 3.5 mg/L)

References	Nominal concentration [mg/L]	Measured concentration [mg/L]	Recovery rate [%]
Standard 6	3.50	3.49	99.8
Standard 7	3.50	3.40	97.3
Standard 8	3.50	3.36	96.1
Standard 9	3.50	3.37	96.2
Standard 10	3.50	3.38	96.7
Mean recovery rate = Accuracy [%]			**97.2**
RSD = Precision [%]			**1.6**

[1] SANTE/2020/12830, p. 12
[2] SANTE/2020/12830, p. 12-13

6 Results

6.1 Main Test (flask method)

For the main test three flasks were prepared with an amount of the pure, dried test item and water, and one flask only with water. After stirring and centrifugation the aqueous phases were diluted with ethanol and measured by means of HPLC. The solution of each flask has been measured twice. The results of these measurements are shown in **Table 5**. The values represented in the table are rounded values. The calculation was carried out with the original not rounded values.

Table 5: Results of the flask method

Flask	1a	2a	3a	4a
measured value [mg/L]	20.93 / 20.70	20.10 / 20.57	21.56 / 22.07	0 / 0
aliquot of the flask [mL]	0.02	0.02	0.02	0.02
total volume after dilution [mL]	1	1	1	1
water solubility [mg/L]	1046 / 1035	1005 / 1028	1078 / 1103	0 / 0
average water solubility [g/L]	1.04	1.02	1.09	0
mean value of the water solubility [c_s*, g/L]	**1.05**			0
standard deviation [mg/L]	0.04			--
relative standard deviation [RSD, %]	**3.6**			--
*c_s = water solubility				

To verify the accuracy of the analytical method a standard solution (prepared with an ethanolic test item standard solution without drying) was measured in between the samples of the flask method. The results of these measurements are shown in **Table 6**.

Table 6: Measurement of a standard solution

Standard solution (nominal value [mg/L])	Measured value [g/L]	Repeatability [%]
Ü-1 (10.0)	10.3	102.7
	10.2	102.3
Ü-2 (20.0)	20.3	101.3
	20.3	101.6

The recovery rate of the measured standard solutions was in the range from 101.3 % to 102.7 %, which shows a good accuracy of the analytical method.

6.2 Final Results

According to OECD Test Guideline 105 (1995) and Regulation (EC) No 440/2008 Method A.6. the water solubility at 20 °C of the pure test item Tafluprost ethyl amide (INCI: Ethyl Tafluprostamide) was determined to be:

c_S = 1.05 g/L
RSD = 3.6 %

7 Figures

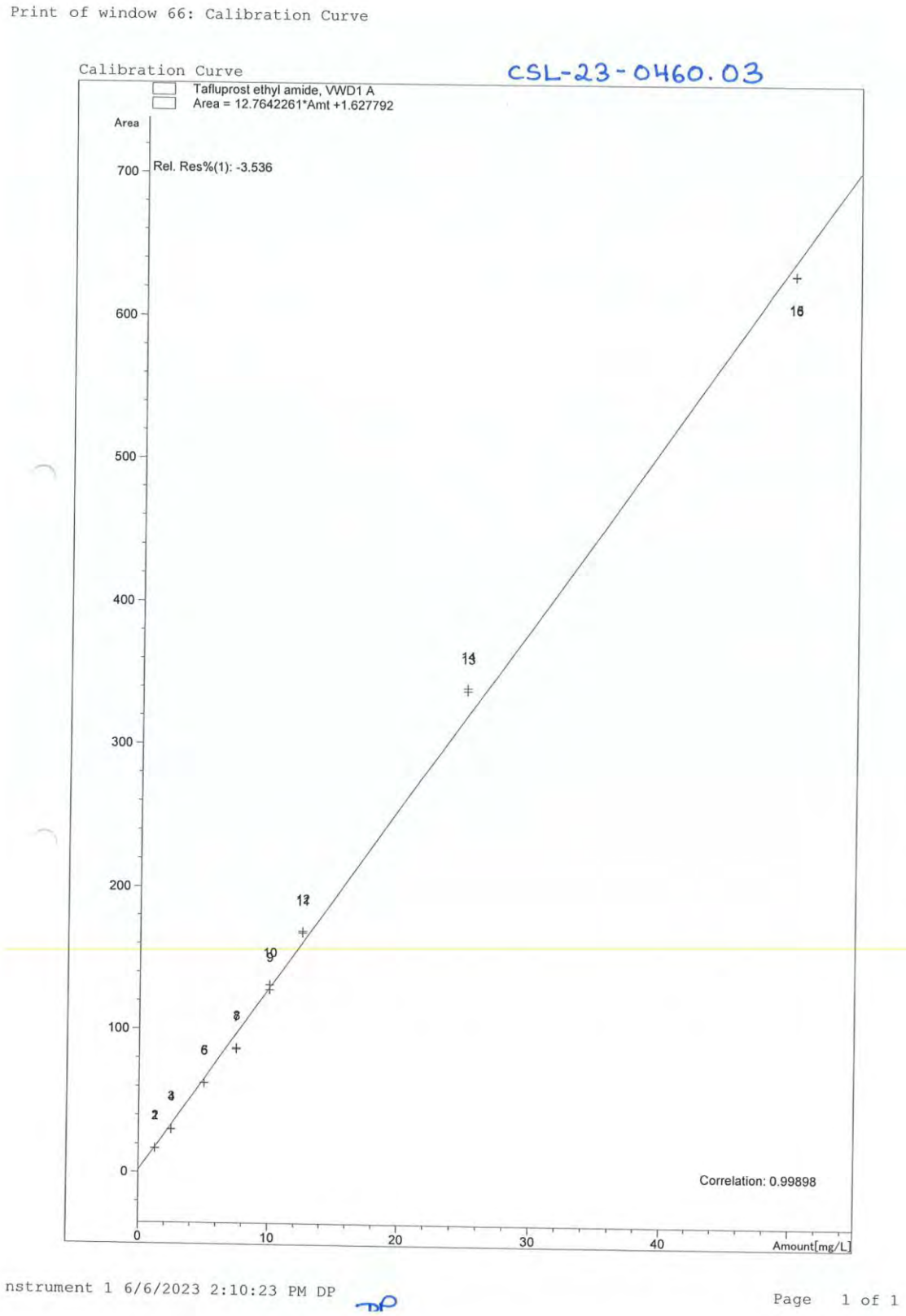


Figure 1: Calibration curve for the HPLC measurements

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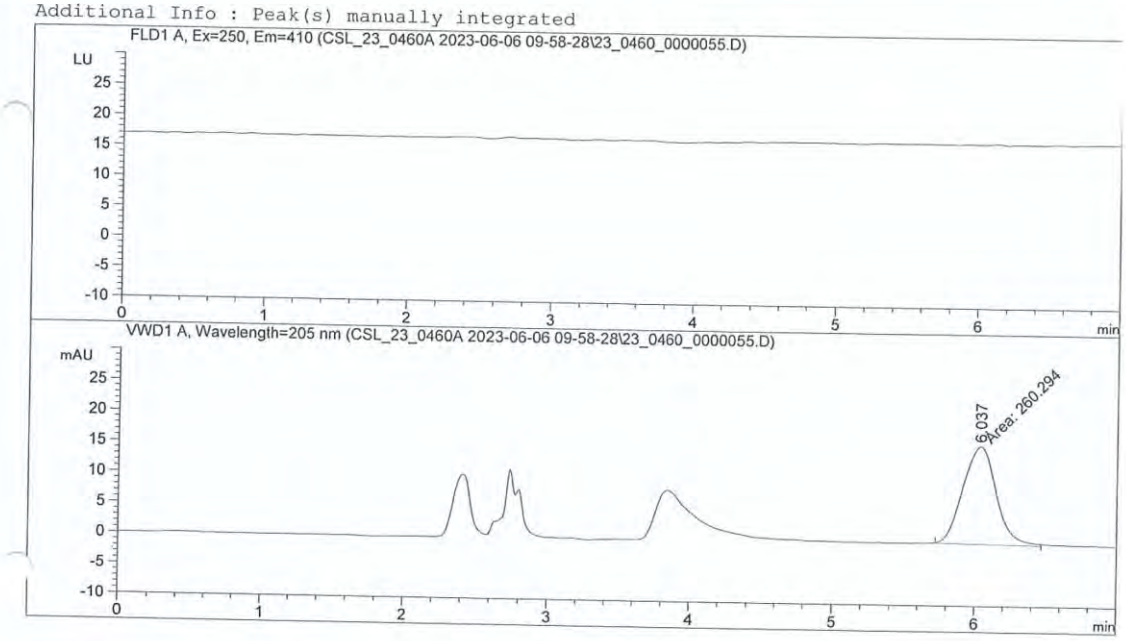
Additional Info : Peak(s) manually integrated



External Standard Report

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Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 2: VWD1 A, Wavelength=205 nm

RetTime [min]	Type	Area mAU *s	Amt/Area	Amount [mg/L]	Grp	Name
6.037	MM	260.29352	7.78540e-2	20.26490		Tafluprost ethyl amide
Totals :				20.26490		

Instrument 1 7/12/2023 12:10:53 PM DP

Figure 2: HPLC chromatogram of the test item (Standard Ü-2, c = 20.0 mg/L)

Data File C:\CHEM32\1\DATA\CSL_23_0460A 2023-06-06 09-58-28\23_0460_0000056.D
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Acq. Instrument	: Instrument 1	Location	: Vial 46
Injection Date	: 7/12/2023 11:39:14 AM	Inj	: 1
		Inj Volume	: 10.0 µl
Acq. Method	: C:\CHEM32\1\DATA\CSL_23_0460A 2023-06-06 09-58-28\CSL_23_0460_03_C.M		
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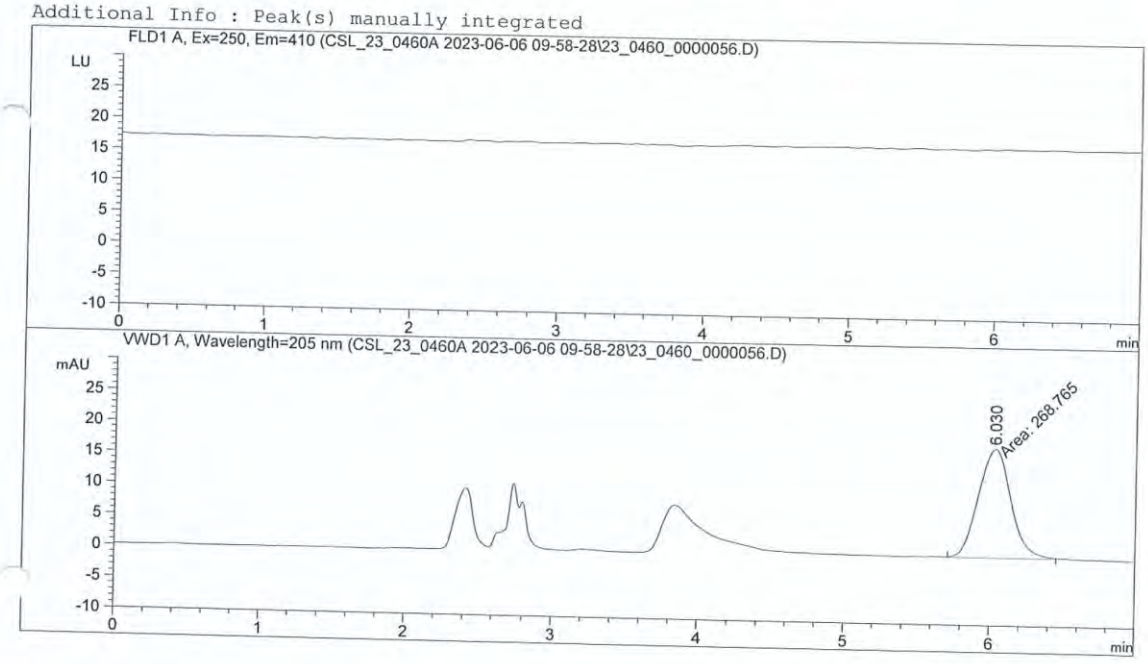
Analysis Method	: C:\CHEM32\1\METHODS\CSL_23_0460_03_C.M		
Last changed	: 7/12/2023 12:09:38 PM by DP (modified after loading)		
Method Info	: CSL-23-0460.04 Kolbenmethode A.6. HPLC-System 1 Säule 13 Säulen Nr. USUXA47267 Lot-Nr. B22032 Zorbax Eclipse Plus C18		

Additional Info : Peak(s) manually integrated



External Standard Report

Sorted By : Signal
Calib. Data Modified : Tuesday, June 06, 2023 2:09:06 PM
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 2: VWD1 A, Wavelength=205 nm

RetTime [min]	Type	Area mAU *s	Amt/Area	Amount [mg/L]	Grp	Name
6.030	MM	268.76538	7.78695e-2	20.92862		Tafluprost ethyl amide
Totals :				20.92862		

Instrument 1 7/12/2023 12:11:16 PM DP

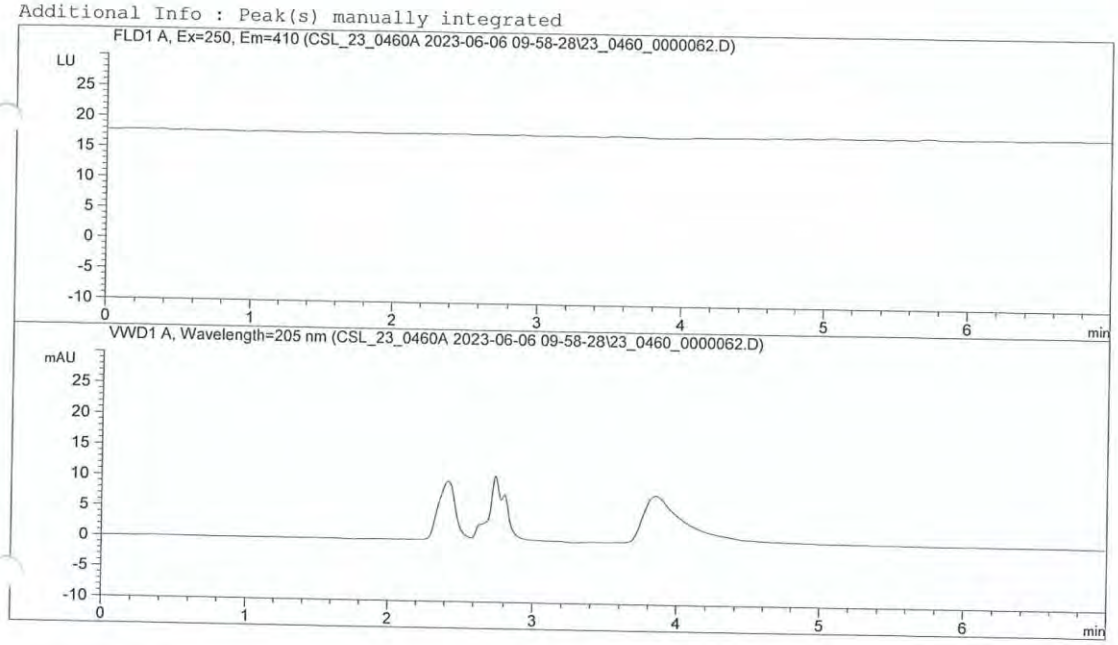
Page 1 of 2

Figure 3: HPLC chromatogram of a test solution (Test 1a)

Data File C:\CHEM32\1\DATA\CSL_23_0460A 2023-06-06 09-58-28\23_0460_0000062.D
Sample Name: Probe 4a-Blind

Acq. Operator	: DP	Seq. Line	: 48
Acq. Instrument	: Instrument 1	Location	: Vial 49
Injection Date	: 7/12/2023 12:32:42 PM	Inj	: 1
		Inj Volume	: 10.0 µl
Acq. Method	: C:\CHEM32\1\DATA\CSL_23_0460A 2023-06-06 09-58-28\CSL_23_0460_03_C.M		
Last changed	: 6/6/2023 2:09:54 PM by DP		
Analysis Method	: C:\CHEM32\1\METHODS\CSL_23_0460_03_C.M		
Last changed	: 7/12/2023 12:09:38 PM by DP (modified after loading)		
Method Info	: CSL-23-0460.04 Kolbenmethode A.6. HPLC-System 1 Säule 13 Säulen Nr. USUXA47267 Lot-Nr. B22032 Zorbax Eclipse Plus C18		

Additional Info : Peak(s) manually integrated



External Standard Report

Sorted By : Signal
Calib. Data Modified : Tuesday, June 06, 2023 2:09:06 PM
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 2: VWD1 A, Wavelength=205 nm

RetTime [min]	Type	Area mAU *s	Amt/Area	Amount [mg/L]	Grp	Name
6.064		-	-	-		Tafluprost ethyl amide

Totals : 0.00000

Instrument 1 7/12/2023 12:41:13 PM DP

Page 1 of 2

Figure 4: HPLC chromatogram of the blank experiment (Test 4a)

8 Appendix

8.1 Certificate of Analysis of the Test Item

CERTIFICATE of ANALYSIS

[redacted]

Tafluprost ethyl amide

N-ethyl-9α,11α-dihydroxy-15,15-difluoro-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide

Item No. 9000843 • Batch No. 0663687

Purity Specification: ≥98%
Molecular Formula : C24H33F2NO4
CAS Number: 1185851-52-8 Formula Weight: 437.5 Expiry date: 08NOV2023

Overview

Tests	Results
HPLC	Purity: 99.2 %
Mass spec	MH+: 437.9
TLC	Purity: 100 %

Reviewed and approved by: [redacted]

[redacted]

WARNING
THIS PRODUCT IS FOR RESEARCH USE - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE. IT IS THE RESPONSIBILITY OF THE PURCHASER TO DETERMINE SUITABILITY FOR OTHER APPLICATIONS.

SAFETY DATA
This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent via email to your institution.

WARRANTY AND LIMITATION OF REMEDY
Buyer agrees to purchase the material subject t[redacted]erms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

[redacted]

[redacted]

8.2 GLP Certificate of the Test Facility

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

consilab Gesellschaft für Anlagensicherheit mbH
Industriepark Höchst, Geb.G 830
65926 Frankfurt am Main

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

1 Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen

1 Physical-chemical testing

5 Prüfungen zum Verhalten im Boden, im Wasser und in der Luft, Prüfungen zur Bioakkumulation und zur Metabolisierung

5 Studies on behaviour in water, soil and air; bioaccumulation

23.11.2022 bis 24.11.2022
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Brandt

Dr. Astrid Brandt, Referentin, Wiesbaden, den **21.März 2023**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hess. Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

Final Report

Original 1 of 1

Determination of the UV/Vis spectrum
Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
(Neat Oil)
according to OECD 101

Study No.: 22120103G0010

Sponsor:

[redacted]

Monitor:
ToxMinds BVBA
Dr. Thomas Petry
Avenue de Broqueville, 116
1200 Brussels
Belgium

Test Facility:
LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler
Germany

Study Director:
Dr. Jörg Johannes

1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following documents:

- OECD Principles of Good Laboratory Practice and Compliance Monitoring, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998 and subsequent advisory/consensus OECD GLP documents (where appropriate).
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemicals Act of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 from 28. Aug. 2013, published in Federal Law Gazette, Germany (BGBl) No. 55/2013 as of 06. Sep. 2013, and further revisions.

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

Affolter — 21 AUG 2023

Dr. Olena Affolter — Date

Deputy Study Director

Information on Study Organisation:

Study Director	Dr. Jörg Johannes
Deputy Study Director	Dr. Olena Affolter
Study Plan dated	05. May 2023
Experimental Starting Date	10. May 2023
Experimental Completion Date	13. Jun. 2023

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice.

All phases of the study (study plan, performance of the study and final report) were checked by the quality assurance. Dates of inspections are given below. Findings are reported to the Study Director and Test Facility Management.

The inspection of the performance of short-term studies (duration less than four weeks) may be carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the final report was written in accordance with the study plan and the Standard Operating Procedures (SOP) of the test facility.

Deviations from the study plan (if any) were acknowledged and assessed by the Study Director and included in the final report.

The reported results reflect the raw data of the study.

Phases of Study	Inspected on	Findings reported on	Audit report no.
Study plan	27. Apr. 2023	27. Apr. 2023	230427-06
Performance of study	10. May 2023	10. May 2023	230510-03
Final report	15. Aug. 2023	15. Aug. 2023	230815-02

C. Bachmann

21 AUG 2023

Dr. Corinna Bachmann
Quality Assurance

Date

Table of Contents

3 SUMMARY

Title of Study: Determination of the UV/Vis spectrum Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) according to OECD 101

Findings and Results:

The test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) was analysed using UV/Visible spectroscopy. The UV/Visible spectrum showed one absorption band in the range 210 – 240 nm with maximum absorption at 226 nm and one absorption band in the range 250 – 285 nm with three maxima at 265 nm, 270 nm and 276 nm. The molar extinction coefficients were in the range 1046.2 to 1306.1 $L*Mol^{-1}cm^{-1}$ for the three maxima. A summary of wavelengths, extinction coefficients and bandwidths is shown in Table 3–a.

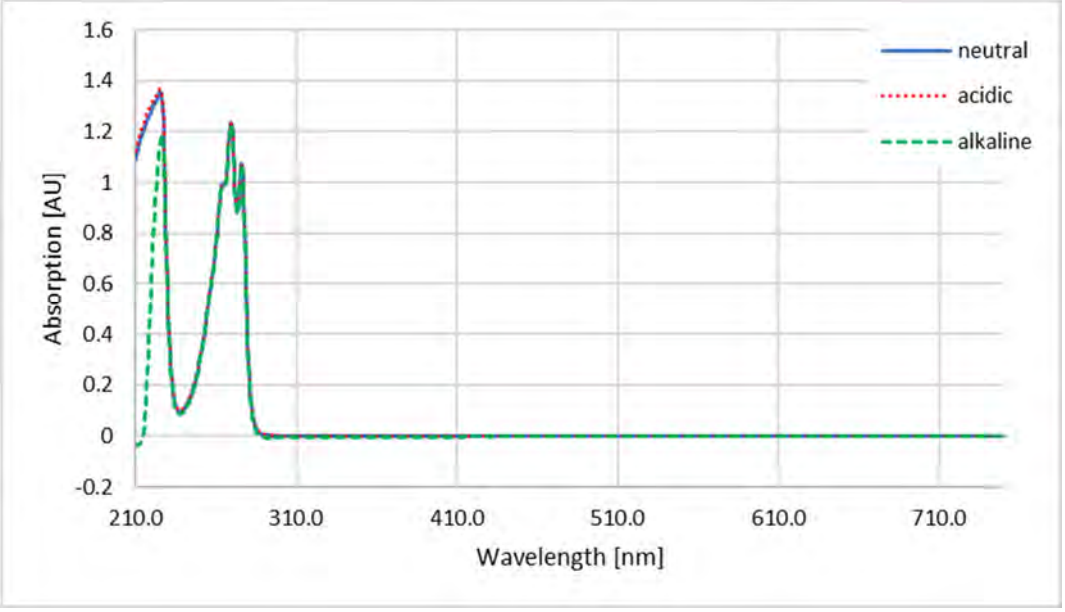


Figure 3–a: UV/Vis spectrum of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) under neutral (solid blue line), acidic (dotted red line) and alkaline (dashed green line) conditions

The spectrum remains essentially unchanged after addition of acid, while the bandwidth of the lower wavelength band is significantly narrowed and the maximum at 226 nm slightly decreases after addition of base.

Table 3–a: Summary of the Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) UV/Vis spectrum

	Absorption band 210 – 240 nm			Absorption band 250 – 285 nm		
	Maximum wavelength [nm]	Extinction coefficient [L*Mol^{-1}cm^{-1}]	Band-width [nm]	Maximum wavelength [nm]	Extinction coefficient [L*Mol^{-1}cm^{-1}]	Band-width [nm]
Alkaline	226.5	1255.8	10	264.5	1039.3	21.5
				269.5	1294.8	
				276.0	1127.0	
Neutral	225.5	1431.2	n.c.	264.5	1046.2	21.5
				269.5	1304.5	
				276.0	1135.0	
Acidic	225.5	1444.6	n.c.	264.5	1049.9	21.5
				269.5	1306.1	
				276.0	1134.5	

n.c. = not calculable, short wavelength side of the peak was truncated

4 PURPOSE OF THE STUDY

This study was performed in order to determine the UV/Vis spectrum of the test item under neutral, acidic and alkaline conditions.

5 LITERATURE

The study was conducted in compliance with the following guideline:

- OECD guideline 101 “UV-VIS Absorption Spectra”, adopted 12. May 1981

6 MATERIAL AND METHODS

6.1 Test Item

Designation in Test Facility:	22120103G
Date of Receipt:	01. Dec. 2022
Condition at Receipt:	cooled, in proper conditions

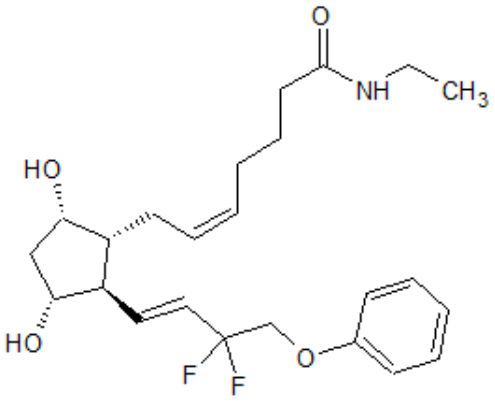
6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Batch no.	TAF-10-1122-01
CAS no.	1185851-52-8
EC no.	867-521-0
Composition	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
Storage	fridge (2 - 8 °C); keep under inert gas
Expiry date	23. Nov. 2026
Stability	stable under storage conditions
Appearance	clear, colorless to light yellow liquid
Purity	99.78 %
Homogeneity	homogeneous
Production date	18. Nov. 2022
EC no.	867-521-0
Molecular formula	$C_{24}H_{33}F_2NO_4$
Molecular weight	437.52 g/mol
Vapour pressure	unknown
Solubility in solvents	water: not stated; ethanol: >1g/L; acetone: not stated; acetonitrile: not stated; DMSO: >1g/L; methanol: >1g/L; DMF: 0.1-1g/L
Stability in solvents	water: not stated; ethanol: not stated; acetone: not stated; acetonitrile: not stated; DMSO: not stated; methanol: not stated; DMF: not stated

A copy of the certificate of analysis of the test item is added in chapter 13.

6.1.2 Structural Formula



O[C@@H]2C[C@H](O)[C@H](C/C=C\CCCC(=O)NCC)[C@H]2/C=C/C(F)(F)COc1ccccc1

6.1.3 Storage

The test item was stored in a closed vessel in the fridge (2 - 8 °C) and kept under inert gas.

6.2 Test System UV/Vis spectrophotometer

Specification:	SPECORD 205
Software:	WinAspect 2.5.0.0
Manufacturer:	Analytik Jena
Cuvettes:	Quartz cuvettes with a cell path length of 10 mm

Usage and calibration followed the corresponding SOP 114 00 241 in the current edition.

6.3 Other Instruments and Devices

Usage and, if applicable, calibration of all instruments followed the corresponding SOPs in the current edition.

- Analytical scales
  Mettler Toledo XSR205DU
- Automatic Pipettes
  Mettler Toledo Rainin, with variable volume
- AutoRep E
  Mettler Toledo Rainin, with variable volume
- pH indicator paper
- Carbon analyser
  Analytik Jena TOC multi N/C 2100S
- Conductometer
  wtw 538
- Ultrasonic bath SONOREX DT 510 H, Bandelin
- Cooled incubator, Memmert ICP 600
- Glass thermometer
- Standard Laboratory Glassware

6.4 Chemicals

- Water
  Demineralised water with TOC < 1 ppm and conductivity below 0.3 µS/cm was used
- Methanol
  CH_3OH, MeOH, HPLC grade
- Sodium hydroxide
  NaOH, 1 M, p.a.
- Hydrochloric acid
  HCl, 2 M, prepared from concentrated HCl (37 %), p.a.
- Hydrochloric acid
  HCl, 1 M, prepared from HCl, 2 M

7 CONDUCT OF THE STUDY

7.1 Sample Preparation

The measurement of UV spectra was performed three times. As the first experiment was not absorbance in the range of 0.5 – 1.5 AU, the measurement was repeated using a 2-fold diluted test item solution. As the final dilution for analysis was prepared using demineralized water instead of double distilled water, these measurements were considered not valid.

The third experiment was then performed using double distilled water, and only the results of this experiment are reported. Raw data and results of the first two determinations are archived together with all other raw data of the study.

Solvent	Methanol
Concentration	492.9 mg/L (0.9463 mmol/L)
Light path	10 mm
Scanned range	210– 750 nm

A test item stock solution of 985.8 mg/L was prepared by weighing 24.7 mg test item into a 25 mL volumetric flask and filling to volume with methanol. 5.0 mL of this stock solution were diluted to volume 10 mL methanol, resulting in a 492.9 mg/L working solution. This solution was used for measurement under neutral, acidic and alkaline conditions after addition of methanol, 1 M HCl or 1 M NaOH to the cuvette, respectively.

All solutions were tempered at 25 °C in a temperature controlled incubation chamber at 25 ± 0.5 °C until use.

- Neutral: 1200 µL of the test item solution were mixed with 120 µL water
- Acidic: 1200 µL of the test item solution were mixed with 120 µL 1 M HCl
- Alkaline: 1200 µL of the test item solution were mixed with 120 µL 1 M NaOH

Due to the dilution step in the cuvette the final concentration used for measurements was 448.1 mg/L (0.9463 mmol/L).

Blank solutions were prepared in the same way using 1200 µL methanol instead of working solution.

8 FINDINGS

8.1 UV/Vis-Spectra

The UV/Vis spectra of blank solutions and test item solutions were recorded separately, and subsequently processed to subtract the blank. The figures in Figure 8.1–a through Figure 8.1–c represent measured spectra of 448.1 mg/L (0.9463 mmol/L) test item solution after blank subtraction (solid blue curves) together with the corresponding blanks (dashed orange curves).

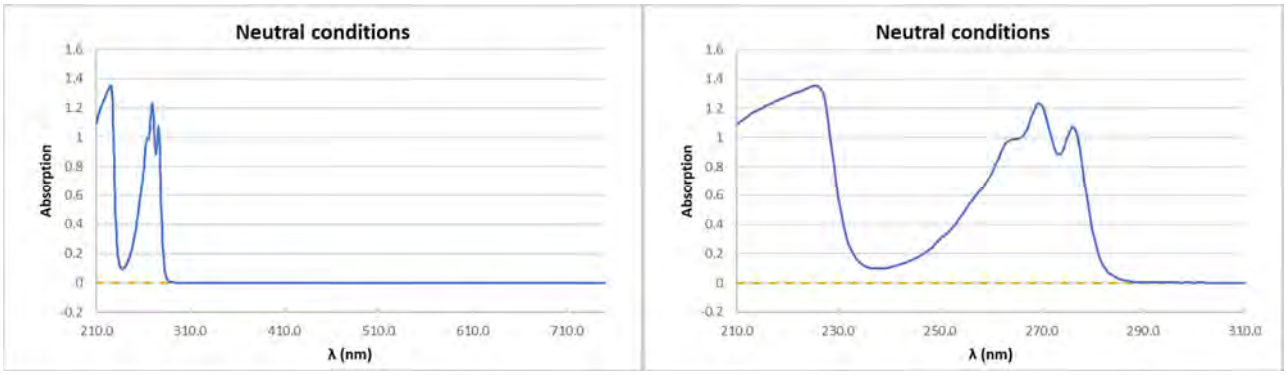


Figure 8.1–a UV/Vis spectrum of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in methanol. Solid blue line: 448.1 mg/L (0.9463 mmol/L) test item in methanol, dashed orange line: Blank methanol.

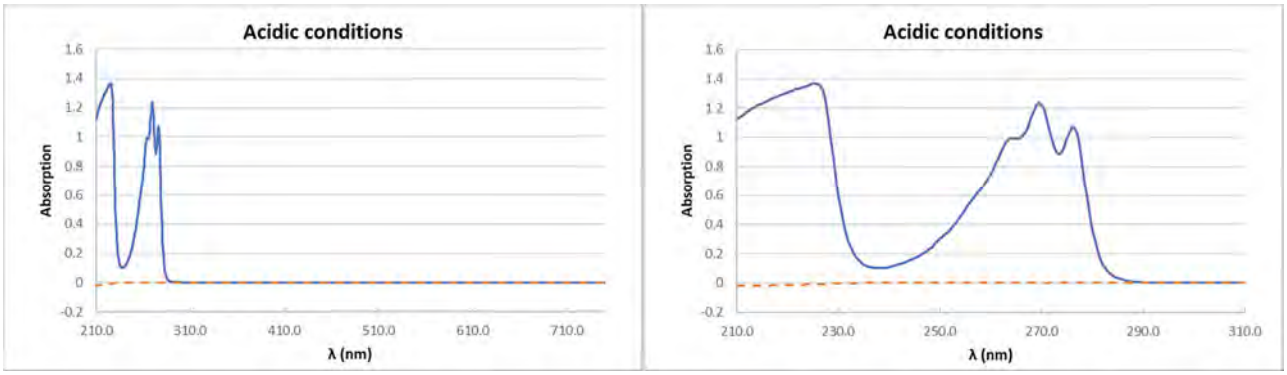


Figure 8.1–b UV/Vis spectrum of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in methanol. Solid blue line: 448.1 mg/L (0.9463 mmol/L) test item in acidified methanol, dashed orange line: Blank acidified methanol.

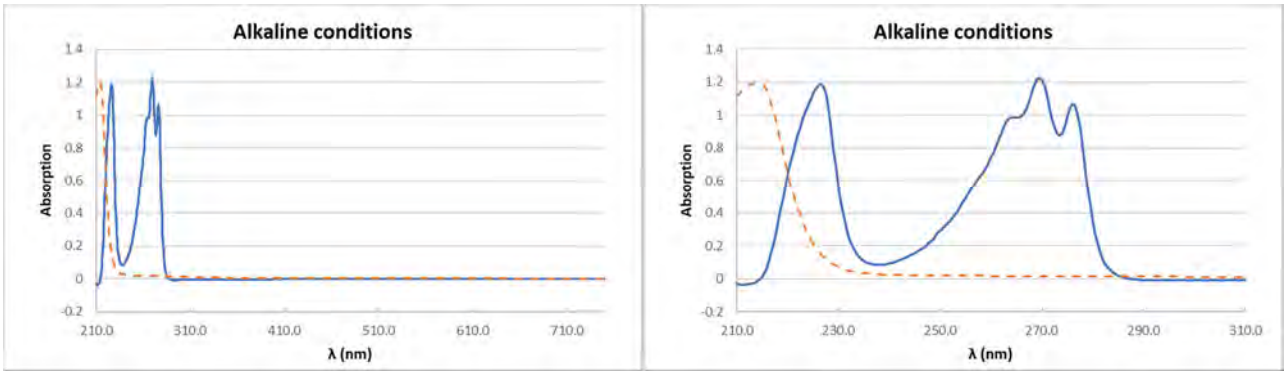


Figure 8.1–c UV/Vis spectrum of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in methanol. Solid blue line: 448.1 mg/L (0.9463 mmol/L) test item in basified methanol, dashed orange line: Blank basified methanol.

8.2 Effects of pH

The test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) was analysed using UV/Visible spectroscopy. The UV/Visible spectrum showed one absorption band in the range 210 – 240 nm with maximum absorption at 226 nm and one absorption band in the range 250 – 285 nm with three maxima at 265 nm, 258 nm and 276 nm.

The molar extinction coefficients for each maximum were calculated using the following equation:

$$\varepsilon_\lambda\ [L*Mol^{-1}cm^{-1}] = \frac{Absorption_\lambda}{c[Mol/L]*d[cm]}$$

With ε_λ = extinction coefficient at wavelength λ [$L*Mol^{-1}cm^{-1}$]
c = concentration of sample [Mol/L]
d = light path in the cuvette [cm]

The absorption maxima and corresponding extinction coefficients at the measurement conditions are shown in Table 8.2–a and Table 8.2–b:

Table 8.2–a UV/VIS Peak maxima of the absorption band in the range 210 – 240 nm determined in a 448.1 mg/L (0.9463 mmol/L) test item solution

pH	Wave-length	Absorption	Extinction coefficient	Bandwidth
	[nm]	[AU]	[$L*Mol^{-1}cm^{-1}$]	[nm]
Alkaline	226.5	1.1884	1255.8	10
Neutral	225.5	1.3544	1431.2	n.c.
Acidic	225.5	1.3671	1444.6	n.c.

n.c. = not calculable, short wavelength side of the peak was truncated

Determination of the bandwidth neutral and acidic conditions was not possible because the short wavelength side of the peak was truncated. Furthermore, absorption of methanol at wavelengths ≤ 210 nm interferes with the UV absorbance of the test item, so no reliable data can be obtained in this wavelength range. The bandwidth under alkaline conditions was determined from the maximum absorbance of the peak at 226.5 nm as follows: The wavelength with the closest absorbance value to half the peak height at the left (low wavelength) and right (high wavelength) side of the peak was extracted from each spectrum. The difference between the wavelengths is the bandwidth.

Table 8.2–b UV/VIS Peak maxima of the absorption band in the range 250 – 285 nm determined in a 448.1 mg/L (0.9463 mmol/L) test item solution

pH	Wavelength	Absorption	Extinction coefficient	Bandwidth
	[nm]	[AU]	[L*Mol^{-1}cm^{-1}]	[nm]
Alkaline	264.5	0.9835	1039.3	21.5
	269.5	1.2253	1294.8	
	276.0	1.0665	1127.0	
Neutral	264.5	0.9935	1046.2	21.5
	269.5	1.2360	1304.5	
	276.0	1.0736	1135.0	
Acidic	264.5	0.9901	1049.9	21.5
	269.5	1.2345	1306.1	
	276.0	1.0741	1134.5	

Determination of the bandwidth of the individual maxima was not possible because they were considered as fine structure of a common peak. Therefore, the bandwidth was determined using the maximum absorbance of the peak at 269.5 nm.

9 DISCUSSION

The test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) was analysed using UV/Visible spectroscopy. The UV/Visible spectrum showed one absorption band in the range 210 – 240 nm with maximum absorption at 226 nm and one absorption band in the range 250 – 285 nm with three maxima at 265 nm, 270 nm and 276 nm. The molar extinction coefficients were in the range 1046.2 to 1306.1 L*Mol^{-1}cm^{-1} for the three maxima.

The spectrum remains essentially unchanged after addition of acid, while the bandwidth of the lower wavelength band is significantly narrowed and the maximum at 226 nm slightly decreases after addition of base.

Determination of the bandwidth neutral and acidic conditions was not possible because the short wavelength side of the peak was truncated. Furthermore, absorption of methanol at wavelengths ≤ 210 nm interferes with the UV absorbance of the test item, so no reliable data can be obtained in this wavelength range.

No observations were made which might cause doubts concerning the validity of the study outcome.

10 DEVIATIONS

10.1 Deviations from the Study Plan

No deviations were ascertained.

10.2 Deviations from the Guidelines

No deviations were ascertained.

11 RECORDING AND ARCHIVING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP-Document-Archive of the test facility for 15 years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP-Substance Archive for 15 years and then discarded.

Number of originals of the final report to be sent to the sponsor: 0, PDF-file only

12 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6, 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien / toxicological in vitro studies on mammalian cells and bacteria)

Datum der Inspektion / Date of Inspection
(Tag.Monat.Jahr / day.month.year)
28. und 29.04.2021

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist spätestens drei Jahre nach der letzten Inspektion zu beantragen. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for not later than three years after the last inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Landesamt für Umwelt Rheinland-Pfalz

Unterschrift, Datum / Signature, Date Mainz, 21.06.21

Sabine Riewenherm - Präsidentin -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

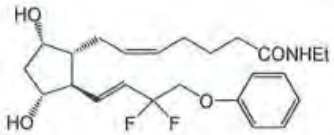
13 ANNEX 2: COPY OF CERTIFICATE OF ANALYSIS OF TEST ITEM

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{33}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications.

Quality Control: [redacted] *Date:* 23NOV2022

Approved: [redacted] *Date:* 23 Nov 2022

Quality Control Laboratory
[redacted]

Client: [redacted]

Approval Date	:	May 11, 2022	**Labeled Age Grade/Size**	:	NA
Date of Receipt	:	May 10, 2022	**Tested Age Grade**	:	NA
Retest	:	No			
# of Samples Submitted	:	1 Set of 7			
Manufacturer's Name	:	NA			
Item Description	:	[redacted] Formula# 042522-07-0001 MFG: 4/25/22			
Item Number	:	NA			
Country of Origin	:	NA			
Country of Import	:	NA			
PO Number	:	NA			
Delivery Conditions	:	Satisfactory, Samples tested as received			
Testing Date Range	:	08-25-2022 to 10-17-2022			

The following test item(s) was/were performed on submitted sample(s) and/or component(s) confirmed by applicant

TEST REQUESTED	RESULT
Dermal: Pre-study tests ADME Bioanalysis (CFR019)**	See Attachment
Dermal: In-vitro absorption study, preparation and experimentation ADME Bioanalysis (CFR019)**	See Attachment

** Analysis completed by Eurofins Subcontract Laboratory

Signed for and on behalf of
Eurofins Product Testing US Inc.

Alexis Klock / Project Coordinator

ATTACHMENT

eurofins | ADME BIOANALYSES

REPORT

Title	*IN-VITRO* SKIN PENETRATION OF RADIOLABELLED ETHYL TAFLUPROSTAMIDE IN 4 DILUTIONS OF TEST ITEM ON HEALTHY HUMAN SKIN
Eurofins|ADME BIOANALYSES study code	22-0148
Name of reference item	^{3}H-Ethyl tafluprostamide (^{3}H-DDDE)
Test Facility	Eurofins|ADME BIOANALYSES 75A Avenue de Pascalet 30310 Vergèze France
Sponsor	[redacted]
Sponsor's Point of Contact	Marty Imler, Director, Business Development, Eurofins Product Testing
Date of the experiment	Study plan signature: September 01st, 2022 Start of the pre-tests: August 25th, 2022 Start of the GLP experiments: September 02nd, 2022 Completion of the experiments: October 17th, 2022
Document status	Final
Date of version	April 20th, 2023

SUMMARY

OBJECTIVE:

The aim of this study was to investigate the rate and extent of the *in vitro* dermal absorption of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE) (*aka* Ethyl tafluprostamide) with a radiolabelled tracer (^{3}H-DDDE) in four test items. These test items were applied to the surface of healthy human skin samples mounted on dynamic cells.

REGULATORY COMPLIANCE:

This study was conducted at Eurofins|ADME BIOANALYSES, 75A Avenue de Pascalet, 30310 Vergèze, France, under the direction of Morgane Delobel.

The study was performed according to:

- Study plan 22-0148,
- the requirements of Good Laboratory Practices (OECD ENV/MC/CHEM (98) 17), 2004/10/EC, arrêté du 10 Août 2004 (France).
- Standard Operating Procedures in use at Eurofins|ADME BIOANALYSES, FRANCE, including but not limited to:

SOP code	SOP title
ETU/RAD/009P	Preparation and control of a radiolabelled test item.
ETU/PEN/004P	Set-up and application for dermal absorption study.
ETU/PEN/005P	Dismantling and treatment of samples from dermal absorption study.

The realization of the technical part of the absorption and distribution following application on human skin was based on:

- OECD guideline for the testing of chemicals, Test No. 428, Skin Absorption: *in vitro* method (13 April 2004)
- SCCS guideline (SCCS/1628/21), basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients, 30-31 March 2021. Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients. SCCS/1628/21
- OECD guidance document for the conduct of skin absorption studies, OECD series on testing and assessment. Number 28, 05-Mar-2004 (ENV/JM/MONO(2004)2).
- OECD guidance notes on dermal absorption Number 156, ENV/JM/MONO(2011)36.
- Cosmetics Europe guidelines for Percutaneous Absorption/Penetration, 1997.

CHOICE OF THE METHOD:

The “OECD guideline for the testing of chemicals: guideline 428, skin absorption: *in vitro* method” recommends to use a radiolabelled substance to perform this absorption study.

EXPERIMENTAL DESIGN:

The test items containing Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE) and the radiolabelled compound ^{3}H-DDDE were prepared at Eurofins|ADME BIOANALYSES. Approximately 1 µCi was applied on each cell (10 µL of test item/cell). Four concentrations of DDDE were tested, two higher and one lower than the concentration (0.018%) in the commercial product [redacted].

Test item	Radiolabelled preparation			
Reference item	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)			
Formulations	DDDE Dilution 0.024%	DDDE Dilution 0.020%	DDDE Dilution 0.018%	DDDE Dilution 0.012%
Theoretical concentration of reference item DDDE at 7.5% in phenoxyethanol	0.32%	0.27%	0.24%	0.16%
Theoretical concentration of active substance DDDE	0.024%	0.020%	0.018%	0.012%
Theoretical test item amount applied per cell (1 cm^2)	10 µL			
Theoretical amount of reference item applied on skin (µg)	2.4 µg	2.0 µg	1.8 µg	1.2 µg
Occlusion condition	No			
Check of the test item stability at 32°C during 24 hours	Yes n=1 (T0) and n=1 after 24 h at 32°C (T24)			
Thickness of the skin (µm)	310 - 400 µm			
Trans epidermal water loss (TEWL)	1.85 – 10.12 g/m²/h			
Number of cell per donor (replicates)	3	3	3	3
Donor ID	1, 2, 3, 4	1, 2, 3, 4	5, 6, 7, 8	5, 6, 7, 8
Total number of donor	8 (each donor was used for 2 test items)			
Total of cells per formulation	12	12	12	12
Total cells	48			
Receptor fluid	5% w/w Bovine serum albumin, 0.9% NaCl in water			
Sampling of Receptor Fluid	0.5h, 2h, 4h, 8h, 12h, 24h			
Washing	24 hours			
Washing of the test item	0.5 mL Tween 80® 5% 1 half cotton swab 3.5 mL of UHQ water (0.5 mL, 7 times) 3 dried half cotton swabs			
Dismantling of the cells	24 hours			
Strips	A maximum of 20 strips was performed*. The strips were pooled as follows: 1-2, 3-6, 7-11, 12-15, 16-20*.			
Separation Epidermis/Dermis	Yes			
Extraction solvent for RCD and RCR, tape strips and cotton-swabs	Ethanol			

* The number of strips generated depended on the donor. This number could be lower than 20 (between 1 and 20). If stripping induces the separation of epidermis and dermis, the stripping was stopped.

RESULTS:

The mean results obtained for test items containing DDDE and the radiolabelled compound ^{3}H-DDDE are presented in following tables:

Distribution of ^{3}H-DDDE after application to human skin (%)

Reference item	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)							
Formulations	DDDE Dilution 0.024%		DDDE Dilution 0.020%		DDDE Dilution 0.018%		DDDE Dilution 0.012%	
	n=12		n=12		n=12		n=12	
Number of strips (mean by test item)	9		9		11		9	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Strips 1-2	5.75	1.99	4.24	2.19	5.18	2.11	5.53	1.57
Strips 3-20	15.86	6.58	15.78***	4.06***	9.27	3.08	9.02	4.12
Total strips	21.61	7.22	18.71	7.05	14.45	4.47	14.55	4.48
Skin Excess*	66.82	6.39	67.70	5.03	70.06	6.60	71.01	5.92
Epidermis	6.63	4.56	5.93	5.11	4.17	1.02	3.63	1.95
Dermis	0.43	0.44	0.21	0.19	0.40	0.30	0.61	0.64
Receptor fluid	3.61	3.36	2.98	2.29	1.94	1.25	2.20	1.27
Epidermis + dermis + receptor fluid**	10.68	7.18	9.12	7.23	6.51	2.16	6.44	2.14
TOTAL RECOVERY	99.11	3.13	95.53	4.26	91.02	2.18	92.00	3.06

*Skin excess corresponds to: Washing + Donor compartment rinsing + Remaining skin
**Absorbed fraction of the applied DDDE according to SCCS guideline
***Mean and SD realized on 11 cells since Cell X has only 1 strip.

Distribution of ^{3}H-DDDE after application to human skin ($\mu g_{eq}/cm^2$)

Reference item	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)							
Formulations	DDDE Dilution 0.024%		DDDE Dilution 0.020%		DDDE Dilution 0.018%		DDDE Dilution 0.012%	
	n=12		n=12		n=12		n=12	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Test substance applied ($\mu Ci/cm^2$)	0.87	0.02	0.88	0.03	0.91	0.03	0.92	0.02
Strips 1-2	0.14	0.05	0.09	0.04	0.10	0.04	0.07	0.02
Strips 3-20	0.37	0.15	0.32***	0.08***	0.17	0.05	0.11	0.05
Total strips	0.51	0.17	0.38	0.14	0.27	0.08	0.18	0.06
Skin Excess*	1.57	0.13	1.38	0.10	1.30	0.14	0.90	0.06
Epidermis	0.16	0.11	0.12	0.11	0.08	0.02	0.05	0.02
Dermis	0.010	0.010	0.004	0.004	0.007	0.006	0.008	0.008
Receptor fluid	0.09	0.08	0.06	0.05	0.04	0.02	0.028	0.016
Epidermis + dermis + receptor fluid**	0.25	0.17	0.19	0.15	0.12	0.04	0.08	0.03

*Skin excess corresponds to: Washing + Donor compartment rinsing + Remaining skin
**Absorbed fraction of the applied DDDE according to SCCS guideline
***Mean and SD realized on 11 cells since Cell X has only 1 strip.

CONCLUSION:

The aim of this study was to investigate the rate and extent of the *in vitro* dermal absorption of DDDE from cosmetic formulation [redacted] as test item using ^{3}H-DDDE radiolabelled tracer. Four test items, with different concentration of DDDE were applied to the surface of healthy human skin samples mounted on dynamic cells. The percentage of DDDE in [redacted] is 0.018%. For comparison,

one test item with a lower percentage of DDDE (0.012%) and two with higher percentages of DDDE (0.020% and 0.024%) were also tested.

A total of 8 donors was used and each donor was used for 2 test items. The donors 1 to 4 were used for the two highest concentration test items (0.024% and 0.020%), and the donors 5 to 8 for the two lowest concentration test items (0.018% and 0.012%).

The mean total recovery for each condition was within the established acceptance criteria (85-115%) according to SCCS guideline, validating the results obtained.

For each test item, the number of strips is variable, ranging from 1 to 20. This intra- and inter-formulation heterogeneity is also found in the results of the dermis, the epidermis and the receptor fluid. The absorption results were presented according to SCCS guideline with Receptor fluid + Rinsing Receptor compartment (RCR) + Epidermis + Dermis during 24 hours:

- 10.68% ± 7.18% of applied dose corresponding to 0.25 ± 0.17 µg/cm² for DDDE Dilution 0.024%.
- 9.12% ± 7.23% of applied dose corresponding to 0.19 ± 0.15 µg/cm² for DDDE Dilution 0.020%.
- 6.51% ± 2.16% of applied dose corresponding to 0.12 ± 0.04 µg/cm² for DDDE Dilution 0.018%.
- 6.44% ± 2.14% of applied dose corresponding to 0.08 ± 0.03 µg/cm² for DDDE Dilution 0.012%.

Donors 1 to 4, which were used to test the two highest concentrations (0.024% and 0.020%) of DDDE, show a substantial greater variability (SD = 7.18% and 7.23%) compared to the donors 5 to 8, which were used to test the two lowest concentrations of DDDE (0.018%, SD = 2.16% and 0.012%, SD = 2.14%).

TABLE OF CONTENTS

LIST OF IN-TEXT TABLES

LIST OF IN-TEXT FIGURES

GLOSSARY

DDDE	:	Ethyl tafluprostamide
BLQ	:	Below the Limit of Quantification (100 dpm)
C	:	Cotton-swabs + tips
D	:	Dermis
E	:	Epidermis
LAV	:	Washing solution
LM	:	Scalpel blade
LR	:	Receptor fluid
Max	:	Maximum value
Min	:	Minimum value
n	:	number of determinations or replicates
NA	:	Not applicable
NC	:	Not Calculated
QA	:	Quality Assurance
RCD	:	Cleaning of donor compartment
RCR	:	Cleaning of receptor compartment
RF	:	Receptor fluid
RS	:	Remaining skin
S	:	Skin = Epidermis + partial dermis
SD	:	Standard Deviation
SI	:	Lower seal
SOPs	:	Standard Operating Procedures
SS	:	Upper seal
TEWL	:	TransEpidermal Water Loss
UHQ	:	Ultra High Quality
UST	:	Upper strips

RESPONSIBILITIES

Responsibility	Name	Contact details
Sponsor	[redacted]	[redacted]
Study Monitor	Marty IMLER MartyImler@eurofins.com Phone: +1 858 243 2464	Eurofins Product Testing 11822 North Creek Parkway, N #110 Bothell, WA 98011
Study Director	Morgane DELOBEL MorganeDelobel@eurofins.com Phone: +33 (0)4 66 73 19 27	Eurofins|ADME BIOANALYSES 75A avenue de Pascalet 30310 Vergèze France Phone : +33 (0)4 66 73 17 73
Deputy Study Director	Hélène MAROT HeleneMarot@eurofins.com Phone: +33 (0)4 66 73 00 62	
Test Facility Management	Laurent BENAZERAF LaurentBenazeraf@eurofins.com Phone: +33 (0)4 66 73 15 53	
Head of QAU	Ophélie VAILLANT OphelieVaillant@eurofins.com Phone: +33 (0)4 66 73 17 52	

STUDY DIRECTOR STATEMENT

I, hereby, confirm that this study was conducted in accordance with the study plan 22-0148, in accordance with the Good Laboratory Practice Standards: OECD ENV/MC/CHEM (98) 17, 2004/10/EC, arrêté du 10 Août 2004 (France), with SCCS guideline (SCCS/1628/21), basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients, 30-31 March 2021, and the Standard Operating Procedures in use at Eurofins|ADME BIOANALYSES.

The pre-tests of preparation of the test item (§7) were out of the scope of 2004/10/EC, arrêté du 10 Août 2004 (France).

The data provided in this report are valid.

Morgane DELOBEL

Signé numériquement par : Morgane DELOBEL
Nom DN : CN = Morgane DELOBEL C = FR O = EUROFINS ADME BIOANALYSES OU = EUROFINS ADME BIOANALYSES, 0002 34146029300044, Dermal unit
Date : 2023.04.20 15:58:52 +02'00'
Motif : J'approuve ce document.

Morgane DELOBEL

Study Director
Eurofins|ADME BIOANALYSES

APPROVAL PAGE

This report has been reviewed and approved by the Test Facility. The following signature documents those approvals:

Test Facility	Eurofins|ADME BIOANALYSES 75A Avenue de Pascalet 30310 Vergèze FRANCE
Deputy Test Facility Management	Jean Paul FRECHE
Date and signature	Jean-Paul FRECHE Signé numériquement par : Jean-Paul FRECHE Nom DN : CN = Jean-Paul FRECHE C = FR O = EUROFINS ADME BIOANALYSES OU = EUROFINS ADME BIOANALYSES, 0002 34146029300044, Biological Therapeutics Date : 2023.04.20 16:17:43 +02'00' Motif : J'approuve ce document.

QUALITY ASSURANCE STATEMENT

Study Title: *IN-VITRO* SKIN PENETRATION OF RADIOLABELLED ETHYL TAFLUPROSTAMIDE IN 4 DILUTIONS OF TEST ITEM ON HEALTHY HUMAN SKIN

Eurofins|ADME BIOANALYSES study code: 22-0148

I, the undersigned, hereby declare that this study was audited in accordance with the Good Laboratory practice standards OECD ENV/MC/CHEM (98) 17, 2004/10/EC, arrêté du 10 Août 2004 (France) and Standard Operating Procedures in use at Eurofins|ADME BIOANALYSES and confirm that this report reflects the raw data generated by Eurofins|ADME BIOANALYSES.

The dates of audits performed by the Quality Assurance Unit, and the dates on which the findings were reported to the Study Director and the Test Facility Management are given below.

AUDIT PHASES AND AUDIT DATES		DATES OF AUDIT REPORT TRANSMISSION TO THE:	
		Study Director	Test Facility Management
Study plan:	27 Jun 2022	27 Jun 2022	27 Jun 2022
Process audit*: Radiolabelled sample analysis	27 Sep 2022	27 Sep 2022	27 Sep 2022
Raw data and Preliminary report:	17 to 25 Nov 2022	28 Nov 2022	25 Nov 2022
Final report:	07 and 11 Apr 2023	11 Apr 2023	11 Apr 2023

* Reported Process-Based Audits related to the study time schedule

Facilities or systems and processes are monitored as part of a regular program. Corresponding audit reports are available at the test facility

Clara PIQUOT

Signé numériquement par : Clara PIQUOT
Nom DN : CN = Clara PIQUOT C = FR O = EUROFINS ADME BIOANALYSES OU = EUROFINS ADME BIOANALYSES, 0002 34146029300044, Assurance Qualité
Date : 2023.04.20 16:23:42 +02'00'
Motif : J'approuve ce document.

Clara PIQUOT
Deputy Quality Assurance Manager
Eurofins|ADME BIOANALYSES

1. Study objective

The aim of this study was to investigate the rate and extent of the in vitro dermal absorption of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE) (*aka* Ethyl tafluprostamide) with a radiolabelled tracer (^{3}H-DDDE) in four test items. These test items were applied to the surface of healthy human skin samples mounted on dynamic cells.

2. Contract laboratory and study location

This study was conducted at Eurofins|ADME BIOANALYSES, 75A, avenue de Pascalet, 30310 Vergèze, France, under the direction of Morgane Delobel.

The study was performed according to:

- Study plan 22-0148,
- the requirements of Good Laboratory Practices (OECD ENV/MC/CHEM (98) 17), 2004/10/EC, arrêté du 10 Août 2004 (France).
- Standard Operating Procedures in use at Eurofins|ADME BIOANALYSES, FRANCE, including but not limited to:

SOP code	SOP title
ETU/RAD/009P	Preparation and control of a radiolabelled test item.
ETU/PEN/004P	Set-up and application for dermal absorption study.
ETU/PEN/005P	Dismantling and treatment of samples from dermal absorption study.

The realization of the technical part of the absorption and distribution following application on human skin was based on:

- OECD guideline for the testing of chemicals, Test No. 428, Skin Absorption: *in vitro* method (13 April 2004)
- SCCS guideline (SCCS/1628/21), basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients, 30-31 March 2021.
- OECD guidance document for the conduct of skin absorption studies, OECD series on testing and assessment. Number 28, 05-Mar-2004 (ENV/JM/MONO(2004)2).
- OECD guidance notes on dermal absorption Number 156, ENV/JM/MONO(2011)36.
- Cosmetics Europe guidelines for Percutaneous Absorption/Penetration, 1997.

3. Deviation(s)

This study is concerned by the deviation 22011. The pre-tests were begun before the signature of the study plan. This minor deviation has no impact on the results.

In the study plan, a typing error has been done on the batch number of the reference item. It is corrected in the present report. This minor deviation has no impact on the results.

In order to apply in volumetry (10 µL) and check the acceptance criteria on the amount applied, the density of each test item was measured during the homogeneity test of the test items. This minor deviation has no impact on the results.

In order to check the radiopurity of the test item, one injection per test item (see §13.2 of the study plan) was realized instead of three (see §11.2 of the study plan). This minor deviation has no impact on the results.

The homogeneity test was realized the day of the formulation, before and during the application. This minor deviation has no impact the results.

Some receptor fluid samples were separated into 2 vials, the sample lists were created outside of the generator. Results were reported on the sample lists. This minor deviation has no impact on the results.

After the end of the experiment, during the treatment of Washing samples of test item 0.018% and test item 0.012%, 5mL of ethanol were added in the Washing samples (liquid part). Unlike the washing cotton samples, ethanol should not have been added to these samples (liquid part). The mix ethanol/water in presence of scintillation liquid leads to milk aspect. In order to avoid opacity of the samples, each sample was separated in two vials and scintillation liquid was added to dilute samples. All samples were counted and the results are added together in the table. This minor deviation has no impact on the results.

On sponsors request, the absorption of the test item should be provided as a mean value along with the standard deviation. Calculation of the MoS shall be the responsibility of the risk assessor. This minor deviation has no impact on the results.

4. Rationale

The “OECD guideline for the testing of chemicals: guideline 428, skin absorption: *in vitro* method” recommends to use a radiolabelled substance to perform this type of study.

5. Reference items and ingredients

5.1. Reference items

Radiolabelled ^{3}H-DDDE was provided by [redacted] informed Eurofins on 10 August 2022 that it had successfully synthesized ^{3}H-DDDE. Eurofins notified Sponsor when the ^{3}H-DDDE was received from [redacted].

Non-radiolabelled item was provided by the Sponsor.

	Reference items	
Name	^{3}H-Ethyl tafluprostamide*(^{3}H-DDDE)	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)
Supplier	[redacted]	[redacted]
Batch	22-0809-93	TAF-F-0122-01
Molecular Weight	437.5	437.52 g/mol
Specific activity (µCi/mg)	138.971 mCi/mg (by calculation)	-
Physical form	Liquid	Solution
Concentration	1 mCi/mL in EtOH	-
Purity by HPLC	> 98%	99.42%
Ethyl Tafluprostamide titer	-	7.5% in 2-phenoxyethanol
Expiry or Re-test date	Not relevant	03/02/2026
Storage conditions	Target temperature -80°C Under inert gas and protected from light	Target temperature +4°C Under inert gas and protected from moisture

*In the absence of stability data a radiopurity check was performed before using.

The certificates of analysis are presented in APPENDIX D.

Note:
DDDE is a lipophilic molecule that is highly insoluble in water at 25°C: 0.09 mg/L (sccs_o_258.pdf (europa.eu)).
Molecular weight (g/mol) = 437.52.
Partition Coefficient (Log Pow) = 5.03.

5.2. Blank formulation

	Blank formulation ([redacted] minus DDDE)
Name	[redacted] Blank formulation
Batch	Formula #042522-07-001
Expiry date	30/04/2025

The final product ([redacted]) is composed of 17 ingredients. For this study, the Sponsor separately provided the [redacted] product minus the DDDE (referred to as the "[redacted] blank formulation") and DDDE for the radiolabelled preparation test items. To make the radiolabelled preparation DDDE (radiolabelled and non-radiolabelled) was added by Eurofins to the [redacted] blank formulation.

5.3. Receipt and handling

On their receipt, all relevant details and remarks relating to the condition of the products were checked and recorded.

All test compounds were handled with a particular care, especially to avoid any injection, swallowing and inhalation, according to Eurofins|ADME BIOANALYSES SOP.

All safety information relative to the manipulation was sent together with the corresponding certificate of analysis (purity, water content, expiry date). Its compliance with this information was the responsibility of the supplier.

6. Rationale for receptor fluid selection

For *in vitro* skin absorption studies, the molecule should be adequately soluble in the selected receptor fluid so that it does not act as a barrier to absorption, and the receptor fluid should maintain the skin barrier integrity. Therefore, the solubility of DDDE in the receptor fluid was demonstrated as not being a rate limiting factor. According to the guideline, ENV/JM/MONO(2004)2, the solubility should be at least 10-fold higher than the maximal concentration expected in the receptor fluid at the end of the *in vitro* study.

Considering the maximal DDDE concentration in test item preparation (0.024% w/w) and the amount applied (10 µL/cm² equivalent to 10 mg/cm² by considering the density approximately equal to 1), the maximal concentration of DDDE in receptor fluid (Receptor fluid circulates at 1.5 mL/h giving a total volume of 36 mL for the volume obtained after a dismantling 24 hours after application on skin) is estimated to be 66.67 ng/mL.

Thus, the DDDE solubility should be at least 666.7 ng/mL (10-fold the maximal estimated concentration) to confirm the suitability of the receptor fluid for the study.

Due to the low solubility of DDDE in water, the selection of the receptor fluid was determined by the determining the solubility of DDDE in the following receptor fluids:

- RF1: 5% w/w Bovine serum albumin, 0.9% NaCl in water
- RF2: 6% polyethylene glycol 20 oleyl ether in PBS 0.01 M* pH* 7.4 (*data given by the supplier)

Detailed results

Solubility test results are presented in Table 5.

The RF1 (5% w/w Bovine serum albumin, 0.9% NaCl in water) and RF2 (6% polyethylene glycol 20 oleyl ether in PBS 0.01 M pH 7.4) presented a CV% inferior to 10% validating the homogeneity between the aliquots. The respective concentrations 2.09 µg/mL and 2.07 µg/mL obtained, for RF1 and RF2 were superior to the threshold 0.67 µg/mL.

Both receptor fluids were suitable for the dermal study. The study director and the Sponsor selected RF1 as the receptor fluid for the study.

7. Pre-test of the preparation of the test items (non GLP)

In order to define the method of preparation of the test items, pre-tests were carried out according to the methods described below:

Radiolabelled preparation n°1: DDDE Dilution 0.018%

Approximately 2 g of test item was prepared.
- Target 20 µCi of the ^{3}H-DDDE was added in a glass flask (this amount is negligible),
- The ethanol was evaporated under a stream of nitrogen gas.
- 1993.35 mg of [redacted] blank formulation ([redacted] placebo) was added in the vial
- The mixture was mixed
- 4.23 mg of non-radiolabelled DDDE were added very slowly and adequate stirring into the vial and mix for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°2: DDDE Dilution 0.018%

Approximately 2 g of test item was prepared.
- Target 20 µCi of the ^{3}H-DDDE was added in a glass flask (this amount is negligible),
- The solvent was evaporated under a stream of nitrogen gas.
- 4.21 mg of non-radiolabelled DDDE into the vial and mix for at least 2 min
- 1995.12 mg of [redacted] blank formulation [redacted] placebo) was added in the vial
- The mixture was mixed for at least 1 hour under magnetic agitation.

Detailed results

Pre-test of the preparation of the test items results are presented below:

Radiolabelled preparation n°1		Radiolabelled preparation n°2	
The day of the preparation	One day after the preparation	The day of the preparation	One day after the preparation
CV (%)			
5.62	1.60	3.22	1.29

The pre-test allowed Eurofins to work out the procedures for preparing the test formulations containing 0.018% of DDDE using

two different methods of preparation. The both radiolabelled preparations were homogeneous.
The method yielding the best outcome, defined by homogeneity tests, was used in the radiolabelled preparations (GLP) for the dermal penetration study. The process of radiolabelled preparation n°2 was used for the preparation of the test items.

8. Preparation of the test item (radiolabelled preparation)

The radiolabelled reference item ^{3}H-DDDE was received as liquid solution at 1 mCi/mL in EtOH.

According to the homogeneity results of the formulation tests carried out during the pre-test, one of the two preparation process described above was chosen and described below.

The following test items were prepared:

- 0.024% (w/w)
- 0.020% (w/w)
- 0.018% (w/w)
- 0.012% (w/w)

Radiolabelled preparation n°1: DDDE Dilution 0.024%

Approximately 2 g of test item was prepared.
- Target 200 µCi of the ^{3}H-DDDE was added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),
- The ethanol was evaporated under a stream of nitrogen gas,
- 6.32 mg of non-radiolabelled DDDE was added into the vial,
- The mixture was mixed at least 2 minutes,
- 1993.71 mg of [redacted] blank formulation ([redacted] placebo) was added in the vial,
- The preparation was stirred for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°2: DDDE Dilution 0.020%

Approximately 2 g of test item was prepared.
- Target 200 µCi of the ^{3}H-DDDE was added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),
- The ethanol was evaporated under a stream of nitrogen gas,
- 5.43 mg of non-radiolabelled DDDE was added into the vial,
- The mixture was mixed at least 2 minutes,
- 1994.32 mg of [redacted] blank formulation ([redacted] placebo) was added in the vial,
- The preparation was stirred for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°3: DDDE Dilution 0.018%

Approximately 2 g of test item was prepared.
- Target 200 µCi of the ^{3}H-DDDE was added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),
- The ethanol was evaporated under a stream of nitrogen gas,
- 4.80 mg of non-radiolabelled DDDE was added into the vial,
- The mixture was mixed at least 2 minutes,
- 1995.16 mg of [redacted] blank formulation ([redacted] placebo) was added in the vial,
- The preparation was stirred for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°4: DDDE Dilution 0.012%

Approximately 2 g of test item was prepared.

- Target 200 µCi of the ^{3}H-DDDE was added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),
- The ethanol was evaporated under a stream of nitrogen gas,
- 3.26 mg of non-radiolabelled DDDE was added into the vial,
- The mixture was mixed at least 2 minutes,
- 1996.71 mg of [redacted] blank formulation ([redacted] placebo) was added in the vial,
- The preparation was stirred for at least 1 hour under magnetic agitation.

Approximately 1 µCi of test item was applied to the skin surface placed in each cell (10 µL/1 cm²).

The radiolabelled preparations (^{3}H-DDDE + DDDE + blank [redacted]) were stored at room temperature under magnetic agitation before use.

One part of the preparations was used for the stability test (after at least 24 hours at 32°C) and the other part for the application.

Table 1: Summary of formulation preparation for DDDE Dilution 0.024%

^{3}H DDDE concentration in Test item (real)	87.48	µCi/g
Specific activity of ^{3}H DDDE (theoretical)	138.971	mCi/mg
Concentration of ^{3}H DDDE (theoretical)	1.00	mCi/mL
Purity of ^{3}H DDDE (between 0-1)	1.00	
% of active susbtance in reference item (DDDE)	7.50	%
Volume of ^{3}H DDDE	0.20	mL
Weight of ^{3}H DDDE by taking account the real concentration in test item	0.001	mg
Weight of DDDE	6.32	mg
Weight of active substance in reference item (DDDE)	0.47	mg
Weight of pure active substance DDDE	0.48	mg
Blank formulation [redacted] placebo	1993.71	mg
Amount of prepared Test item	2000.03	mg
Concentration of DDDE in Test item	0.24	mg of DDDE/g of Test item

Table 2: Summary of formulation preparation for DDDE Dilution 0.020%

^{3}H DDDE concentration in Test item (real)	88.72	µCi/g
Specific activity of ^{3}H DDDE (theoretical)	138.971	mCi/mg
Concentration of ^{3}H DDDE (theoretical)	1.00	mCi/mL
Purity of ^{3}H DDDE (between 0-1)	1.00	
% of active susbtance in reference item (DDDE)	7.50	%
Volume of ^{3}H DDDE	0.20	mL
Weight of ^{3}H DDDE	0.001	mg
Weight of DDDE	5.43	mg
Weight of active substance in reference item (DDDE)	0.41	mg
Weight of pure active substance DDDE	0.41	mg
Blank formulation [redacted] placebo	1994.32	mg
Amount of prepared Test item	1999.75	mg
Concentration of DDDE in Test item	0.20	mg of DDDE/g of Test item

Table 3: Summary of formulation preparation for DDDE Dilution 0.018%

^{3}H DDDE concentration in Test item (real)	88.87	µCi/g
Specific activity of ^{3}H DDDE (theoretical)	138.971	mCi/mg
Concentration of ^{3}H DDDE (theoretical)	1.00	mCi/mL
Purity of ^{3}H DDDE (between 0-1)	1.00	
% of active susbtance in reference item (DDDE)	7.50	%
Volume of ^{3}H DDDE	0.20	mL
Weight of ^{3}H DDDE	0.001	mg
Weight of DDDE	4.80	mg
Weight of active substance in reference item (DDDE)	0.36	mg
Weight of pure active substance DDDE	0.36	mg
Blank formulation [redacted] placebo	1995.16	mg
Amount of prepared Test item	1999.96	mg
Concentration of DDDE in Test item	0.18	mg of DDDE/g of Test item

Table 4: Summary of formulation preparation for DDDE Dilution 0.012%

^{3}H DDDE concentration in Test item (real)	89.61	µCi/g
Specific activity of ^{3}H DDDE (theoretical)	138.971	mCi/mg
Concentration of ^{3}H DDDE (theoretical)	1.00	mCi/mL
Purity of ^{3}H DDDE (between 0-1)	1.00	
% of active susbtance in reference item (DDDE)	7.50	%
Volume of ^{3}H DDDE	0.20	mL
Weight of ^{3}H DDDE	0.001	mg
Weight of DDDE	3.26	mg
Weight of active substance in reference item (DDDE)	0.24	mg
Weight of pure active substance DDDE	0.25	mg
Blank formulation [redacted] placebo	1996.71	mg
Amount of prepared Test item	1999.97	mg
Concentration of DDDE in Test item	0.12	mg of DDDE/g of Test item

9. Design

9.1. Test items

The test items containing DDDE and the radiolabelled compound ^{3}H-DDDE were prepared at Eurofins|ADME BIOANALYSES. Approximately 1 µCi was applied on each cell (10 µL of test item /cell).

9.2. Cell design

Four donors were included and three diffusion cells per donor were measured for test items, a total of twelve cells was used by each formulation. 48 cells were used for this study.

A total of 8 donors was used with each donor was used for 2 test items.

The same donors for the test items 0.024% and 0.020%, and the same donors for the test items 0.018% and 0.012%.

The test items remained on the skin for 24 hours before removal by an appropriate washing solution.

The radioactivity was measured in washing solution, *Stratum Corneum* by tape-stripping, epidermis, dermis and receptor fluid samples, as well as in all materials used in dosing preparation.

Test item	Radiolabelled preparation			
Reference item	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)			
Formulations	DDDE Dilution 0.024%	DDDE Dilution 0.020%	DDDE Dilution 0.018%	DDDE Dilution 0.012%
Theoretical concentration of reference item DDDE at 7.5% in phenoxyethanol	0.32%	0.27%	0.24%	0.16%
Theoretical concentration of active substance DDDE	0.024%	0.020%	0.018%	0.012%
Theoretical test item amount applied per cell (1 cm^2)	10 µL			
Theoretical amount of reference item applied on skin (µg)	2.4 µg	2.0 µg	1.8 µg	1.2 µg
Occlusion condition	No			
Check of the test item stability at 32°C during 24 hours	Yes n=1 (T0) and n=1 after 24 h at 32°C (T24)			
Thickness of the skin (µm)	310 - 400 µm			
Trans epidermal water loss (TEWL)	1.85 – 10.12 $g/m^2/h$			
Number of cell per donor (replicates)	3	3	3	3
Donor ID	1, 2, 3, 4	1, 2, 3, 4	5, 6, 7, 8	5, 6, 7, 8
Total number of donor	8 (each donor was used for 2 test items)			
Total of cells per formulation	12	12	12	12
Total cells	48			
Receptor fluid	5% w/w Bovine serum albumin, 0.9% NaCl in water			
Sampling of Receptor Fluid	0.5h, 2h, 4h, 8h, 12h, 24h			
Washing	24 hours			
Washing of the test item	0.5 mL Tween 80® 5% 1 half cotton swab 3.5 mL of UHQ water (0.5 mL, 7 times) 3 dried half cotton swabs			
Dismantling of the cells	24 hours			
Strips	A maximum of 20 strips was performed*. The strips were pooled as follows: 1-2, 3-6, 7-11, 12-15, 16-20*.			
Separation Epidermis/Dermis	Yes			
Extraction solvent for RCD and RCR, tape strips and cotton-swabs	Ethanol			

* The number of strips generated depended on the donor. This number could be lower than 20 (between 1 and 20). If stripping induces the separation of epidermis and dermis, the stripping was stopped.

10. Skin preparation

10.1. Preparation of human skin samples

The human skin samples used were obtained from abdominal surgery. Just after receipt, excess subcutaneous fat was removed if necessary. The storage of the skin at -20°C was less than 9 months.

Detailed results Skin details are presented in Table 6.

10.2. Measurement of skin samples thickness

Skin samples were excised and cut into pieces of 1.8 cm x 1.8 cm and the skin was dermatomed to a thickness of 300-400 µm. The thickness was measured using Oditest calipers according to the SOP in use in the laboratory.

Detailed results The thickness of the prepared skin samples used is presented in Table 8 to Table 11.

All skin sections had a thickness comprised between 310 to 400 µm.

10.3. Measurement of cutaneous sample integrity

The room temperature should be maintained between 20 and 25°C and the relative humidity between 30 and 70%.

Approximately 30 minutes after the set-up of the cells, the integrity of the *stratum corneum* was determined for each dermatomed skin sample by measuring the TEWL using evaporimeter.

There should be no water on the skin and in the cell donor compartment and the measurement were taken away from any heating source and air stream.

The human skin was included in the study if the TEWL was determined to be between 0.5 and 13 g/m^2/h.

Detailed results The results are presented in Table 8 to Table 11.

During measurement of TEWL, room temperature was comprised between 23.2°C and 24.2°C for the first application (test items 0.024% and 0.020%) and between 21.6°C and 22.4°C for the second application (test items 0.018% and 0.012%). Relative humidity was comprised between 46.3% and 55.4% for the first application and between 45.0% and 46.5% for the second application.

The TEWL values were within the acceptable range.

10.4. Measurement of temperature of cutaneous sample surface

The passive diffusion of chemicals (and therefore their skin absorption) is affected by temperature. Therefore, the diffusion chamber and skin samples were maintained at a constant temperature of 32 ± 1°C. The temperature was measured for each cell just before the application.

Detailed results

The results are presented in Table 8 to Table 11.

The skin temperatures were within the acceptable range.

11. Before application

11.1. Test items homogeneity

The homogeneity of the test items was checked on 10 µL of the test item (n=6: 2 on the top, 2 on the middle and 2 on the bottom) using the same pipette than those used during the application.

10 µL was weighed in the scintillation vial and scintillation liquid was added.

The homogeneity of the test items was checked the day of preparation, before and during the application (see §deviations).

The homogeneity of the test items before the application was considered acceptable if the obtained coefficient of variation (CV) on the 6 values is less or equal to 5%.

The CV measured during application was stated as a measure of variability. The homogeneity of the test items obtained during the application should be used to calculate the recovery.

Detailed results

The homogeneity results are presented in Table 12 to Table 15.

	DDDE Dilution 0.024%	DDDE Dilution 0.020%	DDDE Dilution 0.018%	DDDE Dilution 0.012%
The day of preparation				
Mean (µCi/g)	84.54	83.27	88.07	89.59
CV (%)	4.07	4.76	3.90	1.49
Before the application				
Mean (µCi/g)	86.34	88.49	88.55	90.33
CV (%)	1.09	0.95	1.10	1.71
During the application				
Mean (µCi/g)	87.48	88.72	88.87	89.61
CV (%)	3.79	1.52	1.64	1.62

The CV (%) measured before application was inferior to 5% and allowed the application of the test item on skin.

These specific activities during application were used to calculate the recovery.

11.2. Radiochemical purity and stability of the test item

The radiochemical purity was tested by HPLC-radioactive analysis (Berthold Technologies: FlowStar LB 514, RadioStar 5.0.12.6).

The chromatographic conditions provided by [redacted] (presented in APPENDIX B) or equivalent, were used. The radiochemical purity of the test items was checked before and after storage at 32°C for at least 24 hours (one sample injected one time in each condition) and corresponded to the stability of the reconstituted test items.

The radiochemical purity of the radiolabelled preparation was tested by checking the absence of radiochemical peak higher than 2% of the total radioactivity.

Detailed results

Chromatograms of radiochemical purity are presented in APPENDIX B.

The radiopurity of the test items presented a purity of 100% all the day of use and all formulations.

Radiochemical purity was checked the day of test items preparation (T0) and after storage for 24 hours at 32°C.

Radiopurity was 100% for both test items at T0, before application and after storage for 24 hours at 32°C.

Therefore, the test items were stable during the experiment.

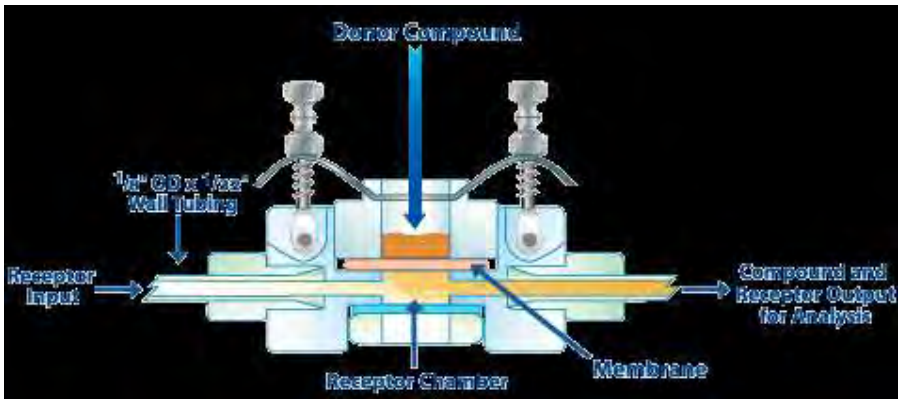
12. Application

12.1. Preparation of dynamic cells

The dynamic cells used corresponded to:

Application area = 1 cm²

Flow of receptor fluid = 1.5 mL/h



Cells were identified by a letter.

The skin samples were placed on the receptor compartment. The donor compartment was then placed onto the skin samples. A clamp was placed to link both compartments and the cell was tilted before being placed in the chamber in order to evacuate the presence of air bubbles in the flexible.

The skin surface temperature was maintained at 32°C ± 1°C, with a fixed water bath integrated in the dynamic system.

After at least 30 minutes of stabilisation, the integrity of the skin was verified using the TEWL method.

The donor compartment was not closed (non-occlusive system).

12.2.Application of the test item

The test item preparations were applied homogeneously at 10 µL/cm² (10 µL/cell) without massage on each skin sample.

The application was performed using a positive displacement pipette and the exact amount applied was determined by weight then recorded.

Before application, the positive displacement pipette containing the amount to be applied was weighed (P1). Then the empty pipette was weighed after application (P2). The applied amount (Px) corresponded to: Px = P1 – P2.

12.3.Start of the experiment

The experiment started immediately after application of the preparation on the skin surface.

The total experiment was stopped 24 hours after application.

Start and end of experiment were noted in the raw data.

12.4.Receptor fluid sampling

The receptor fluid was collected continuously and was pass through the dynamic system at a flow rate of 1.5 mL/h.

At each interval time, the receptor fluid was placed into one or some vials. When more than one vial is used, the result taken into account was the sum of results in each vial.

The sampling time are specified in the paragraph 9.2 Cells design.

12.5.Washing and dismantling of cell and sample treatment

24 hours after application, the cells were washed and dismantled.

The remaining formulation preparation was washed after 24 hours with:

- 0.5 mL Tween 80® 5% with 1 half cotton swab
- 3.5 mL of UHQ water (0.5 mL, 7 times)
- 3 dried half cotton swabs.

The washing solutions were added to the skin surface (with half cotton) then removed with a pipette and was collected for analysis. The skin surface was carefully dried with three half cotton swabs before/during or after this washing procedure.

The washing solution (W) was added into one vial and the half cotton-swabs and tip were placed in another vial. Half cotton-swabs and tip (C) was extracted by 5 mL of ethanol. Vials were shaken for a maximum of 26 days and sonicated.

For the Washing samples Y to AV (test items 0.018% and 0.012%), the liquid part were separated into two vials (see §Deviation).

The cells were dismantled and skin samples were treated as follows.

- Rinsing Donor compartment (RCD)

The donor compartment, the upper seal and the scalpel blade (LM) was placed in a flask and 10 mL of ethanol was added. The closed flask was shaken for a maximum of 6 days.

- *Stratum corneum* treatment: Upper strips (STx)

The skin was taken with tweezers and each skin sample was placed between seal and aluminium foil.

The *stratum corneum* was taken off from the skin sample using adhesive scotch Magic 3M® by stripping. In order to standardize stripping, a pressure of 50 g/cm² from device pressure was applied on top of the Scotch tape for 10 s before removing it.

A maximum of 20 tape strips should be performed. However, the total number of strips removed depends on the skin. Consequently, the stripping was stopped if an epidermis/dermis separation is observed.

The strips were pooled for analysis as follow: 1-2, 3-6, 7-10, 11-15, 16-20. The exact number of strips performed is presented in Table 7.

Tape strips were extracted by 5 mL of ethanol. Vials were shaken for a maximum of 6 days.

- Epidermis (E), Dermis (D) and remaining skin (RS) treatment

Using the scalpel blade, the skin corresponding to the application area was separated from the remaining (surrounding) skin. The remaining skin is the ring of skin surrounding the application site (RS is needed to place the skin on the cell), not directly in contact with the applied formulation and with the receptor fluid. The remaining skin was cut in four parts. After separation, epidermis and dermis were placed in corresponding vials.

For epidermis, dermis and remaining skin, Solvable™ or equivalent (3 mL) were added.

The E and D vials were placed in a heat chamber for a maximum of 6 days for total dissolution.

The RS vials were placed in a heat chamber for a maximum of 13 days for total dissolution.

The scalpel blade and seal were added with the donor compartment for extraction.

Aluminium foil was destroyed.

- Rinsing of receptor compartment (RCR)

The part of the receptor compartment in contact with the receptor fluid was rinsing according to the process described below:

- 1 dried half cotton swab for the chamber of the receptor fluid (to absorb the remaining receptor fluid)
- 1 half cotton swab soaked with the ethanol for the entrance and the exit of the receptor fluid flow
- 1 half cotton swab soaked with the ethanol for the chamber of the receptor fluid
- 1 dried half cotton swab for entrance and the exit of the receptor fluid flow then for the chamber of the receptor fluid

The 4 half cotton swabs were placed in a flask called RCR, then 5 mL of the ethanol was added in the vial. The vial was mix for a maximum of 6 days.

13. Radioactivity measurement

For receptor fluid and RCR, about 10 mL of Ultima Gold XR scintillation liquid was added.

For washing solution, half cotton-swab and tip vials and strips vial, approximately 10 mL of Ultima Gold XR scintillation liquid was added.

For the Epidermis, Dermis and remaining skin after dissolution with Solvable™, approximately 10 mL of Hionic Fluor scintillation liquid was added.

The RCD vial (corresponding to RCD + SS + LM), contains ethanol. 1 aliquot of 1 mL was taken. In each aliquot, approximately 10 mL of Ultima Gold XR scintillation liquid was added.

Samples were analyzed for radiolabel content by scintillation counting (Tricarb 2910TR, version 2.06 Perkin). Calculations were performed using Excel directly from the raw data obtained with the scintillation counter (QuantaSmart 4.00 software). Conversion of the counts per minute (cpm) to disintegrations per minute (dpm) were performed directly by the microprocessor in the instrument using a quench curve of the appropriate scintillation cocktail stored in memory.

14. Results

14.1. Calculations

Calculation was performed using Excel. Results were expressed as:

1. $\mu g_{eq}/cm^2$ (μg_{eq}: µg equivalent of ^{3}H-DDDE).
2. % of ^{3}H-DDDE / applied dose.

The limit of quantitation was 100 dpm *minus* blank value. Results below the limit of quantitation were noted as “BLQ” in result tables and were considered as 0 for calculation.

Results were presented with 2 digits according to the studied compartments and/or recovered amount.

14.2. Recovery

For each experiment and each cell, a recovery balance was calculated. The mean value had to be 100 ± 15%.

Detailed results

The detailed results are presented in Table 16, Table 19, Table 22 and Table 25.

The individual total recovery was between:

- 93.51% and 105.17% leading to a mean recovery of 99.11% for DDDE Dilution 0.024% formulation,
- 89.81% and 103.23% leading to a mean recovery of 95.53% for DDDE Dilution 0.020% formulation,
- 87.40% and 95.55% leading to a mean recovery of 91.02% for DDDE Dilution 0.018% formulation,
- 86.35% and 96.25% leading to a mean recovery of 92.00% for DDDE Dilution 0.012% formulation,

The mean recoveries for each condition is within the acceptance criteria (85-115%) validating the experiment.

All the cells presented a recovery within the acceptance criteria.

14.3.Absorption

The absorption was equal to:

Receptor fluid + Rinsing Receptor compartment (RCR) + Epidermis + Dermis (according to the SCCS guideline).

Detailed results Detailed results are presented in Table 16, Table 19, Table 22 and Table 25.

The results obtained in this study are presented in following tables:

Distribution of ^{3}H-DDDE after application to human skin (%)

Reference item	**Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)**							
Formulations	DDDE Dilution 0.024%		DDDE Dilution 0.020%		DDDE Dilution 0.018%		DDDE Dilution 0.012%	
	n=12		n=12		n=12		n=12	
Number of strips (mean by test item)	9		9		11		9	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Strips 1-2	5.75	1.99	4.24	2.19	5.18	2.11	5.53	1.57
Strips 3-20	15.86	6.58	15.78***	4.06***	9.27	3.08	9.02	4.12
Total strips	21.61	7.22	18.71	7.05	14.45	4.47	14.55	4.48
Skin Excess*	66.82	6.39	67.70	5.03	70.06	6.60	71.01	5.92
Epidermis	6.63	4.56	5.93	5.11	4.17	1.02	3.63	1.95
Dermis	0.43	0.44	0.21	0.19	0.40	0.30	0.61	0.64
Receptor fluid	3.61	3.36	2.98	2.29	1.94	1.25	2.20	1.27
Epidermis + dermis + receptor fluid**	10.68	7.18	9.12	7.23	6.51	2.16	6.44	2.14
TOTAL RECOVERY	99.11	3.13	95.53	4.26	91.02	2.18	92.00	3.06

*Skin excess corresponds to: Washing + Donor compartment rinsing + Remaining skin
**Absorbed fraction of the applied DDDE according to SCCS guideline
***Mean and SD realized on 11 cells since Cell X has only 1 strip.

Distribution of ^{3}H-DDDE after application to human skin ($\mu g_{eq}/cm^2$)

Reference item	**Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)**							
Formulations	DDDE Dilution 0.024%		DDDE Dilution 0.020%		DDDE Dilution 0.018%		DDDE Dilution 0.012%	
	n=12		n=12		n=12		n=12	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Test substance applied ($\mu Ci/cm^2$)	0.87	0.02	0.88	0.03	0.91	0.03	0.92	0.02
Strips 1-2	0.14	0.05	0.09	0.04	0.10	0.04	0.07	0.02
Strips 3-20	0.37	0.15	0.32***	0.08***	0.17	0.05	0.11	0.05
Total strips	0.51	0.17	0.38	0.14	0.27	0.08	0.18	0.06
Skin Excess*	1.57	0.13	1.38	0.10	1.30	0.14	0.90	0.06
Epidermis	0.16	0.11	0.12	0.11	0.08	0.02	0.05	0.02
Dermis	0.010	0.010	0.004	0.004	0.007	0.006	0.008	0.008
Receptor fluid	0.09	0.08	0.06	0.05	0.04	0.02	0.028	0.016
Epidermis + dermis + receptor fluid**	0.25	0.17	0.19	0.15	0.12	0.04	0.08	0.03

*Skin excess corresponds to: Washing + Donor compartment rinsing + Remaining skin
**Absorbed fraction of the applied DDDE according to SCCS guideline
***Mean and SD realized on 11 cells since Cell X has only 1 strip.

15. Conclusion

The aim of this study was to investigate the rate and extent of the *in vitro* dermal absorption of DDDE from cosmetic formulation [redacted]) as test item using ^{3}H-DDDE radiolabelled tracer. Four test items, with different concentration of DDDE were applied to the surface of healthy human skin samples mounted on dynamic cells. The percentage of DDDE in [redacted] is 0.018%. For comparison, one test item with a lower percentage of DDDE (0.012%) and two with higher percentages of DDDE (0.020% and 0.024%) were also tested.

A total of 8 donors was used and each donor was used for 2 test items. The donors 1 to 4 were used for the two highest concentration test items (0.024% and 0.020%), and the donors 5 to 8 for the two lowest concentration test items (0.018% and 0.012%).

The mean total recovery for each condition was within the established acceptance criteria (85-115%) according to SCCS guideline, validating the results obtained.

For each test item, the number of strips is variable, ranging from 1 to 20. This intra- and inter-formulation heterogeneity is also found in the results of the dermis, the epidermis and the receptor fluid. The absorption results were presented according to SCCS guideline with Receptor fluid + Rinsing Receptor compartment (RCR) + Epidermis + Dermis during 24 hours:

- 10.68% ± 7.18% of applied dose corresponding to 0.25 ± 0.17 µg/cm² for DDDE Dilution 0.024%.
- 9.12% ± 7.23% of applied dose corresponding to 0.19 ± 0.15 µg/cm² for DDDE Dilution 0.020%.
- 6.51% ± 2.16% of applied dose corresponding to 0.12 ± 0.04 µg/cm² for DDDE Dilution 0.018%.
- 6.44% ± 2.14% of applied dose corresponding to 0.08 ± 0.03 µg/cm² for DDDE Dilution 0.012%.

Donors 1 to 4, which were used to test the two highest concentrations (0.024% and 0.020%) of DDDE, show a substantial greater variability (SD = 7.18% and 7.23%) compared to the donors 5 to 8, which were used to test the two lowest concentrations of DDDE (0.018%, SD = 2.16% and 0.012%, SD = 2.14%).

16. Archiving procedure

All the documentation relative to this study will be archived in accordance with the Study Plan (APPENDIX C).

APPENDIX A

RESULTS

Table 5: Solubility test results

Radiolabelled reference item	^{3}H- Ethyl tafluprostamide
Real tested amount (µg/mL)	2.0144
Radioactivity (µCi)	2
RF 1	5% w/w Bovine serum albumin, 0.9% NaCl in water
RF 2	PBS 0.01M pH 7.4 containing 6% polyoxyethylene 20 oleyl ether

^{3}H- Ethyl tafluprostamide in 5% w/w Bovine serum albumin, 0.9% NaCl in water

Replicate	dpm	µCi	Weight (g)	µCi/mL	Recovery (%)	Concentration in RF (µg/mL)
1	84738	0.0382	0.0197	1.95	97.5	2.0
2	87448	0.0394	0.0203	1.95	97.6	2.0
3	86551	0.0390	0.0199	1.97	98.6	2.0
4	98629	0.0444	0.0210	2.13	106.4	2.1
5	100141	0.0451	0.0204	2.22	111.2	2.2
6	102722	0.0463	0.0209	2.23	111.4	2.2
Mean					103.8	**2.09**
SD					6.7	0.14
CV (%)					6.5	6.5

Density taken into account: 1.006

^{3}H- Ethyl tafluprostamide in PBS 0.01M pH 7.4 containing 6% polyoxyethylene 20 oleyl ether

Replicate	dpm	µCi	Weight (g)	µCi/mL	Recovery (%)	Concentration in RF (µg/mL)
1	88365	0.0398	0.0200	2.00	99.8	2.0
2	94563	0.0426	0.0204	2.09	104.7	2.1
3	92711	0.0418	0.0207	2.02	101.2	2.0
4	90542	0.0408	0.0197	2.08	103.8	2.1
5	94010	0.0423	0.0207	2.05	102.6	2.1
6	102112	0.0460	0.0220	2.10	104.9	2.1
Mean					102.8	**2.07**
SD					2.0	0.04
CV (%)					2.0	2.0

Density taken into account: 1.003

Table 6: Human skin donors' identification

Donor identification	Origin	Date of removal	Age	Gender	Supplier
1425	Caucasian	02/12/2021	53	F	Tissue Bank
1431	Caucasian	26/01/2022	35	F	Private hospital
RA-22-02-A01005	Caucasian	03/02/2022	58	F	Tissue Bank
1465	Caucasian	18/05/2022	36	F	Tissue Bank
MRS-22-02-A01041	Caucasian	07/02/2022	59	F	Tissue Bank
1441	Caucasian	23/02/2022	41	F	Tissue Bank
1446	Caucasian	27/01/2022	57	F	Tissue Bank
1459	Caucasian	28/03/2022	45	F	Tissue Bank

Table 7: Number of strips performed

Formulations	Cell	Number of strips
DDDE Dilution 0.024%	A	5
	B	6
	C	4
	D	14
	E	5
	F	10
	G	16
	H	6
	I	10
	J	10
	K	10
	L	8
DDDE Dilution 0.020%	M	9
	N	5
	O	11
	P	18
	Q	15
	R	13
	S	4
	T	5
	U	11
	V	8
	W	5
	X	1

Formulations	Cell	Number of strips
DDDE Dilution 0.018%	Y	7
	Z	8
	AA	7
	AB	12
	AC	11
	AD	11
	AE	14
	AF	20
	AG	19
	AH	6
	AI	11
	AJ	6
DDDE Dilution 0.012%	AK	3
	AL	6
	AM	13
	AN	12
	AO	11
	AP	8
	AQ	13
	AR	12
	AS	15
	AT	5
	AU	5
	AV	4

Table 8: Skin thickness, TEWL and temperature for DDDE 0.024% test item

Test item	DDDE Dilution 0.024%											
Matrix	Human dermatomed skin											
Cells	A	B	C	D	E	F	G	H	I	J	K	L
Room Temperature (ºC)	min: 23.20°C max: 24.20°C											
Relative Humidity (%)	min: 46.30 % max: 55.40%											
TEWL (g/m²/h)	6.64	6.29	3.51	2.68	1.85	3.20	6.96	2.54	3.72	8.38	6.46	3.73
Mean	4.66											
SD	2.14											
Cutaneous temperature (°C)	31.8	31.9	32.3	32.7	31.8	32.3	31.8	32.5	32.6	32.5	32.4	32.3
Mean	32.2											
SD	0.3											
Thickness µm	338	388	332	344	354	374	324	318	392	368	398	394
Mean	360											
SD	29											

Table 9: Skin thickness, TEWL and temperature for DDDE 0.020% test item

Test item	DDDE Dilution 0.02%											
Matrix	Human dermatomed skin											
Cells	M	N	O	P	Q	R	S	T	U	V	W	X
Room Temperature (ºC)	min: 23.20°C max: 24.20°C											
Relative Humidity (%)	min: 46.30 % max: 55.40%											
TEWL (g/m²/h)	2.34	7.89	7.63	5.49	5.62	4.15	10.12	5.93	4.78	3.40	3.76	7.62
Mean	5.73											
SD	2.24											
Cutaneous temperature (°C)	32.4	32.5	32.5	32.3	32.4	32.6	31.8	31.1	31.2	32.2	31.9	31.7
Mean	32.1											
SD	0.5											
Thickness µm	330	348	364	310	368	328	400	388	324	388	400	370
Mean	360											
SD	31											

Table 10: Skin thickness, TEWL and temperature for DDDE 0.018% test item

Test item	DDDE Dilution 0.018%											
Matrix	Human dermatomed skin											
Cells	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ
Room Temperature (ºC)	min: 21.60, max: 22.40											
Relative Humidity (%)	min: 45.00, max: 46.50											
TEWL (g/m²/h)	4.35	3.81	2.86	3.61	3.27	1.86	7.29	9.06	4.83	4.95	4.63	6.40
Mean	4.74											
SD	2.01											
Cutaneous temperature (°C)	31.3	31.4	31.3	31.1	31.4	31.4	31.3	32.7	32.7	33.0	32.8	32.6
Mean	31.9											
SD	0.8											
Thickness µm	316	394	400	392	380	398	310	392	388	330	400	388
Mean	374											
SD	34											

Table 11: Skin thickness, TEWL and temperature for DDDE 0.012% test item

Test item	DDDE Dilution 0.012%											
Matrix	Human dermatomed skin											
Cells	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV
Room Temperature (ºC)	min: 21.60, max: 22.40											
Relative Humidity (%)	min: 45.00, max: 46.50											
TEWL (g/m²/h)	3.40	3.78	3.34	3.35	3.54	3.51	3.82	3.37	5.31	3.29	5.25	8.33
Mean	4.19											
SD	1.48											
Cutaneous temperature (°C)	32.8	33.0	32.2	31.8	31.9	32.6	32.5	31.9	32.3	32.8	32.8	32.7
Mean	32.4											
SD	0.4											
Thickness µm	330	400	398	388	400	394	330	388	324	356	388	400
Mean	375											
SD	31											

Table 12: ^{3}H-DDDE activities for DDDE 0.024% test item

The day of the preparation

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.00	1739163	0.78	1	78.34
2	9.30	1822186	0.82	1	88.26
3	10.10	1871751	0.84	1	83.48
4	10.00	1905135	0.86	1	85.82
5	10.00	1921664	0.87	1	86.56
6	10.10	1900766	0.86	1	84.77
Mean	9.92		0.84		84.54
SD	0.31		0.03		3.44
CV (%)	3.09		3.70		4.07

Before application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.10	1913900	0.86	1	85.36
2	10.00	1930337	0.87	1	86.95
3	9.90	1930340	0.87	1	87.83
4	10.10	1935984	0.87	1	86.34
5	10.00	1912130	0.86	1	86.13
6	10.20	1934713	0.87	1	85.44
Mean	10.05		0.87		86.34
SD	0.10		0.00		0.94
CV (%)	1.04		0.55		1.09

During application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.40	1907264	0.86	1	82.61
2	10.10	1926669	0.87	1	85.93
3	9.40	1932029	0.87	1	92.58
4	9.90	1912283	0.86	1	87.01
5	10.10	1965846	0.89	1	87.67
6	9.80	1938151	0.87	1	89.09
Mean	9.95		0.87		87.48
SD	0.34		0.01		3.32
CV (%)	3.41		1.09		3.79

Table 13: ^{3}H-DDDE activities for DDDE 0.020% test item

The day of the preparation

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.10	1725002	0.78	1	76.93
2	10.00	1972279	0.89	1	88.84
3	9.90	1824990	0.82	1	83.04
4	9.90	1881450	0.85	1	85.61
5	10.00	1818507	0.82	1	81.91
6	10.10	1867061	0.84	1	83.27
Mean	10.00		0.83		83.27
SD	0.09		0.04		3.96
CV (%)	0.89		4.43		4.76

Before application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	9.80	1923138	0.87	1	88.40
2	9.80	1951787	0.88	1	89.71
3	9.90	1958864	0.88	1	89.13
4	10.00	1943851	0.88	1	87.56
5	10.00	1964843	0.89	1	88.51
6	10.10	1964486	0.88	1	87.61
Mean	9.93		0.88		88.49
SD	0.12		0.01		0.84
CV (%)	1.22		0.81		0.95

During application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.00	1937953	0.87	1	87.30
2	10.00	1962649	0.88	1	88.41
3	10.10	1975418	0.89	1	88.10
4	10.00	1951823	0.88	1	87.92
5	9.70	1959632	0.88	1	91.00
6	10.00	1988644	0.90	1	89.58
Mean	9.97		0.88		88.72
SD	0.14		0.01		1.35
CV (%)	1.37		0.90		1.52

Table 14: ^{3}H-DDDE activities for DDDE 0.018% test item

The day of the preparation

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.60	1943922	0.88	1	82.61
2	10.40	2043657	0.92	1	88.52
3	9.70	1991957	0.90	1	92.50
4	10.40	2008597	0.90	1	87.00
5	9.90	1994217	0.90	1	90.74
6	10.30	1991166	0.90	1	87.08
Mean	10.22		0.90		88.07
SD	0.34		0.01		3.43
CV (%)	3.36		1.61		3.90

Before application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.40	2041663	0.92	1	88.43
2	10.00	1997960	0.90	1	90.00
3	10.20	2000592	0.90	1	88.35
4	10.30	1992136	0.90	1	87.12
5	10.10	1999869	0.90	1	89.19
6	10.20	1996946	0.90	1	88.19
Mean	10.20		0.90		88.55
SD	0.14		0.01		0.97
CV (%)	1.39		0.91		1.10

During application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.80	2106433	0.95	1	87.86
2	10.30	2012443	0.91	1	88.01
3	10.30	2012085	0.91	1	87.99
4	10.00	2033953	0.92	1	91.62
5	10.70	2099598	0.95	1	88.39
6	10.20	2023879	0.91	1	89.38
Mean	10.38		0.92		88.87
SD	0.31		0.02		1.45
CV (%)	2.95		2.12		1.64

Table 15: ^{3}H-DDDE activities for DDDE 0.012% test item

The day of the preparation

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	9.90	1951122	0.88	1	88.78
2	10.10	2017050	0.91	1	89.96
3	10.00	2007136	0.90	1	90.41
4	10.30	1996097	0.90	1	87.30
5	9.90	1998343	0.90	1	90.92
6	10.10	2022333	0.91	1	90.19
Mean	10.05		0.90		89.59
SD	0.15		0.01		1.33
CV (%)	1.51		1.27		1.49

Before application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.10	1967995	0.89	1	87.77
2	10.10	2039699	0.92	1	90.97
3	10.20	2087154	0.94	1	92.17
4	10.00	2025039	0.91	1	91.22
5	10.20	2048065	0.92	1	90.45
6	10.50	2084229	0.94	1	89.41
Mean	10.18		0.92		90.33
SD	0.17		0.02		1.55
CV (%)	1.69		2.15		1.71

During application

Replicate	Measured quantity mg	Total rad dpm	Total rad µCi	Dilution Factor	Activity µCi/g
1	10.30	2009363	0.91	1	87.88
2	10.50	2052879	0.92	1	88.07
3	10.30	2051254	0.92	1	89.71
4	10.50	2113650	0.95	1	90.68
5	10.00	2033694	0.92	1	91.61
6	10.30	2051675	0.92	1	89.73
Mean	10.32		0.92		89.61
SD	0.18		0.02		1.45
CV (%)	1.78		1.68		1.62

Table 16: Individual results obtained after DDDE application on human skin for DDDE 0.024% test item

Test item activity : 87.48 µCi/g
Concentration of DDDE in test item : 0.24 mg/g

Cell	A	B	C	D	E	F	G	H	I	J	K	L	Mean	SD	CV (%)
Skin donor identification	RA22-02-A1005	RA22-02-A1005	RA22-02-A1005	1431	1431	1431	1425	1425	1425	1465	1465	1465			
Application area (cm²)	1	1	1	1	1	1	1	1	1	1	1	1			
Test item Amount applied (mg)	10.20	10.00	9.70	9.60	10.10	9.90	9.90	10.10	9.50	10.10	10.10	9.90	**9.92**	**0.22**	**2.24**
Test item Amount applied (mg/cm²)	10.20	10.00	9.70	9.60	10.10	9.90	9.90	10.10	9.50	10.10	10.10	9.90	**9.92**	**0.22**	**2.24**
Test item Amount applied (µCi)	0.89	0.87	0.85	0.84	0.88	0.87	0.87	0.88	0.83	0.88	0.88	0.87	**0.87**	**0.02**	**2.24**
Test item Amount applied (µCi/cm²)	0.89	0.87	0.85	0.84	0.88	0.87	0.87	0.88	0.83	0.88	0.88	0.87	**0.87**	**0.02**	**2.24**
Concentration of Test substance (mg/g Test item)	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24			
Test substance applied (µgeq/cm²)	2.42	2.38	2.31	2.28	2.40	2.35	2.35	2.40	2.26	2.40	2.40	2.35			
Strip 1-2 (µCi)	0.04	0.06	0.04	0.04	0.08	0.07	0.03	0.02	0.04	0.06	0.06	0.06	**0.05**	**0.02**	**35.19**
Strip 3-6 (µCi)	0.07	0.09	0.02	0.09	0.12	0.13	0.10	0.13	0.17	0.10	0.11	0.14	**0.11**	**0.04**	**35.58**
Strip 7-11 (µCi)	NC	NC	NC	0.05	NC	0.05	0.05	NC	0.05	0.07	0.03	0.04	**0.05**	**0.01**	**25.27**
Strip 12-15 (µCi)	NC	NC	NC	0.023	NC	NC	0.004	NC	NC	NC	NC	NC	**0.013**	**NC**	**NC**
Strip 16-20 (µCi)	NC	NC	NC	NC	NC	NC	0.003	NC	NC	NC	NC	NC	**0.003**	**NC**	**NC**
Total strips (µgeq/cm²)	0.29	0.40	0.17	0.53	0.53	0.71	0.51	0.41	0.71	0.64	0.57	0.64	**0.51**	**0.17**	**32.70**
Total strips (%)	11.97	16.86	7.20	23.27	22.24	30.08	21.71	17.12	31.26	26.56	23.71	27.39	**21.61**	**7.22**	**33.41**
RCD (µCi)	0.001	0.002	0.001	0.001	BLQ	BLQ	0.003	0.012	BLQ	0.003	0.002	0.002	**0.002**	**0.003**	**158.15**
Washing 24h (µCi)	0.55	0.55	0.60	0.67	0.62	0.59	0.65	0.56	0.54	0.49	0.55	0.55	**0.58**	**0.05**	**8.76**
Remaining Skin (µCi)	0.0005	0.0003	0.0003	0.0002	0.0002	0.0001	0.0004	0.0002	0.0003	0.0040	0.0006	0.0038	**0.0009**	**0.0014**	**154.85**
Skin Excess (µCi)	0.56	0.55	0.60	0.67	0.62	0.59	0.65	0.58	0.54	0.50	0.56	0.55	**0.58**	**0.05**	**8.52**
Skin Excess (µCi/cm²)	0.56	0.55	0.60	0.67	0.62	0.59	0.65	0.58	0.54	0.50	0.56	0.55	**0.58**	**0.05**	**8.52**
Skin Excess (µgeq/cm²)	1.51	1.50	1.62	1.83	1.68	1.59	1.78	1.57	1.47	1.36	1.51	1.50	**1.57**	**0.13**	**8.52**
Skin Excess (%)	62.23	62.92	70.28	80.13	69.85	67.59	75.53	65.26	65.03	56.52	62.82	63.64	**66.82**	**6.39**	**9.56**
Epidermis (µCi)	0.090	0.080	0.104	0.008	0.056	0.026	0.002	0.114	0.014	0.095	0.077	0.032	**0.058**	**0.040**	**68.98**
Epidermis (µCi/cm²)	0.090	0.080	0.104	0.008	0.056	0.026	0.002	0.114	0.014	0.095	0.077	0.032	**0.058**	**0.040**	**68.98**
Epidermis (µgeq/cm²)	0.25	0.22	0.28	0.02	0.15	0.07	0.01	0.31	0.04	0.26	0.21	0.09	**0.16**	**0.11**	**68.98**
Epidermis (%)	10.11	9.15	12.21	0.96	6.28	3.01	0.28	12.86	1.65	10.70	8.70	3.68	**6.63**	**4.56**	**68.70**
Dermis (µCi)	0.0022	0.0024	0.0098	0.0002	0.0005	0.0003	0.0004	0.0039	0.0018	0.0088	0.0103	0.0047	**0.0038**	**0.0038**	**101.11**
Dermis (µCi/cm²)	0.0022	0.0024	0.0098	0.0002	0.0005	0.0003	0.0004	0.0039	0.0018	0.0088	0.0103	0.0047	**0.0038**	**0.0038**	**101.11**
Dermis (µgeq/cm²)	0.006	0.006	0.027	0.001	0.001	0.001	0.001	0.011	0.005	0.024	0.028	0.013	**0.010**	**0.010**	**101.11**
Dermis (%)	0.25	0.27	1.15	0.02	0.06	0.03	0.05	0.44	0.22	1.00	1.16	0.54	**0.43**	**0.44**	**101.11**
Epidermis + Strip (µgeq/cm²)	0.54	0.62	0.45	0.55	0.68	0.78	0.52	0.72	0.74	0.89	0.78	0.73	**0.67**	**0.13**	**19.85**
Epidermis + Strip (%)	22.08	26.01	19.41	24.23	28.53	33.09	22.00	29.98	32.91	37.26	32.42	31.06	**28.25**	**5.49**	**19.42**
Epidermis + Dermis (µgeq/cm²)	0.25	0.22	0.31	0.02	0.15	0.07	0.01	0.32	0.04	0.28	0.24	0.10	**0.17**	**0.12**	**68.82**
Epidermis + Dermis (%)	10.36	9.42	13.36	0.98	6.34	3.05	0.33	13.30	1.87	11.70	9.87	4.22	**7.07**	**4.85**	**68.60**
Epidermis + Dermis + Strip (µgeq/cm²)	0.54	0.62	0.47	0.55	0.69	0.78	0.52	0.73	0.75	0.92	0.81	0.74	**0.68**	**0.14**	**19.94**
Epidermis + Dermis + Strip (%)	22.33	26.28	20.56	24.25	28.58	33.12	22.04	30.42	33.13	38.26	33.58	31.60	**28.68**	**5.58**	**19.46**
Receptor liquid 24h (µCi)	0.08	0.07	0.08	0.01	0.01	0.01	0.01	0.02	0.04	0.02	0.01	0.01	**0.03**	**0.03**	**94.15**
RCR (µCi)	0.0002	0.0004	0.0001	0.0001	0.0001	0.0001	0.0017	0.0002	0.0001	0.0006	0.0002	0.0001	**0.0003**	**0.0005**	**146.37**
Receptor fluid (µCi)	0.08	0.07	0.08	0.01	0.01	0.01	0.01	0.02	0.04	0.02	0.01	0.01	**0.03**	**0.03**	**93.03**
Receptor fluid (µCi/cm²)	0.08	0.07	0.08	0.01	0.01	0.01	0.01	0.02	0.04	0.02	0.01	0.01	**0.03**	**0.03**	**93.03**
Receptor fluid (µgeq/cm²)	0.22	0.18	0.23	0.02	0.02	0.02	0.04	0.06	0.11	0.06	0.04	0.03	**0.09**	**0.08**	**93.03**
Receptor fluid (%)	8.96	7.62	9.87	0.79	0.77	0.79	1.69	2.60	4.82	2.36	1.61	1.45	**3.61**	**3.36**	**93.05**
Epidermis + dermis + receptor fluid (µCi)	0.17	0.15	0.20	0.01	0.06	0.03	0.02	0.14	0.06	0.12	0.10	0.05	**0.09**	**0.06**	**67.28**
Epidermis + dermis + receptor fluid (µgeq/cm²)	0.47	0.41	0.54	0.04	0.17	0.09	0.05	0.38	0.15	0.34	0.28	0.13	**0.25**	**0.17**	**67.28**
Epidermis + dermis + receptor fluid (%)	19.32	17.04	23.23	1.77	7.11	3.84	2.02	15.90	6.68	14.06	11.48	5.67	**10.68**	**7.18**	**67.24**
Strips + Epidermis + Dermis + Receptor Fluid (µCi)	0.28	0.30	0.26	0.21	0.26	0.29	0.21	0.29	0.32	0.36	0.31	0.29	**0.28**	**0.04**	**15.34**
Strips + Epidermis + Dermis + Receptor Fluid (µgeq/cm²)	0.76	0.81	0.70	0.57	0.70	0.80	0.56	0.79	0.86	0.98	0.84	0.78	**0.76**	**0.12**	**15.34**
Strips + Epidermis + Dermis + Receptor Fluid (%)	31.29	33.90	30.43	25.04	29.36	33.92	23.73	33.02	37.94	40.62	35.19	33.06	**32.29**	**4.81**	**14.91**
TOTAL RECOVERY (%)	93.51	96.82	100.71	105.17	99.21	101.51	99.26	98.28	102.97	97.14	98.01	96.70	**99.11**	**3.13**	**3.16**

Strips 1-2 (µgeq/cm²)	0.11	0.16	0.11	0.10	0.20	0.19	0.08	0.06	0.12	0.16	0.17	0.18	**0.14**	**0.05**	**35.19**
Strips 1-2 (%)	4.57	6.76	4.62	4.17	8.51	8.23	3.41	2.31	5.16	6.84	7.01	7.47	**5.75**	**1.99**	**34.50**
Strips 3-20 (µgeq/cm²)	0.18	0.24	0.06	0.44	0.33	0.51	0.43	0.36	0.59	0.47	0.40	0.47	**0.37**	**0.15**	**40.37**
Strips 3-20 (%)	7.40	10.09	2.58	19.10	13.73	21.84	18.30	14.82	26.10	19.72	16.70	19.92	**15.86**	**6.58**	**41.47**

BLQ: Below the Limit of Quantification NC: Not Calculated

Table 17: Cumulative % of applied dose in the receptor fluid for DDDE 0.024% test item

	A	B	C	D	E	F	G	H	I	J	K	L	Mean	SD
LR 0.5h	0.13	0.09	0.07	0.02	0.01	BLQ	BLQ	0.07	0.02	BLQ	BLQ	BLQ	0.03	0.05
LR 2h	2.74	2.13	3.08	0.22	0.17	0.18	0.25	0.59	0.65	0.14	0.15	0.16	0.87	1.11
LR 4h	4.33	3.53	4.15	0.32	0.28	0.29	0.49	0.94	1.72	0.38	0.37	0.34	1.43	1.61
LR 8h	5.13	4.45	5.13	0.45	0.42	0.43	0.79	1.34	2.90	0.86	0.76	0.65	1.94	1.91
LR 12h	5.93	5.11	6.12	0.55	0.52	0.53	0.99	1.64	3.49	1.30	1.02	0.89	2.34	2.20
LR 24h	8.94	7.58	9.86	0.78	0.77	0.78	1.50	2.58	4.80	2.29	1.59	1.44	3.58	3.37

Table 18: Cumulative $\mu g_{eq}/cm^2$ of applied dose in the receptor fluid for DDDE 0.024% test item

	A	B	C	D	E	F	G	H	I	J	K	L	Mean	SD
LR 0.5h	0.0031	0.0022	0.0015	0.0004	0.0002	BLQ	BLQ	0.0018	0.0005	BLQ	BLQ	BLQ	0.0008	0.0011
LR 2h	0.066	0.051	0.071	0.005	0.004	0.004	0.006	0.014	0.015	0.003	0.004	0.004	0.021	0.026
LR 4h	0.10	0.08	0.10	0.01	0.01	0.01	0.01	0.02	0.04	0.01	0.01	0.01	0.03	0.04
LR 8h	0.12	0.11	0.12	0.01	0.01	0.01	0.02	0.03	0.07	0.02	0.02	0.02	0.05	0.05
LR 12h	0.14	0.12	0.14	0.01	0.01	0.01	0.02	0.04	0.08	0.03	0.02	0.02	0.06	0.05
LR 24h	0.22	0.18	0.23	0.02	0.02	0.02	0.04	0.06	0.11	0.06	0.04	0.03	0.08	0.08

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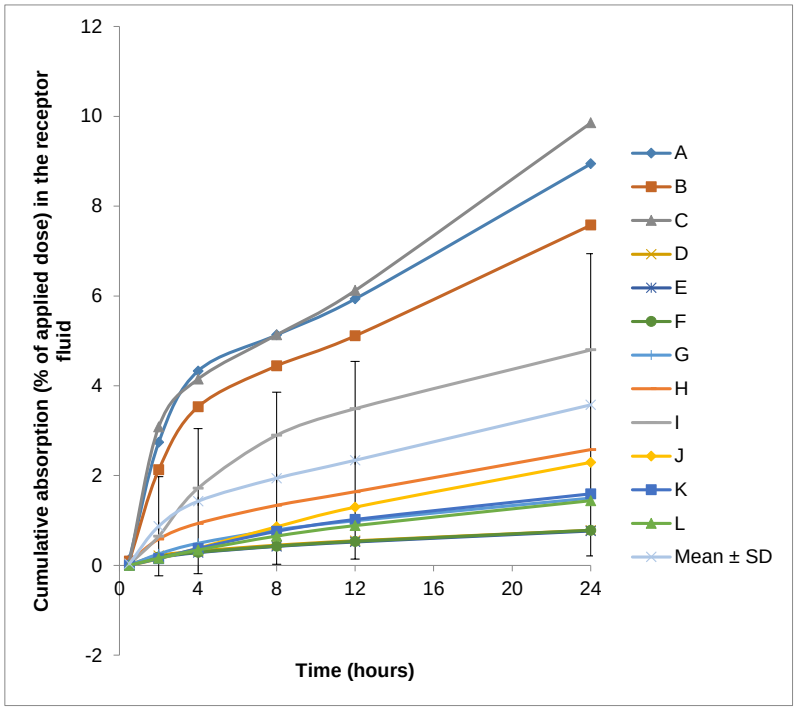
	Eurofins|ADME BIOANALYSES study code: 22-0148	

Figure 1: Cumulative absorption (% of applied dose and $\mu g_{eq}/cm^2$) in the receptor fluid for DDDE 0.024% test item



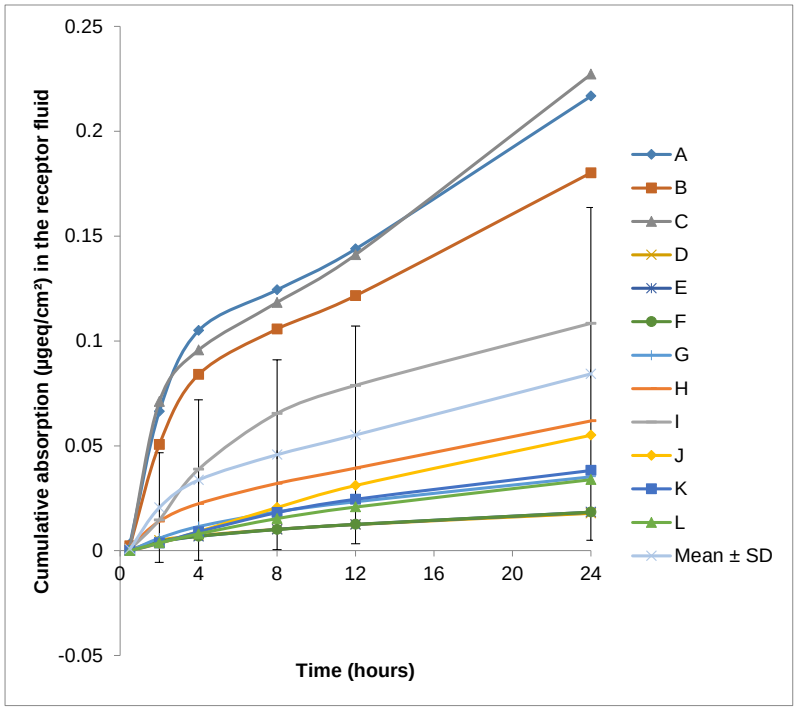


Table 19: Individual results obtained after DDDE application on human skin for DDDE 0.020% test item

Test item activity : 88.72 µCi/g
Concentration of DDDE in test item : 0.20 mg/g

Cell	M	N	O	P	Q	R	S	T	U	V	W	X	Mean	SD	CV (%)
Skin donor identification	RA22-02-A1005	RA22-02-A1005	RA22-02-A1005	1431	1431	1431	1425	1425	1425	1465	1465	1465			
Application area (cm²)	1	1	1	1	1	1	1	1	1	1	1	1			
Test item Amount applied (mg)	10.40	9.90	9.90	9.70	9.50	9.90	10.50	10.00	10.20	9.40	10.00	10.20	**9.97**	**0.33**	**3.32**
Test item Amount applied (mg/cm²)	10.40	9.90	9.90	9.70	9.50	9.90	10.50	10.00	10.20	9.40	10.00	10.20	**9.97**	**0.33**	**3.32**
Test item Amount applied (µCi)	0.92	0.88	0.88	0.86	0.84	0.88	0.93	0.89	0.90	0.83	0.89	0.90	**0.88**	**0.03**	**3.32**
Test item Amount applied (µCi/cm²)	0.92	0.88	0.88	0.86	0.84	0.88	0.93	0.89	0.90	0.83	0.89	0.90	**0.88**	**0.03**	**3.32**
Concentration of Test substance (mg/g Test item)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20			
Test substance applied (µgeq/cm²)	2.13	2.02	2.02	1.98	1.94	2.02	2.15	2.04	2.08	1.92	2.04	2.08			
Strip 1-2 (µCi)	0.04	0.06	0.06	0.01	0.04	0.03	0.02	0.03	0.03	0.07	0.05	0.02	**0.04**	**0.02**	**49.52**
Strip 3-6 (µCi)	0.12	0.10	0.13	0.05	0.11	0.10	0.07	0.11	0.11	0.13	0.11	NC	**0.10**	**0.02**	**22.23**
Strip 7-11 (µCi)	0.03	NC	0.04	0.07	0.04	0.06	NC	NC	0.05	0.05	NC	NC	**0.05**	**0.01**	**26.85**
Strip 12-15 (µCi)	NC	NC	NC	0.031	0.003	0.002	NC	NC	NC	NC	NC	NC	**0.012**	**0.017**	**135.11**
Strip 16-20 (µCi)	NC	NC	NC	0.005	NC	NC	NC	NC	NC	NC	NC	NC	**0.005**	**NC**	**NC**
Total strips (µgeq/cm²)	0.43	0.37	0.53	0.37	0.44	0.45	0.22	0.32	0.43	0.56	0.37	0.05	**0.38**	**0.14**	**36.22**
Total strips (%)	20.34	18.04	26.19	18.54	22.74	22.18	10.38	15.90	20.66	29.28	17.87	2.41	**18.71**	**7.05**	**37.68**
RCD (µCi)	BLQ	0.0010	0.0025	0.0005	BLQ	0.0013	BLQ	0.0005	0.0006	0.0008	0.0037	0.0008	**0.0010**	**0.0011**	**114.05**
Washing 24h (µCi)	0.61	0.60	0.59	0.65	0.60	0.61	0.57	0.50	0.65	0.55	0.60	0.61	**0.60**	**0.04**	**6.91**
Remaining Skin (µCi)	0.0001	0.0004	0.0005	0.0002	0.0004	0.0029	0.0008	0.0003	0.0002	0.0004	0.0020	0.0012	**0.0008**	**0.0009**	**108.41**
Skin Excess (µCi)	0.61	0.60	0.60	0.65	0.60	0.62	0.57	0.50	0.65	0.55	0.61	0.61	**0.60**	**0.04**	**6.91**
Skin Excess (µCi/cm²)	0.61	0.60	0.60	0.65	0.60	0.62	0.57	0.50	0.65	0.55	0.61	0.61	**0.60**	**0.04**	**6.91**
Skin Excess (µgeq/cm²)	1.40	1.39	1.38	1.50	1.38	1.42	1.32	1.15	1.51	1.28	1.40	1.41	**1.38**	**0.10**	**6.91**
Skin Excess (%)	65.98	68.74	67.97	75.86	71.33	70.09	61.51	56.38	72.22	66.55	68.28	67.45	**67.70**	**5.03**	**7.43**
Epidermis (µCi)	0.03	0.07	0.01	0.01	0.01	0.05	0.11	0.10	0.00	0.05	0.05	0.15	**0.05**	**0.05**	**87.69**
Epidermis (µCi/cm²)	0.03	0.07	0.01	0.01	0.01	0.05	0.11	0.10	0.00	0.05	0.05	0.15	**0.05**	**0.05**	**87.69**
Epidermis (µgeq/cm²)	0.07	0.17	0.03	0.01	0.02	0.13	0.25	0.23	0.01	0.11	0.10	0.34	**0.12**	**0.11**	**87.69**
Epidermis (%)	3.08	8.32	1.32	0.73	1.00	6.19	11.78	11.45	0.41	5.56	5.13	16.18	**5.93**	**5.11**	**86.22**
Dermis (µCi)	0.0007	0.0022	0.0016	0.0002	0.0002	0.0029	0.0028	0.0038	0.0003	0.0010	0.0012	0.0060	**0.0019**	**0.0017**	**90.40**
Dermis (µCi/cm²)	0.0007	0.0022	0.0016	0.0002	0.0002	0.0029	0.0028	0.0038	0.0003	0.0010	0.0012	0.0060	**0.0019**	**0.0017**	**90.40**
Dermis (µgeq/cm²)	0.002	0.005	0.004	0.001	0.000	0.007	0.006	0.009	0.001	0.002	0.003	0.014	**0.004**	**0.004**	**90.40**
Dermis (%)	0.07	0.25	0.19	0.03	0.02	0.33	0.30	0.43	0.04	0.13	0.13	0.66	**0.21**	**0.19**	**89.29**
Epidermis + Strip (µgeq/cm²)	0.50	0.53	0.56	0.38	0.46	0.57	0.48	0.56	0.44	0.67	0.47	0.39	**0.50**	**0.08**	**16.53**
Epidermis + Strip (%)	23.42	26.36	27.51	19.27	23.74	28.37	22.16	27.35	21.08	34.84	23.00	18.59	**24.64**	**4.53**	**18.39**
Epidermis + Dermis (µgeq/cm²)	0.07	0.17	0.03	0.01	0.02	0.13	0.26	0.24	0.01	0.11	0.11	0.35	**0.13**	**0.11**	**87.55**
Epidermis + Dermis (%)	3.15	8.57	1.51	0.75	1.03	6.52	12.08	11.89	0.45	5.69	5.27	16.84	**6.14**	**5.29**	**86.08**
Epidermis + Dermis + Strip (µgeq/cm²)	0.50	0.54	0.56	0.38	0.46	0.58	0.48	0.57	0.44	0.67	0.47	0.40	**0.50**	**0.08**	**16.37**
Epidermis + Dermis + Strip (%)	23.49	26.62	27.70	19.30	23.76	28.70	22.46	27.78	21.11	34.97	23.14	19.24	**24.86**	**4.52**	**18.19**
Receptor liquid 24h (µCi)	0.02	0.05	0.03	0.01	0.01	0.01	0.05	0.07	0.01	0.01	0.02	0.04	**0.03**	**0.02**	**78.16**
RCR (µCi)	0.0001	0.0001	0.0002	0.0001	0.0001	0.0001	0.0003	0.0002	0.0001	0.0002	0.0001	0.0015	**0.0002**	**0.0004**	**160.96**
Receptor fluid (µCi)	0.02	0.05	0.03	0.01	0.01	0.01	0.05	0.07	0.01	0.01	0.02	0.04	**0.03**	**0.02**	**77.97**
Receptor fluid (µCi/cm²)	0.02	0.05	0.03	0.01	0.01	0.01	0.05	0.07	0.01	0.01	0.02	0.04	**0.03**	**0.02**	**77.97**
Receptor fluid (µgeq/cm²)	0.04	0.10	0.06	0.01	0.02	0.02	0.13	0.15	0.03	0.03	0.04	0.10	**0.06**	**0.05**	**77.97**
Receptor fluid (%)	1.69	5.18	2.98	0.71	0.98	0.92	5.85	7.54	1.30	1.71	2.02	4.83	**2.98**	**2.29**	**76.96**
Epidermis + dermis + receptor fluid (µCi)	0.04	0.12	0.04	0.01	0.02	0.07	0.17	0.17	0.02	0.06	0.06	0.20	**0.08**	**0.07**	**80.71**
Epidermis + dermis + receptor fluid (µgeq/cm²)	0.10	0.28	0.09	0.03	0.04	0.15	0.38	0.40	0.04	0.14	0.15	0.45	**0.19**	**0.15**	**80.71**
Epidermis + dermis + receptor fluid (%)	4.84	13.75	4.49	1.46	2.01	7.43	17.92	19.43	1.75	7.39	7.29	21.66	**9.12**	**7.23**	**79.28**
Strips + Epidermis + Dermis + Receptor Fluid (µCi)	0.23	0.28	0.27	0.17	0.21	0.26	0.26	0.31	0.20	0.31	0.22	0.22	**0.25**	**0.04**	**17.57**
Strips + Epidermis + Dermis + Receptor Fluid (µgeq/cm²)	0.54	0.64	0.62	0.40	0.48	0.60	0.61	0.72	0.47	0.70	0.51	0.50	**0.57**	**0.10**	**17.57**
Strips + Epidermis + Dermis + Receptor Fluid (%)	25.18	31.79	30.68	20.01	24.74	29.61	28.31	35.32	22.41	36.68	25.16	24.07	**27.83**	**5.13**	**18.45**
TOTAL RECOVERY (%)	91.17	100.53	98.66	95.86	96.07	99.71	89.81	91.70	94.63	103.23	93.44	91.52	**95.53**	**4.26**	**4.46**

Strips 1-2 (µgeq/cm²)	0.09	0.13	0.13	0.01	0.08	0.08	0.05	0.07	0.06	0.16	0.11	0.05	**0.09**	**0.04**	**49.52**
Strips 1-2 (%)	4.19	6.55	6.40	0.65	4.18	3.87	2.37	3.40	2.83	8.56	5.50	2.41	**4.24**	**2.19**	**51.66**
Strips 3-20 (µgeq/cm²)	0.34	0.23	0.40	0.35	0.36	0.37	0.17	0.26	0.37	0.40	0.25	NC	**0.32**	**0.08**	**24.15**
Strips 3-20 (%)	16.15	11.50	19.79	17.90	18.55	18.31	8.01	12.49	17.83	20.72	12.37	NC	**15.78**	**4.06**	**25.70**

BLQ: Below the Limit of Quantification NC: Not Calculated

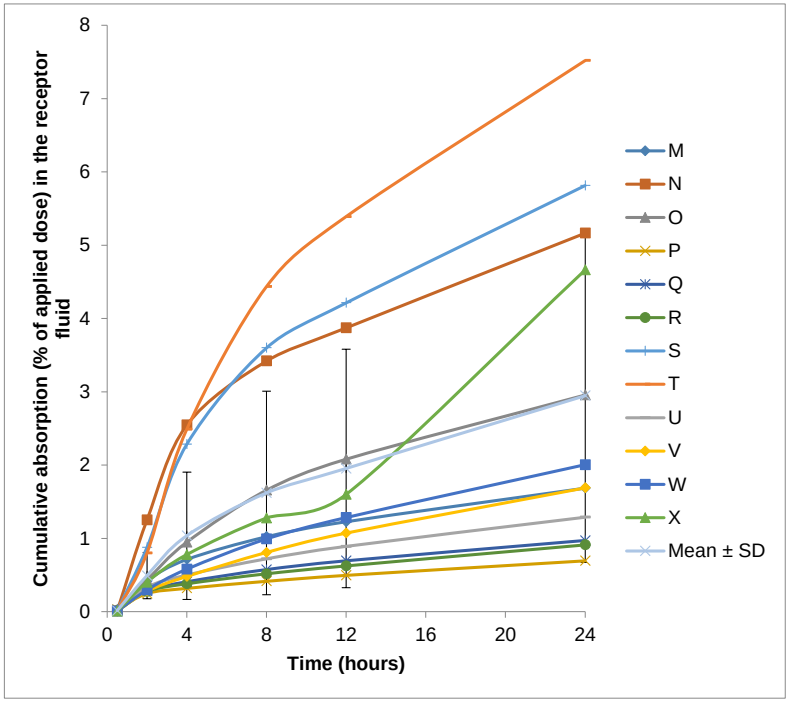
Table 20: Cumulative % of applied dose in the receptor fluid for DDDE 0.020% test item

	M	N	O	P	Q	R	S	T	U	V	W	X	Mean	SD
LR 0.5h	0.01	0.01	0.03	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
LR 2h	0.42	1.25	0.43	0.25	0.28	0.28	0.88	0.80	0.34	0.28	0.30	0.40	0.49	0.32
LR 4h	0.71	2.55	0.95	0.32	0.41	0.38	2.29	2.49	0.50	0.48	0.58	0.78	1.04	0.87
LR 8h	1.02	3.42	1.66	0.42	0.57	0.52	3.60	4.44	0.72	0.81	0.99	1.28	1.62	1.39
LR 12h	1.23	3.87	2.08	0.50	0.69	0.63	4.21	5.39	0.89	1.07	1.28	1.60	1.95	1.63
LR 24h	1.69	5.17	2.96	0.70	0.97	0.91	5.81	7.52	1.29	1.69	2.01	4.66	2.95	2.27

Table 21: Cumulative $\mu g_{eq}/cm^2$ of applied dose in the receptor fluid for DDDE 0.020% test item

	M	N	O	P	Q	R	S	T	U	V	W	X	Mean	SD
LR 0.5h	0.0002	0.0002	0.0007	0.0006	0.0005	0.0003	0.0003	0.0001	0.0002	0.0002	0.0001	0.0002	0.0003	0.0002
LR 2h	0.009	0.025	0.009	0.005	0.006	0.006	0.019	0.016	0.007	0.005	0.006	0.008	0.010	0.007
LR 4h	0.02	0.05	0.02	0.01	0.01	0.01	0.05	0.05	0.01	0.01	0.01	0.02	0.02	0.02
LR 8h	0.02	0.07	0.03	0.01	0.01	0.01	0.08	0.09	0.02	0.02	0.02	0.03	0.03	0.03
LR 12h	0.03	0.08	0.04	0.01	0.01	0.01	0.09	0.11	0.02	0.02	0.03	0.03	0.04	0.03
LR 24h	0.04	0.10	0.06	0.01	0.02	0.02	0.12	0.15	0.03	0.03	0.04	0.10	0.06	0.05

Figure 2: Cumulative absorption (% of applied dose and $\mu g_{eq}/cm^2$) in the receptor fluid for DDDE 0.020% test item



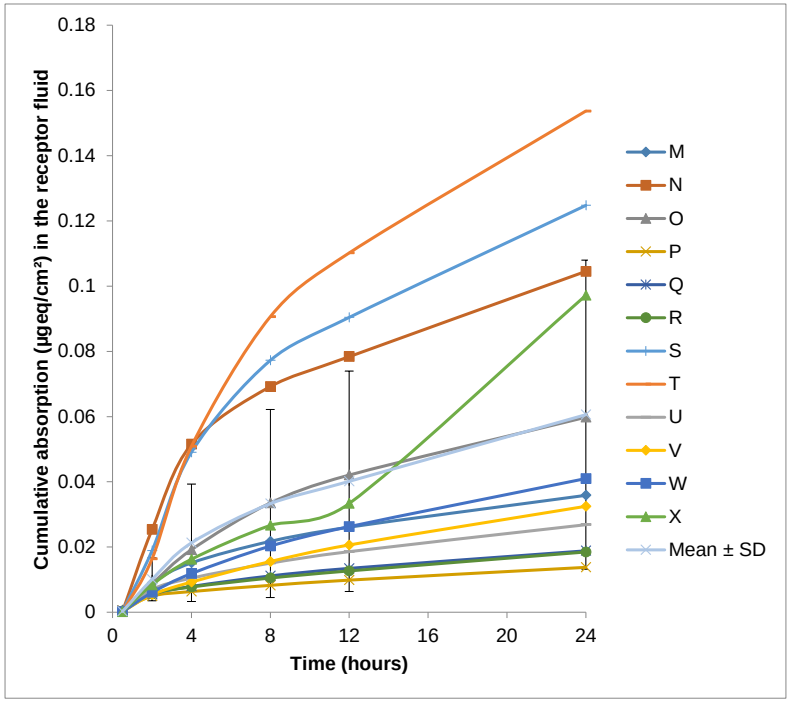


Table 22: Individual results obtained after DDDE application on human skin for DDDE 0.018% test item

Test item activity : 88.87 µCi/g
Concentration of DDDE in test item : 0.18 mg/g

Cell	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	**Mean**	**SD**	**CV (%)**
Skin donor identification	MRS-22-02-A01041	MRS-22-02-A01041	MRS-22-02-A01041	1459	1459	1459	1441	1441	1441	1446	1446	1446			
Application area (cm²)	1	1	1	1	1	1	1	1	1	1	1	1			
Test item Amount applied (mg)	10.20	10.40	11.20	10.10	9.90	9.60	10.20	10.20	10.00	10.30	10.30	10.30	**10.23**	**0.38**	**3.69**
Test item Amount applied (mg/cm²)	10.20	10.40	11.20	10.10	9.90	9.60	10.20	10.20	10.00	10.30	10.30	10.30	**10.23**	**0.38**	**3.69**
Test item Amount applied (µCi)	0.91	0.92	1.00	0.90	0.88	0.85	0.91	0.91	0.89	0.92	0.92	0.92	**0.91**	**0.03**	**3.69**
Test item Amount applied (µCi/cm²)	0.91	0.92	1.00	0.90	0.88	0.85	0.91	0.91	0.89	0.92	0.92	0.92	**0.91**	**0.03**	**3.69**
Concentration of Test substance (mg/g Test item)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18			
Test substance applied (µgeq/cm²)	1.84	1.88	2.02	1.83	1.79	1.73	1.84	1.84	1.81	1.86	1.86	1.86			
Strip 1-2 (µCi)	0.01	0.03	0.04	0.06	0.06	0.07	0.05	0.07	0.03	0.03	0.03	0.06	**0.05**	**0.02**	**39.11**
Strip 3-6 (µCi)	0.03	0.05	0.05	0.06	0.04	0.07	0.07	0.05	0.05	0.06	0.07	0.07	**0.06**	**0.01**	**24.97**
Strip 7-11 (µCi)	0.01	0.02	0.01	0.03	0.03	0.02	0.04	0.02	0.03	NC	0.04	NC	**0.02**	**0.01**	**49.07**
Strip 12-15 (µCi)	NC	NC	NC	0.01	NC	NC	0.02	0.01	0.01	NC	NC	NC	**0.01**	**0.01**	**55.11**
Strip 16-20 (µCi)	NC	NC	NC	NC	NC	NC	NC	0.02	0.01	NC	NC	NC	**0.01**	**NC**	**NC**
Total strips (µgeq/cm²)	0.10	0.20	0.22	0.32	0.27	0.33	0.39	0.33	0.28	0.19	0.29	0.26	**0.27**	**0.08**	**29.52**
Total strips (%)	5.50	10.64	10.72	17.78	14.84	19.26	21.06	18.13	15.54	10.34	15.60	14.05	**14.45**	**4.47**	**30.91**
RCD (µCi)	0.001	0.002	0.001	0.002	0.018	0.004	0.002	0.001	0.002	0.001	0.001	0.004	**0.003**	**0.005**	**145.69**
Washing 24h (µCi)	0.74	0.69	0.75	0.60	0.59	0.60	0.55	0.55	0.56	0.69	0.60	0.64	**0.63**	**0.07**	**11.21**
Remaining Skin (µCi)	0.0004	0.0170	0.0001	0.0050	0.0048	0.0006	0.0002	0.0008	0.0018	0.0029	0.0008	0.0086	**0.0036**	**0.0049**	**137.95**
Skin Excess (µCi)	0.74	0.71	0.75	0.61	0.61	0.61	0.55	0.55	0.57	0.69	0.61	0.66	**0.64**	**0.07**	**11.11**
Skin Excess (µCi/cm²)	0.74	0.71	0.75	0.61	0.61	0.61	0.55	0.55	0.57	0.69	0.61	0.66	**0.64**	**0.07**	**11.11**
Skin Excess (µgeq/cm²)	1.51	1.43	1.52	1.24	1.25	1.24	1.12	1.12	1.15	1.40	1.23	1.33	**1.30**	**0.14**	**11.11**
Skin Excess (%)	81.95	76.33	75.25	68.03	69.79	71.30	60.69	60.51	63.67	75.43	66.17	71.65	**70.06**	**6.60**	**9.43**
Epidermis (µCi)	0.04	0.04	0.03	0.03	0.02	0.03	0.03	0.05	0.05	0.05	0.05	0.04	**0.04**	**0.01**	**24.70**
Epidermis (µCi/cm²)	0.04	0.04	0.03	0.03	0.02	0.03	0.03	0.05	0.05	0.05	0.05	0.04	**0.04**	**0.01**	**24.70**
Epidermis (µgeq/cm²)	0.07	0.09	0.07	0.06	0.05	0.06	0.06	0.10	0.10	0.10	0.09	0.08	**0.08**	**0.02**	**24.70**
Epidermis (%)	3.99	4.65	3.49	3.03	2.54	3.38	3.49	5.18	5.80	5.32	5.01	4.11	**4.17**	**1.02**	**24.50**
Dermis (µCi)	0.003	0.003	0.001	0.003	0.002	0.002	0.010	0.002	0.008	0.002	0.005	0.003	**0.004**	**0.003**	**74.94**
Dermis (µCi/cm²)	0.003	0.003	0.001	0.003	0.002	0.002	0.010	0.002	0.008	0.002	0.005	0.003	**0.004**	**0.003**	**74.94**
Dermis (µgeq/cm²)	0.007	0.006	0.002	0.006	0.004	0.004	0.020	0.004	0.016	0.004	0.011	0.006	**0.007**	**0.006**	**74.94**
Dermis (%)	0.36	0.30	0.08	0.31	0.24	0.21	1.09	0.24	0.88	0.21	0.59	0.30	**0.40**	**0.30**	**75.21**
Epidermis + Strip (µgeq/cm²)	0.17	0.29	0.29	0.38	0.31	0.39	0.45	0.43	0.39	0.29	0.38	0.34	**0.34**	**0.08**	**22.41**
Epidermis + Strip (%)	9.49	15.30	14.21	20.81	17.38	22.63	24.55	23.32	21.33	15.66	20.61	18.17	**18.62**	**4.41**	**23.68**
Epidermis + Dermis (µgeq/cm²)	0.08	0.09	0.07	0.06	0.05	0.06	0.08	0.10	0.12	0.10	0.10	0.08	**0.08**	**0.02**	**24.68**
Epidermis + Dermis (%)	4.35	4.95	3.56	3.35	2.78	3.58	4.58	5.42	6.67	5.53	5.60	4.41	**4.57**	**1.13**	**24.72**
Epidermis + Dermis + Strip (µgeq/cm²)	0.18	0.29	0.29	0.39	0.32	0.40	0.47	0.43	0.40	0.30	0.39	0.34	**0.35**	**0.08**	**22.73**
Epidermis + Dermis + Strip (%)	9.85	15.60	14.28	21.12	17.62	22.84	25.64	23.56	22.21	15.87	21.19	18.46	**19.02**	**4.56**	**23.98**
Receptor liquid 24h (µCi)	0.003	0.006	0.006	0.014	0.015	0.012	0.024	0.029	0.042	0.016	0.023	0.015	**0.017**	**0.011**	**63.91**
RCR (µCi)	0.00005	0.00005	BLQ	0.00080	0.00008	0.00023	0.00014	0.00074	0.00076	0.00029	0.00047	0.00126	**0.00041**	**0.00040**	**98.52**
Receptor fluid (µCi)	0.003	0.006	0.006	0.015	0.015	0.012	0.024	0.030	0.042	0.016	0.024	0.016	**0.018**	**0.011**	**63.56**
Receptor fluid (µCi/cm²)	0.003	0.006	0.006	0.015	0.015	0.012	0.024	0.030	0.042	0.016	0.024	0.016	**0.018**	**0.011**	**63.56**
Receptor fluid (µgeq/cm²)	0.01	0.01	0.01	0.03	0.03	0.02	0.05	0.06	0.09	0.03	0.05	0.03	**0.04**	**0.02**	**63.56**
Receptor fluid (%)	0.37	0.70	0.61	1.62	1.73	1.41	2.63	3.34	4.77	1.73	2.61	1.77	**1.94**	**1.25**	**64.32**
Epidermis + dermis + receptor fluid (µCi)	0.04	0.05	0.04	0.04	0.04	0.04	0.07	0.08	0.10	0.07	0.08	0.06	**0.06**	**0.02**	**32.56**
Epidermis + dermis + receptor fluid (µgeq/cm²)	0.09	0.11	0.08	0.09	0.08	0.09	0.13	0.16	0.21	0.14	0.15	0.12	**0.12**	**0.04**	**32.56**
Epidermis + dermis + receptor fluid (%)	4.72	5.65	4.18	4.96	4.51	5.00	7.21	8.76	11.44	7.26	8.21	6.18	**6.51**	**2.16**	**33.25**
Strips + Epidermis + Dermis + Receptor Fluid (µCi)	0.09	0.15	0.15	0.20	0.17	0.21	0.26	0.24	0.24	0.16	0.22	0.19	**0.19**	**0.05**	**25.15**
Strips + Epidermis + Dermis + Receptor Fluid (µgeq/cm²)	0.19	0.31	0.30	0.42	0.35	0.42	0.52	0.50	0.49	0.33	0.44	0.38	**0.39**	**0.10**	**25.15**
Strips + Epidermis + Dermis + Receptor Fluid (%)	10.22	16.29	14.90	22.74	19.35	24.25	28.27	26.89	26.98	17.60	23.80	20.23	**20.96**	**5.52**	**26.34**
TOTAL RECOVERY (%)	92.17	92.62	90.15	90.77	89.14	95.55	88.96	87.40	90.64	93.03	89.97	91.88	**91.02**	**2.18**	**2.39**

Strips 1-2 (µgeq/cm²)	0.03	0.06	0.09	0.13	0.13	0.14	0.11	0.15	0.07	0.06	0.07	0.11	**0.10**	**0.04**	**39.11**
Strips 1-2 (%)	1.49	3.38	4.46	7.20	7.03	7.97	5.93	7.90	3.92	3.37	3.53	6.03	**5.18**	**2.11**	**40.69**
Strips 3-20 (µgeq/cm²)	0.07	0.14	0.13	0.19	0.14	0.20	0.28	0.19	0.21	0.13	0.22	0.15	**0.17**	**0.05**	**32.20**
Strips 3-20 (%)	4.01	7.26	6.26	10.57	7.80	11.29	15.13	10.24	11.62	6.97	12.07	8.02	**9.27**	**3.08**	**33.19**

BLQ: Below the Limit of Quantification NC: Not Calculated

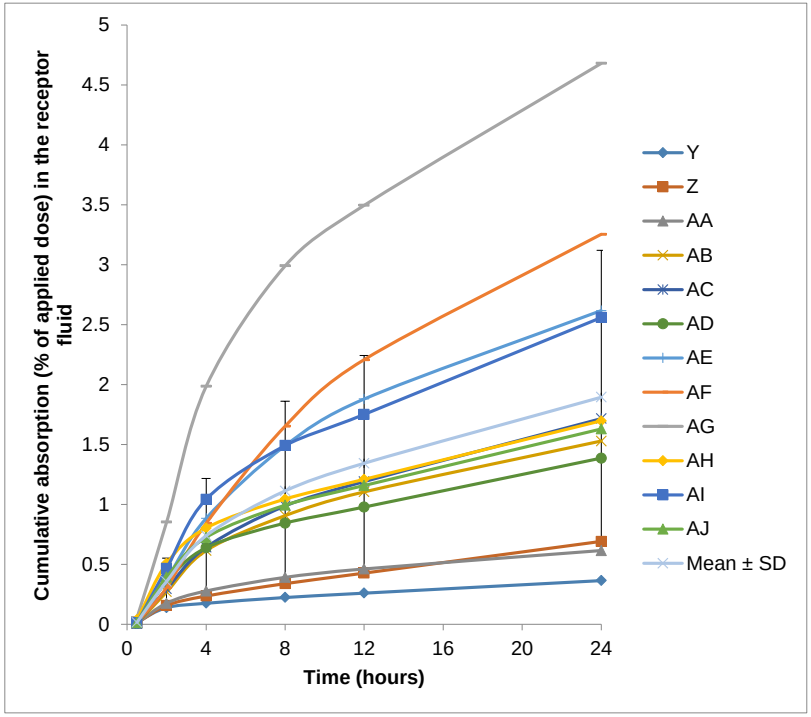
Table 23: Cumulative % of applied dose in the receptor fluid for DDDE 0.018% test item

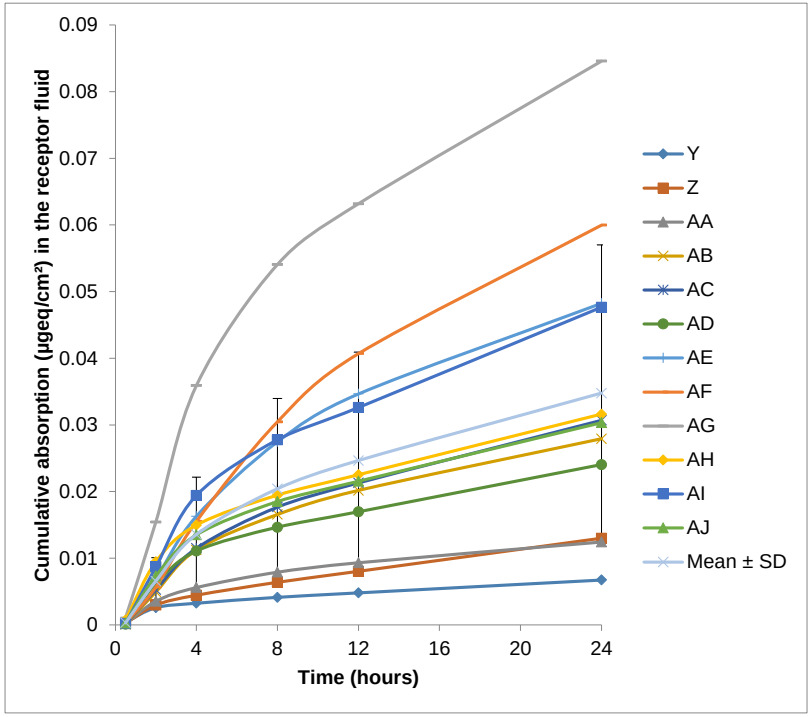
	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	Mean	SD
LR 0.5h	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.06	0.04	0.01	0.01	0.02	0.02
LR 2h	0.14	0.16	0.18	0.27	0.30	0.36	0.37	0.31	0.85	0.51	0.47	0.39	0.36	0.19
LR 4h	0.18	0.24	0.28	0.62	0.64	0.64	0.88	0.84	1.99	0.80	1.04	0.73	0.74	0.48
LR 8h	0.22	0.34	0.39	0.91	0.99	0.84	1.49	1.65	2.99	1.05	1.49	0.99	1.11	0.75
LR 12h	0.26	0.43	0.46	1.10	1.19	0.98	1.88	2.21	3.50	1.21	1.75	1.16	1.34	0.90
LR 24h	0.36	0.69	0.61	1.53	1.72	1.39	2.62	3.25	4.68	1.70	2.56	1.63	1.90	1.23

Table 24: Cumulative $\mu g_{eq}/cm^2$ of applied dose in the receptor fluid for DDDE 0.018% test item

	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	Mean	SD
LR 0.5h	0.0004	0.0002	0.0002	0.0001	0.0001	0.0001	0.0001	0.0002	0.0010	0.0007	0.0002	0.0003	0.0003	0.0003
LR 2h	0.003	0.003	0.004	0.005	0.005	0.006	0.007	0.006	0.015	0.009	0.009	0.007	0.007	0.004
LR 4h	0.003	0.004	0.006	0.011	0.012	0.011	0.016	0.016	0.036	0.015	0.019	0.014	0.014	0.009
LR 8h	0.004	0.006	0.008	0.017	0.018	0.015	0.027	0.030	0.054	0.019	0.028	0.019	0.020	0.014
LR 12h	0.005	0.008	0.009	0.020	0.021	0.017	0.035	0.041	0.063	0.023	0.033	0.022	0.025	0.016
LR 24h	0.007	0.013	0.012	0.028	0.031	0.024	0.048	0.060	0.085	0.032	0.048	0.030	0.035	0.022

Figure 3: Cumulative absorption (% of applied dose and $\mu g_{eq}/cm^2$) in the receptor fluid for DDDE 0.018% test item





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	Eurofins|ADME BIOANALYSES study code: 22-0148	

Table 25: Individual results obtained after DDDE application on human skin for DDDE 0.012% test item

Test item activity : 89.61 µCi/g
Concentration of DDDE in test item : 0.12 mg/g

Cell	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	**Mean**	**SD**	**CV (%)**
Skin donor identification	MRS-22-02-A01041	MRS-22-02-A01041	MRS-22-02-A01041	1459	1459	1459	1441	1441	1441	1446	1446	1446			
Application area (cm²)	1	1	1	1	1	1	1	1	1	1	1	1			
Test item Amount applied (mg)	10.10	10.00	10.30	10.40	10.20	10.60	10.80	10.40	10.10	10.20	10.00	10.10	**10.27**	**0.25**	**2.40**
Test item Amount applied (mg/cm²)	10.10	10.00	10.30	10.40	10.20	10.60	10.80	10.40	10.10	10.20	10.00	10.10	**10.27**	**0.25**	**2.40**
Test item Amount applied (µCi)	0.91	0.90	0.92	0.93	0.91	0.95	0.97	0.93	0.91	0.91	0.90	0.91	**0.92**	**0.02**	**2.40**
Test item Amount applied (µCi/cm²)	0.91	0.90	0.92	0.93	0.91	0.95	0.97	0.93	0.91	0.91	0.90	0.91	**0.92**	**0.02**	**2.40**
Concentration of Test substance (mg/g Test item)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12			
Test substance applied (µgeq/cm²)	1.24	1.23	1.27	1.28	1.25	1.30	1.33	1.28	1.24	1.25	1.23	1.24			
Strip 1-2 (µCi)	0.05	0.05	0.04	0.06	0.07	0.05	0.05	0.06	0.04	0.03	0.04	0.08	**0.05**	**0.01**	**28.16**
Strip 3-6 (µCi)	0.02	0.06	0.04	0.08	0.07	0.06	0.07	0.05	0.07	0.03	0.06	0.04	**0.06**	**0.02**	**32.27**
Strip 7-11 (µCi)	NC	NC	0.03	0.03	0.04	0.02	0.05	0.03	0.04	NC	NC	NC	**0.03**	**0.01**	**30.57**
Strip 12-15 (µCi)	NC	NC	0.03	0.01	NC	NC	0.01	0.01	0.01	NC	NC	NC	**0.02**	**0.01**	**48.92**
Strip 16-20 (µCi)	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	**NC**	**NC**	**NC**
Total strips (µgeq/cm²)	0.10	0.16	0.19	0.24	0.25	0.17	0.26	0.22	0.22	0.09	0.14	0.16	**0.18**	**0.06**	**31.92**
Total strips (%)	7.86	12.99	15.25	19.04	20.17	13.04	19.94	17.18	17.90	6.78	11.58	12.82	**14.55**	**4.48**	**30.81**
RCD (µCi)	0.001	BLQ	0.003	0.001	0.002	0.003	0.002	0.001	0.006	0.001	0.004	0.002	**0.002**	**0.002**	**74.81**
Washing 24h (µCi)	0.71	0.68	0.66	0.63	0.63	0.66	0.59	0.59	0.59	0.73	0.67	0.66	**0.65**	**0.05**	**7.20**
Remaining Skin (µCi)	0.0002	0.0002	0.0007	0.0003	0.0003	0.0005	0.0036	0.0007	0.0013	0.0009	0.0009	0.0005	**0.0008**	**0.0009**	**109.32**
Skin Excess (µCi)	0.71	0.68	0.67	0.63	0.63	0.66	0.59	0.59	0.59	0.73	0.68	0.66	**0.65**	**0.05**	**7.00**
Skin Excess (µCi/cm²)	0.71	0.68	0.67	0.63	0.63	0.66	0.59	0.59	0.59	0.73	0.68	0.66	**0.65**	**0.05**	**7.00**
Skin Excess (µgeq/cm²)	0.98	0.94	0.91	0.87	0.86	0.91	0.81	0.81	0.82	1.00	0.93	0.90	**0.90**	**0.06**	**7.00**
Skin Excess (%)	78.57	76.39	72.22	68.11	68.82	69.64	61.10	63.52	65.70	79.90	75.34	72.80	**71.01**	**5.92**	**8.34**
Epidermis (µCi)	0.06	0.06	0.01	0.02	0.02	0.04	0.01	0.04	0.03	0.02	0.03	0.06	**0.03**	**0.02**	**52.62**
Epidermis (µCi/cm²)	0.06	0.06	0.01	0.02	0.02	0.04	0.01	0.04	0.03	0.02	0.03	0.06	**0.03**	**0.02**	**52.62**
Epidermis (µgeq/cm²)	0.09	0.08	0.02	0.03	0.03	0.06	0.02	0.05	0.04	0.03	0.04	0.08	**0.05**	**0.02**	**52.62**
Epidermis (%)	6.94	6.19	1.39	2.08	2.71	4.27	1.20	4.16	2.83	2.38	3.16	6.27	**3.63**	**1.95**	**53.60**
Dermis (µCi)	0.001	0.001	0.001	0.001	0.001	0.006	0.016	0.012	0.005	0.017	0.007	0.002	**0.006**	**0.006**	**105.00**
Dermis (µCi/cm²)	0.001	0.001	0.001	0.001	0.001	0.006	0.016	0.012	0.005	0.017	0.007	0.002	**0.006**	**0.006**	**105.00**
Dermis (µgeq/cm²)	0.002	0.001	0.001	0.001	0.001	0.008	0.022	0.016	0.007	0.023	0.009	0.003	**0.008**	**0.008**	**105.00**
Dermis (%)	0.14	0.06	0.06	0.08	0.12	0.62	1.65	1.24	0.53	1.83	0.75	0.27	**0.61**	**0.64**	**103.99**
Epidermis + Strip (µgeq/cm²)	0.18	0.24	0.21	0.27	0.29	0.23	0.28	0.27	0.26	0.11	0.18	0.24	**0.23**	**0.05**	**22.05**
Epidermis + Strip (%)	14.80	19.18	16.65	21.12	22.88	17.31	21.14	21.34	20.74	9.17	14.74	19.09	**18.18**	**3.88**	**21.33**
Epidermis + Dermis (µgeq/cm²)	0.09	0.08	0.02	0.03	0.04	0.06	0.04	0.07	0.04	0.05	0.05	0.08	**0.05**	**0.02**	**41.78**
Epidermis + Dermis (%)	7.07	6.25	1.45	2.17	2.83	4.89	2.85	5.40	3.36	4.21	3.91	6.53	**4.24**	**1.81**	**42.62**
Epidermis + Dermis + Strip (µgeq/cm²)	0.19	0.24	0.21	0.27	0.29	0.23	0.30	0.29	0.26	0.14	0.19	0.24	**0.24**	**0.05**	**20.79**
Epidermis + Dermis + Strip (%)	14.94	19.24	16.71	21.20	23.00	17.93	22.79	22.58	21.27	10.99	15.49	19.36	**18.79**	**3.72**	**19.79**
Receptor liquid 24h (µCi)	0.003	0.005	0.008	0.013	0.016	0.019	0.023	0.025	0.032	0.019	0.026	0.031	**0.018**	**0.010**	**52.19**
RCR (µCi)	BLQ	0.0001	0.0008	0.0003	0.0010	0.0005	0.0004	0.0008	0.0041	0.0010	0.0125	0.0005	**0.0018**	**0.0035**	**192.25**
Receptor fluid (µCi)	0.003	0.005	0.008	0.014	0.017	0.020	0.024	0.026	0.036	0.020	0.038	0.031	**0.020**	**0.011**	**56.75**
Receptor fluid (µCi/cm²)	0.003	0.005	0.008	0.014	0.017	0.020	0.024	0.026	0.036	0.020	0.038	0.031	**0.020**	**0.011**	**56.75**
Receptor fluid (µgeq/cm²)	0.004	0.008	0.011	0.019	0.023	0.027	0.033	0.036	0.049	0.028	0.053	0.043	**0.028**	**0.016**	**56.75**
Receptor fluid (%)	0.35	0.61	0.90	1.46	1.81	2.09	2.46	2.79	3.93	2.24	4.29	3.47	**2.20**	**1.27**	**57.76**
Epidermis + dermis + receptor fluid (µCi)	0.07	0.06	0.02	0.03	0.04	0.07	0.05	0.08	0.07	0.06	0.07	0.09	**0.06**	**0.02**	**32.43**
Epidermis + dermis + receptor fluid (µgeq/cm²)	0.09	0.08	0.03	0.05	0.06	0.09	0.07	0.10	0.09	0.08	0.10	0.12	**0.08**	**0.03**	**32.43**
Epidermis + dermis + receptor fluid (%)	7.42	6.86	2.35	3.63	4.64	6.98	5.31	8.19	7.29	6.45	8.20	10.01	**6.44**	**2.14**	**33.17**
Strips + Epidermis + Dermis + Receptor Fluid (µCi)	0.14	0.18	0.16	0.21	0.23	0.19	0.24	0.24	0.23	0.12	0.18	0.21	**0.19**	**0.04**	**20.33**
Strips + Epidermis + Dermis + Receptor Fluid (µgeq/cm²)	0.19	0.24	0.22	0.29	0.31	0.26	0.34	0.32	0.31	0.17	0.24	0.28	**0.27**	**0.05**	**20.33**
Strips + Epidermis + Dermis + Receptor Fluid (%)	15.29	19.86	17.60	22.67	24.81	20.02	25.25	25.37	25.20	13.23	19.78	22.83	**20.99**	**4.09**	**19.47**
TOTAL RECOVERY (%)	93.86	96.25	89.82	90.78	93.63	89.66	86.35	88.89	90.90	93.13	95.13	95.63	**92.00**	**3.06**	**3.33**

Strips 1-2 (µgeq/cm²)	0.07	0.07	0.05	0.08	0.10	0.07	0.07	0.08	0.05	0.04	0.05	0.10	**0.07**	**0.02**	**28.16**
Strips 1-2 (%)	5.58	5.84	3.99	6.16	7.83	5.00	5.46	6.40	4.30	2.98	4.37	8.44	**5.53**	**1.57**	**28.38**
Strips 3-20 (µgeq/cm²)	0.03	0.09	0.14	0.16	0.15	0.10	0.19	0.14	0.17	0.05	0.09	0.05	**0.11**	**0.05**	**46.72**
Strips 3-20 (%)	2.29	7.16	11.27	12.88	12.34	8.04	14.48	10.78	13.60	3.81	7.21	4.38	**9.02**	**4.12**	**45.67**

BLQ: Below the Limit of Quantification NC: Not Calculated

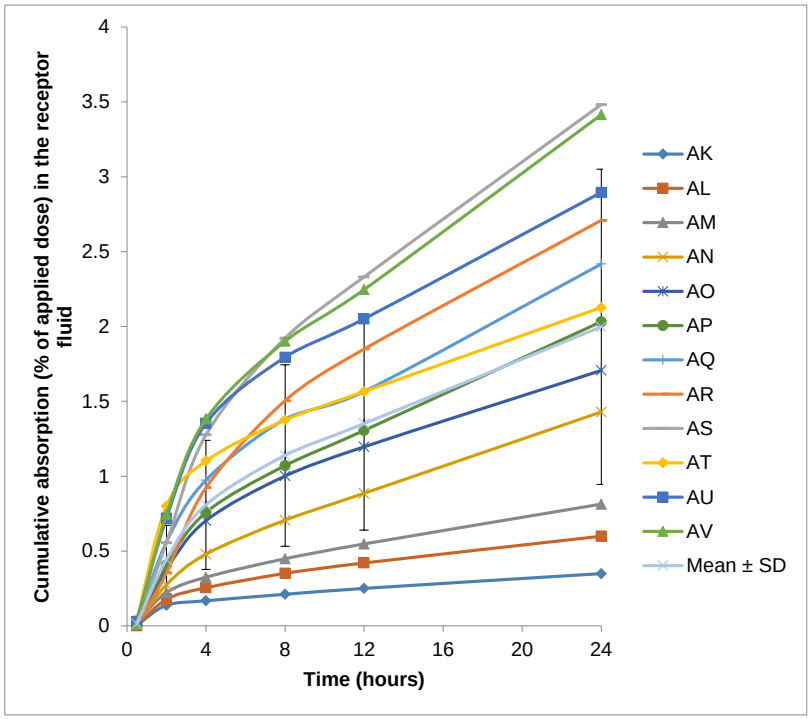
Table 26: Cumulative % of applied dose in the receptor fluid for DDDE 0.012% test item

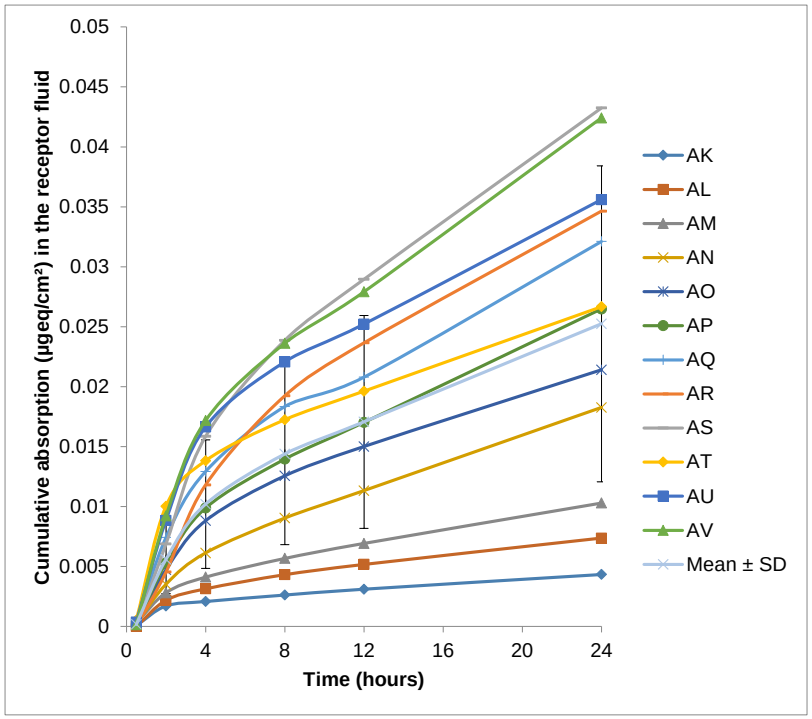
	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	Mean	SD
LR 0.5h	0.01	BLQ	0.02	0.02	0.01	0.01	0.02	BLQ	BLQ	0.04	0.03	0.01	0.01	0.01
LR 2h	0.14	0.18	0.22	0.28	0.38	0.41	0.56	0.35	0.55	0.80	0.72	0.74	0.44	0.23
LR 4h	0.17	0.26	0.32	0.48	0.70	0.76	0.97	0.92	1.28	1.10	1.35	1.38	0.81	0.43
LR 8h	0.21	0.35	0.45	0.71	1.00	1.07	1.38	1.50	1.92	1.37	1.79	1.90	1.14	0.61
LR 12h	0.25	0.42	0.55	0.89	1.20	1.31	1.57	1.85	2.33	1.56	2.05	2.25	1.35	0.71
LR 24h	0.35	0.60	0.81	1.43	1.71	2.03	2.42	2.71	3.48	2.13	2.89	3.41	2.00	1.05

Table 27: Cumulative $\mu g_{eq}/cm^2$ of applied dose in the receptor fluid for DDDE 0.012% test item

	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	Mean	SD
LR 0.5h	0.0001	BLQ	0.0003	0.0002	0.0002	0.0001	0.0002	BLQ	BLQ	0.0005	0.0004	0.0001	0.0002	0.0002
LR 2h	0.002	0.002	0.003	0.004	0.005	0.005	0.007	0.005	0.007	0.010	0.009	0.009	0.006	0.003
LR 4h	0.002	0.003	0.004	0.006	0.009	0.010	0.013	0.012	0.016	0.014	0.017	0.017	0.010	0.005
LR 8h	0.003	0.004	0.006	0.009	0.013	0.014	0.018	0.019	0.024	0.017	0.022	0.024	0.014	0.008
LR 12h	0.003	0.005	0.007	0.011	0.015	0.017	0.021	0.024	0.029	0.020	0.025	0.028	0.017	0.009
LR 24h	0.004	0.007	0.010	0.018	0.021	0.026	0.032	0.035	0.043	0.027	0.036	0.042	0.025	0.013

Figure 4: Cumulative absorption (% of applied dose and $\mu g_{eq}/cm^2$) in the receptor fluid for DDDE 0.012% test item





APPENDIX B

METHOD FOR DETERMINATION OF RADIOPURITY AND CHROMATOGRAMS

1. MATERIAL AND REAGENTS

Material used for the determination of radiopurity

	Material	Reference	Supplier
Reagent	H_2O	Milli Q	Eurofins|ADME BIOANALYSES
	Ultima-Flo	6013579	Perkin Elmer
	Ultima-Gold XR	6013119	Perkin Elmer
	Hionic-Fluor	6013319	Perkin Elmer
	Solvable	6NE9100	Perkin Elmer
	Formic acid	F/1900/PB08	Fisher Scientific
	Acetonitrile	401242	Carbo Erba
	Ethanol	20821.330	VWR Chimicals
	NaCl (Sodium Chloride)	27810.295	VWR Chimicals
	PBS (Phosphate Buffer Saline)	P4417-100TAB	Sigma Aldrich
	Polyoxyethylene 20 oleyl ether (Brij® 98)	347185000	Accros organics
	BSA (Bovine Seum Albumine)	240401000	Accros organics
	Tween® 80	28830.291	VWR Chimicals

Apparatus	Automatic sampler	SIL-20AC HT	Shimadzu
	Pump	LC-20AD	Shimadzu
	Detector	FlowStar LB 514	Berthold technologies
	Acquisition data	RadioStar 5.0.12.6	Berthold technologies
	Column	C18 Kromasil 5µm 250 x 4.6 mm (M05CLA25)	NOURYON

Material	Balance
	Tewameter
	Hygrometer
	Bronaugh cells
	Oven
	Oditest calipers
	Thermometer
	Scalpel
	Scintillation vial
	Falcon tube
	Pipettes
	Vortex
	Magnetic agitator
	Evaporimeter
	Thermometer

Data acquisition and calculation	Data acquisition (scintillation counting)	Tricarb 2910TR
	Analysis software	QuantaSmart 4.0 software
	Statistical calculation	Excel®
	Weighting software	LABX® 2020 (11.0.0.731)

2. OPERATING CONDITIONS

- Column temperature: room temperature
- Automatic sampler temperature: room temperature
- Mobile phase: Gradient

Time (min)	Flow rate (mL/min)	channel A	channel B
0	1	97	3
16	1	0	100
21	1	0	100
21.1	1	70	30
23	1	97	3
30	1	97	3

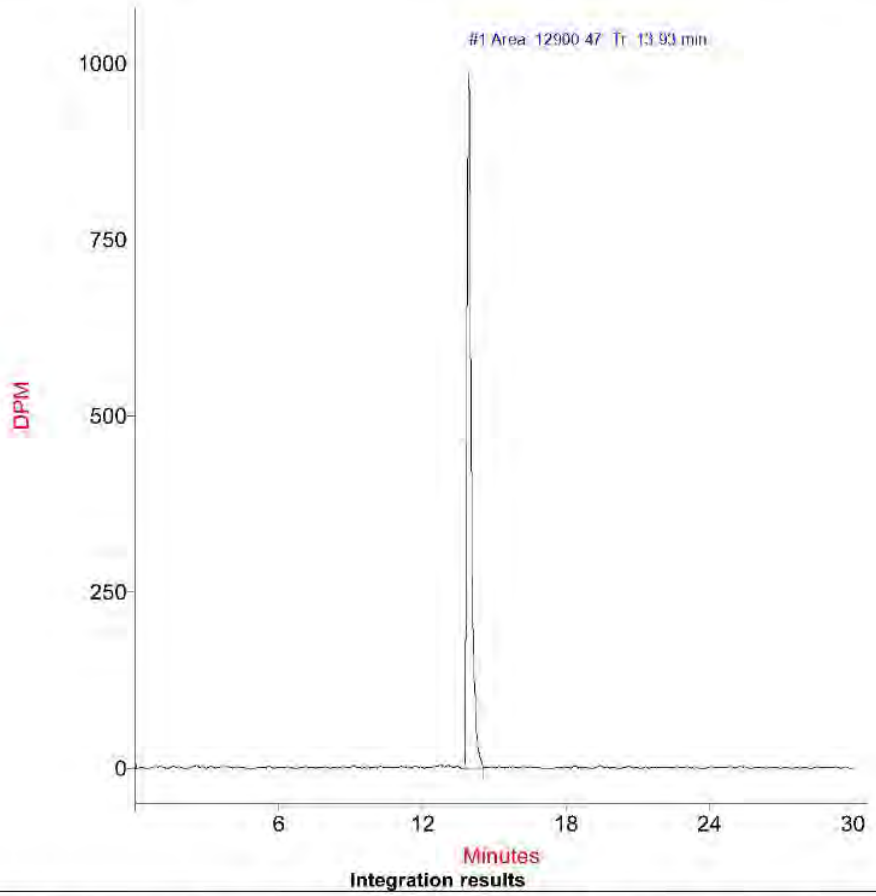
Channel A: UHQ water + 0.1% formic acid
Channel B: Acetonitrile + 0.1% formic acid

- Scintillation flow rate: 2 mL/min
- Injection volume: 10 µL
- Analysis time: about 30 min
- Retention time: ^{3}H-DDDE: about 12.9 min

Figure 5: Reference item radiopurity: the day of solubility test

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 test item radiopurity - day of solubility



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		13.93	12900.47	100.00	0.00	0.00	15348.77	12279.02
SUM			**12900.47**	**100.00**	**0.00**	**0.00**		**12279.02**

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Figure 6: Reference item radiopurity: the day of formulation of 1 and 2 radiolabelled preparations

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 radiopureté item - jour de formulation

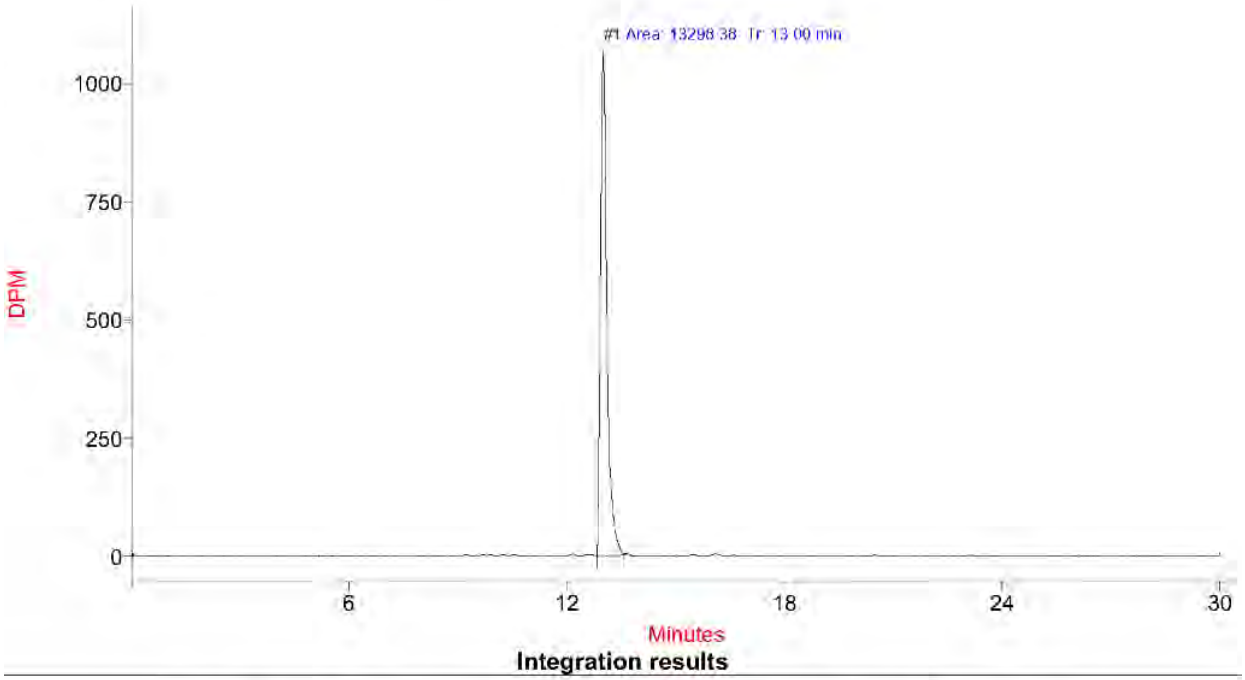
Sample information

Name	22-0148 radiopureté item - jour de for	Sample type	Sample
Vial #	1		
Amount	0.000000 mg	Injected volume	10.00 µl
Dilution	1	Division factor	1

(* = original value has been modified)
Information :
C18 - Grad init eau + 0.1AF 97/3 ACN + 0.1AF - up 16 to 0/100 - plat 5 - down 0.1 to 70/30 - down 1.9 to 97/3 stop 30

Acquisition information

Acquisition date	9/14/2022 9:35:35 AM (+01:00) (RadioStar 5.0.12.6)		
Acquisition source :			
	Radioflow Detector FlowstarFlowstar Channel 1		
Run length	30.00 Minutes	Nb of points	1801
Acquisition rate	1.00 points/second		



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		13.00	13298.38	100.00	0.00	0.00	17096.02	12822.02
SUM			13298.38	100.00	0.00	0.00		12822.02

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Chromatographic Method

Flowstar method

Channel 1 Nuclide :	H-3	Time Cst :	1.0
Analog Range	0	Fixed Background	0.00 CPM
Counting Efficiency	100.00 %	Spillover	0.00 %
Halflife correction	No		
Channel 2	Off		
PeakWidth Half	0.5		
Automatic background substraction	Off		
Scintillator pump	On	Flow :	2.000 ml/min
Active Cell Volume	500.000 µl	HPLC Flow	1.000 ml/mn
Flow Correction Factor	Inactive		
Fraction Collector	Off		
Waste Valve	On	Delay Time	15 s
ON-Level	4.00	OFF-Level	2.00
Cell Type	Z500-6	Serial Number	6019

Radioactivity results

Parameters

Cell type	Z	Cell Volume	500 µl
HPLC Pump Flow	1.000 ml/min	Scintillator Pump Flow	2.000 ml/min
Low energy channel	Inactive		
High energy channel	H-3	Analysis	22-0148 radiopureté item ...
Signal in	DPM	Half life [(D) HH:MM:SS]	4489 - 12:00:00
Fixed background subtract	0 CPM		
Counting efficiency	100.00 %		

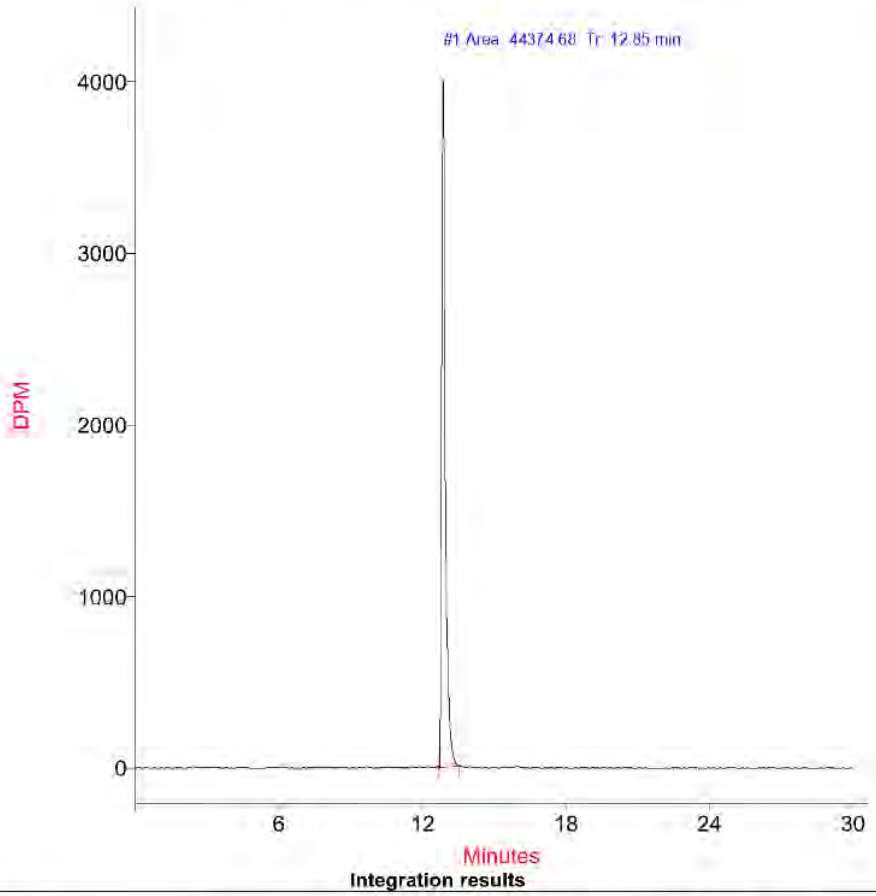
Results

Gross counts total	14979 DPM	Net counts total	14979 DPM
Gross counts of ROIs	12822 DPM	Net counts of ROIs	12822 DPM
Area of ROIs	85.60 %	Unresolved	14.40 %

Figure 7: Reference item radiopurity: the day of formulation of 3 and 4 radiolabelled preparations

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 - Test item radiopurity - Day of formulation



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.85	44374.68	100.00	0.00	0.00	52522.57	42018.06
SUM			**44374.68**	**100.00**	**0.00**	**0.00**		**42018.06**

Monday, September 19, 2022 11:22:24 AM Page 1/1

Figure 8: Radiopurity of the radiolabelled preparation at T0 for DDDE Dilution 0.024%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 radiopureté formulation 1 0.024% a T0

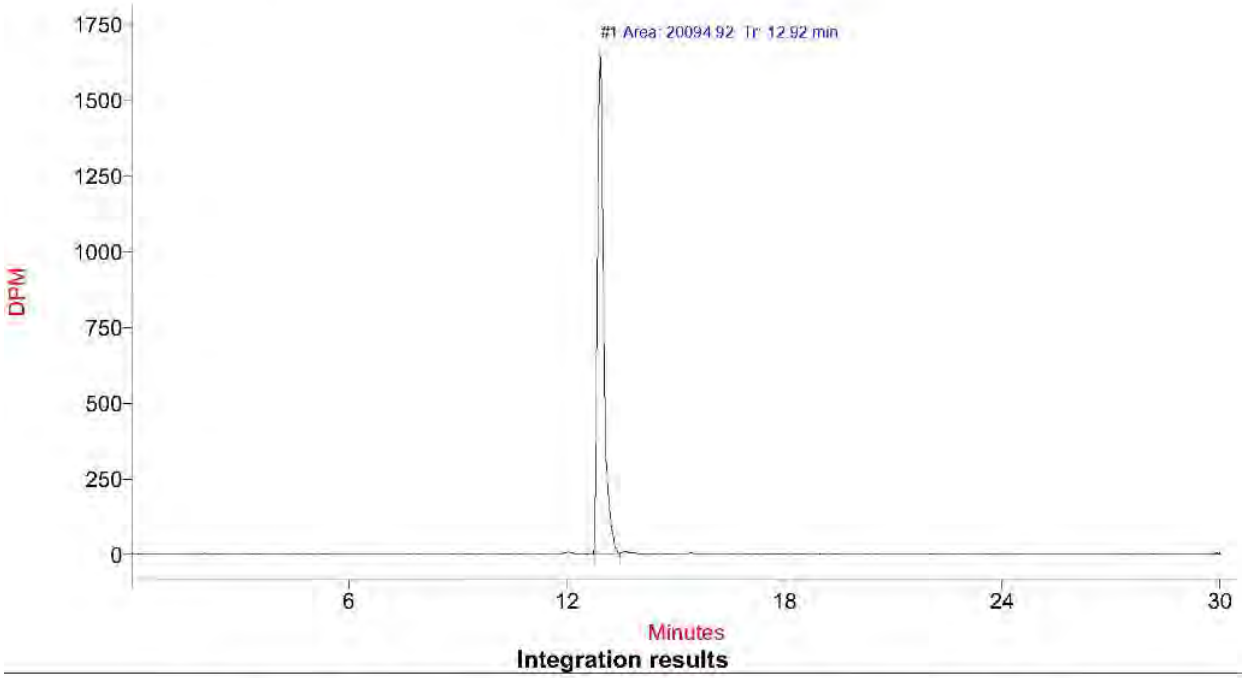
Sample information

Name	22-0148 radiopureté formulation 1 0.(	Sample type	Sample
Vial #	1		
Amount	0.000000 mg	Injected volume	5.00 µl
Dilution	1	Division factor	1

(* = original value has been modified)
Information :
C18 - Grad init eau + 0.1AF 97/3 ACN + 0.1AF - up 16 to 0/100 - plat 5 - down 0.1 to 70/30 - down 1.9 to 97/3 stop 30

Acquisition information

Acquisition date	9/14/2022 12:20:51 PM (+01:00) (RadioStar 5.0.12.6)		
Acquisition source :			
	Radioflow Detector FlowstarFlowstar Channel 1		
Run length	30.00 Minutes	Nb of points	1801
Acquisition rate	1.00 points/second		



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.92	20094.92	100.00	0.00	0.00	26012.13	18642.03
SUM			**20094.92**	**100.00**	**0.00**	**0.00**		**18642.03**

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Chromatographic Method

Flowstar method

Channel 1 Nuclide :	H-3	Time Cst :	1.0
Analog Range	0	Fixed Background	0.00 CPM
Counting Efficiency	100.00 %	Spillover	0.00 %
Halflife correction	No		
Channel 2	Off		
PeakWidth Half	0.5		
Automatic background substraction	Off		
Scintillator pump	On	Flow :	2.000 ml/min
Active Cell Volume	500.000 µl	HPLC Flow	1.000 ml/mn
Flow Correction Factor	Inactive		
Fraction Collector	Off		
Waste Valve	On	Delay Time	15 s
ON-Level	4.00	OFF-Level	2.00
Cell Type	Z500-6	Serial Number	6019

Radioactivity results

Parameters

Cell type	Z	Cell Volume	500 µl
HPLC Pump Flow	1.000 ml/min	Scintillator Pump Flow	2.000 ml/min
Low energy channel	Inactive		
High energy channel	H-3	Analysis	22-0148 radiopureté formu...
Signal in	DPM	Half life [(D) HH:MM:SS]	4489 - 12:00:00
Fixed background subtract	0 CPM		
Counting efficiency	100.00 %		

Results

Gross counts total	21977 DPM	Net counts total	21977 DPM
Gross counts of ROIs	18642 DPM	Net counts of ROIs	18642 DPM
Area of ROIs	84.83 %	Unresolved	15.17 %

Figure 9: Radiopurity of the radiolabelled preparation after 24 hours at 32°C for DDDE Dilution 0.024%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 radiopurity of formulation 1 (0.024%) - T24h00

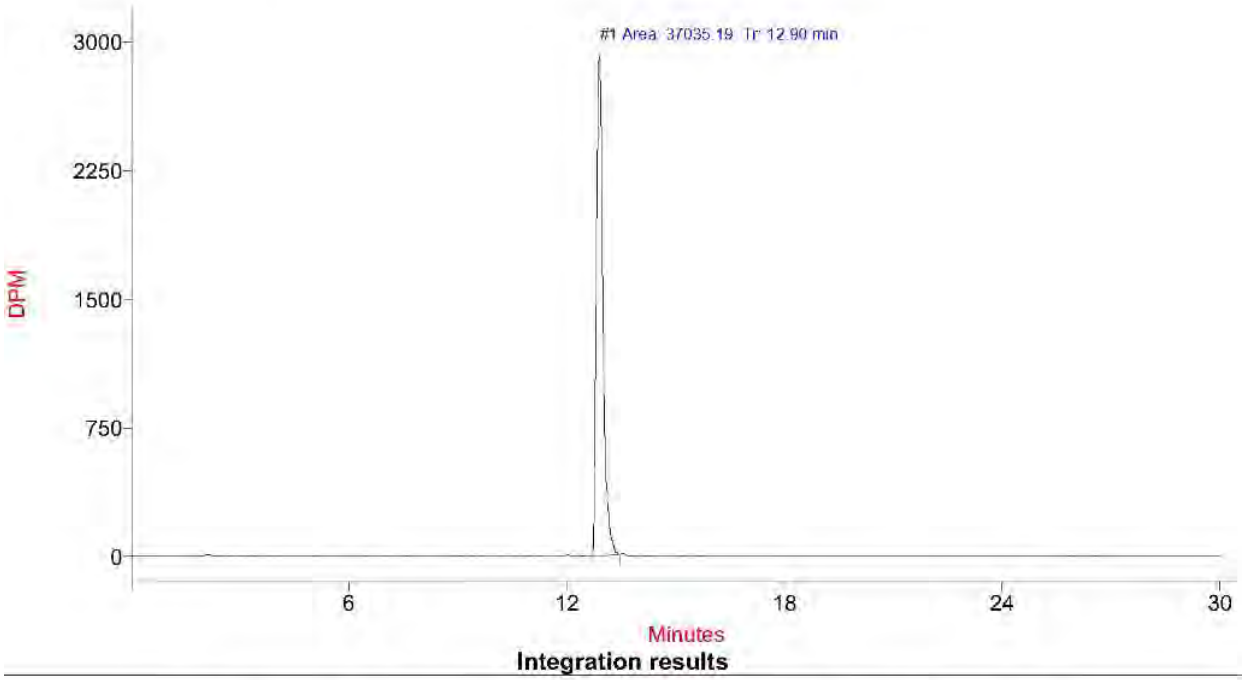
Sample information

Name	22-0148 radiopurity of formulation 1 (0.024%) - T24h00	Sample type	Sample
Vial #	1		
Amount	0.000000 mg	Injected volume	5.00 µl
Dilution	1	Division factor	1

(* = original value has been modified)
Information :
C18 - Grad init eau + 0.1AF 97/3 ACN + 0.1AF - up 16 to 0/100 - plat 5 - down 0.1 to 70/30 - down 1.9 to 97/3 stop 30

Acquisition information

Acquisition date	9/15/2022 12:28:31 PM (+01:00) (RadioStar 5.0.12.6)		
Acquisition source :	Radioflow Detector FlowstarFlowstar Channel 1		
Run length	30.00 Minutes	Nb of points	1801
Acquisition rate	1.00 points/second		



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.90	37035.19	100.00	0.00	0.00	48105.07	35277.05
SUM			37035.19	100.00	0.00	0.00		35277.05

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Chromatographic Method

Flowstar method

Channel 1 Nuclide :	H-3	Time Cst :	1.0
Analog Range	0	Fixed Background	0.00 CPM
Counting Efficiency	100.00 %	Spillover	0.00 %
Halflife correction	No		
Channel 2	Off		
PeakWidth Half	0.5		
Automatic background substraction	Off		
Scintillator pump	On	Flow :	2.000 ml/min
Active Cell Volume	500.000 µl	HPLC Flow	1.000 ml/mn
Flow Correction Factor	Inactive		
Fraction Collector	Off		
Waste Valve	On	Delay Time	15 s
ON-Level	4.00	OFF-Level	2.00
Cell Type	Z500-6	Serial Number	6019

Radioactivity results

Parameters

Cell type	Z	Cell Volume	500 µl
HPLC Pump Flow	1.000 ml/min	Scintillator Pump Flow	2.000 ml/min
Low energy channel	Inactive		
High energy channel	H-3	Analysis	22-0148 radiopurity of fo...
Signal in	DPM	Half life [(D) HH:MM:SS]	4489 - 12:00:00
Fixed background subtract	0 CPM		
Counting efficiency	100.00 %		

Results

Gross counts total	39216 DPM	Net counts total	39216 DPM
Gross counts of ROIs	35277 DPM	Net counts of ROIs	35277 DPM
Area of ROIs	89.96 %	Unresolved	10.04 %

Figure 10: Radiopurity of the radiolabelled preparation at T0 for DDDE Dilution 0.02%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 radiopureté formulation 2 0.020% a T0

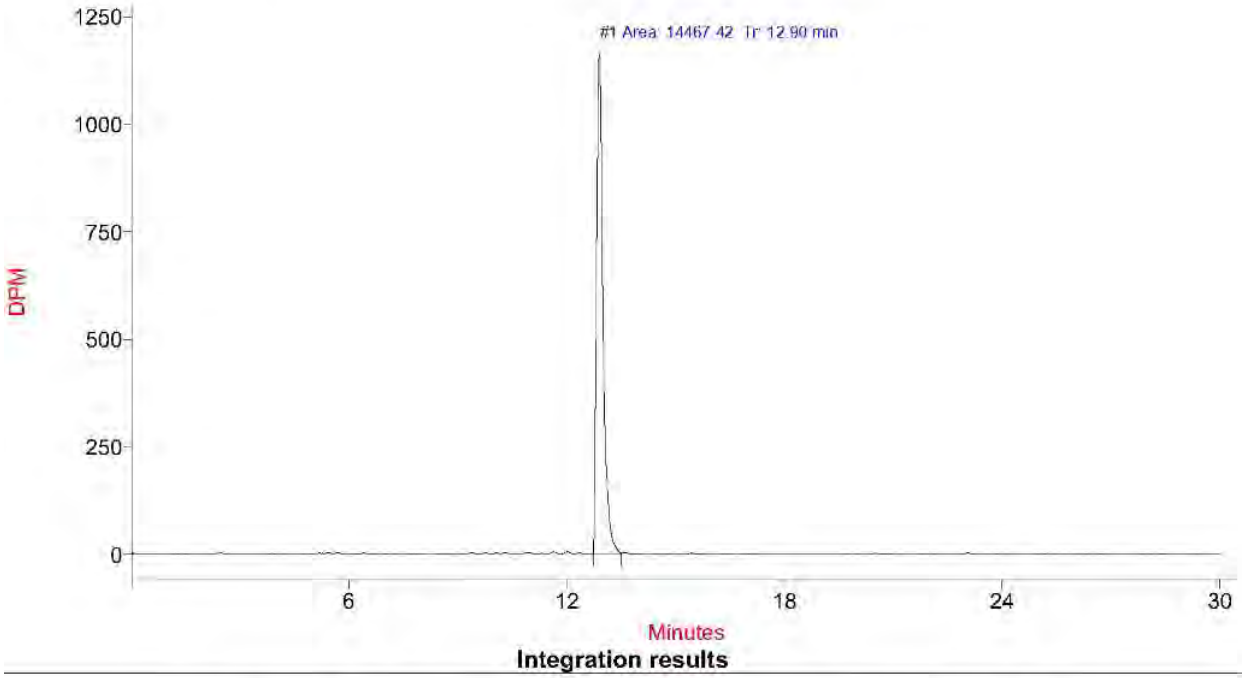
Sample information

Name	22-0148 radiopureté formulation 2 0.(	Sample type	Sample
Vial #	1		
Amount	0.000000 mg	Injected volume	5.00 µl
Dilution	1	Division factor	1

(* = original value has been modified)
Information :
C18 - Grad init eau + 0.1AF 97/3 ACN + 0.1AF - up 16 to 0/100 - plat 5 - down 0.1 to 70/30 - down 1.9 to 97/3 stop 30

Acquisition information

Acquisition date	9/14/2022 12:52:09 PM (+01:00) (RadioStar 5.0.12.6)		
Acquisition source :			
	Radioflow Detector FlowstarFlowstar Channel 1		
Run length	30.00 Minutes	Nb of points	1801
Acquisition rate	1.00 points/second		



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.90	14467.42	100.00	0.00	0.00	17929.59	13746.02
SUM			**14467.42**	**100.00**	**0.00**	**0.00**		**13746.02**

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Chromatographic Method

Flowstar method

Channel 1 Nuclide :	H-3	Time Cst :	1.0
Analog Range	0	Fixed Background	0.00 CPM
Counting Efficiency	100.00 %	Spillover	0.00 %
Halflife correction	No		
Channel 2	Off		
PeakWidth Half	0.5		
Automatic background substraction	Off		
Scintillator pump	On	Flow :	2.000 ml/min
Active Cell Volume	500.000 µl	HPLC Flow	1.000 ml/mn
Flow Correction Factor	Inactive		
Fraction Collector	Off		
Waste Valve	On	Delay Time	15 s
ON-Level	4.00	OFF-Level	2.00
Cell Type	Z500-6	Serial Number	6019

Radioactivity results

Parameters

Cell type	Z	Cell Volume	500 µl
HPLC Pump Flow	1.000 ml/min	Scintillator Pump Flow	2.000 ml/min
Low energy channel	Inactive		
High energy channel	H-3	Analysis	22-0148 radiopureté formu...
Signal in	DPM	Half life [(D) HH:MM:SS]	4489 - 12:00:00
Fixed background subtract	0 CPM		
Counting efficiency	100.00 %		

Results

Gross counts total	16234 DPM	Net counts total	16234 DPM
Gross counts of ROIs	13746 DPM	Net counts of ROIs	13746 DPM
Area of ROIs	84.67 %	Unresolved	15.33 %

Figure 11: Radiopurity of the radiolabelled preparation after 24 hours at 32°C for DDDE Dilution 0.02%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 radiopurity of formulation 2 (0.020%) - T24h00

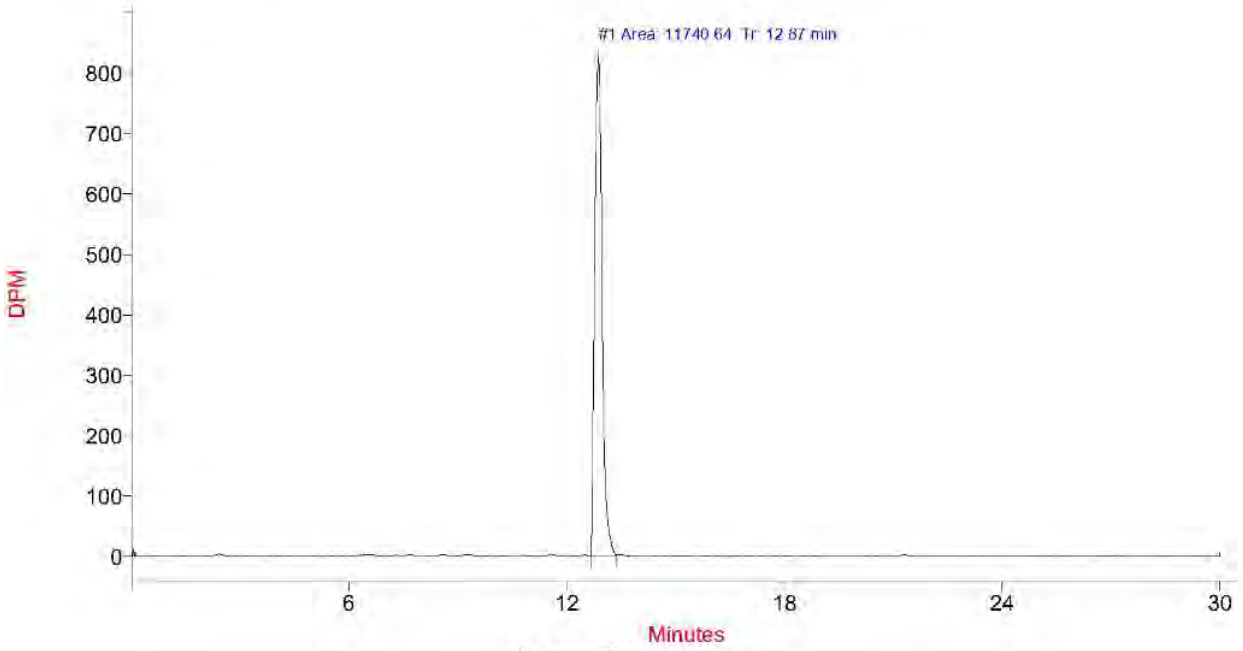
Sample information

Name	22-0148 radiopurity of formulation 2 (0.020%) - T24h00	Sample type	Sample
Vial #	1		
Amount	0.000000 mg	Injected volume	5.00 µl
Dilution	1	Division factor	1

(* = original value has been modified)
Information :
C18 - Grad init eau + 0.1AF 97/3 ACN + 0.1AF - up 16 to 0/100 - plat 5 - down 0.1 to 70/30 - down 1.9 to 97/3 stop 30

Acquisition information

Acquisition date	9/15/2022 12:59:49 PM (+01:00) (RadioStar 5.0.12.6)		
Acquisition source :	Radioflow Detector FlowstarFlowstar Channel 1		
Run length	30.00 Minutes	Nb of points	1801
Acquisition rate	1.00 points/second		



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.87	11740.64	100.00	0.00	0.00	16481.00	11262.02
SUM			11740.64	100.00	0.00	0.00		11262.02

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Chromatographic Method

Flowstar method

Channel 1 Nuclide :	H-3	Time Cst :	1.0
Analog Range	0	Fixed Background	0.00 CPM
Counting Efficiency	100.00 %	Spillover	0.00 %
Halflife correction	No		
Channel 2	Off		
PeakWidth Half	0.5		
Automatic background substraction	Off		
Scintillator pump	On	Flow :	2.000 ml/min
Active Cell Volume	500.000 µl	HPLC Flow	1.000 ml/mn
Flow Correction Factor	Inactive		
Fraction Collector	Off		
Waste Valve	On	Delay Time	15 s
ON-Level	4.00	OFF-Level	2.00
Cell Type	Z500-6	Serial Number	6019

Radioactivity results

Parameters

Cell type	Z	Cell Volume	500 µl
HPLC Pump Flow	1.000 ml/min	Scintillator Pump Flow	2.000 ml/min
Low energy channel	Inactive		
High energy channel	H-3	Analysis	22-0148 radiopurity of fo...
Signal in	DPM	Half life [(D) HH:MM:SS]	4489 - 12:00:00
Fixed background subtract	0 CPM		
Counting efficiency	100.00 %		

Results

Gross counts total	12927 DPM	Net counts total	12927 DPM
Gross counts of ROIs	11262 DPM	Net counts of ROIs	11262 DPM
Area of ROIs	87.12 %	Unresolved	12.88 %

Figure 12: Radiopurity of the radiolabelled preparation at T0 for DDDE Dilution 0.018%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 - Diluted 3 formulation T0

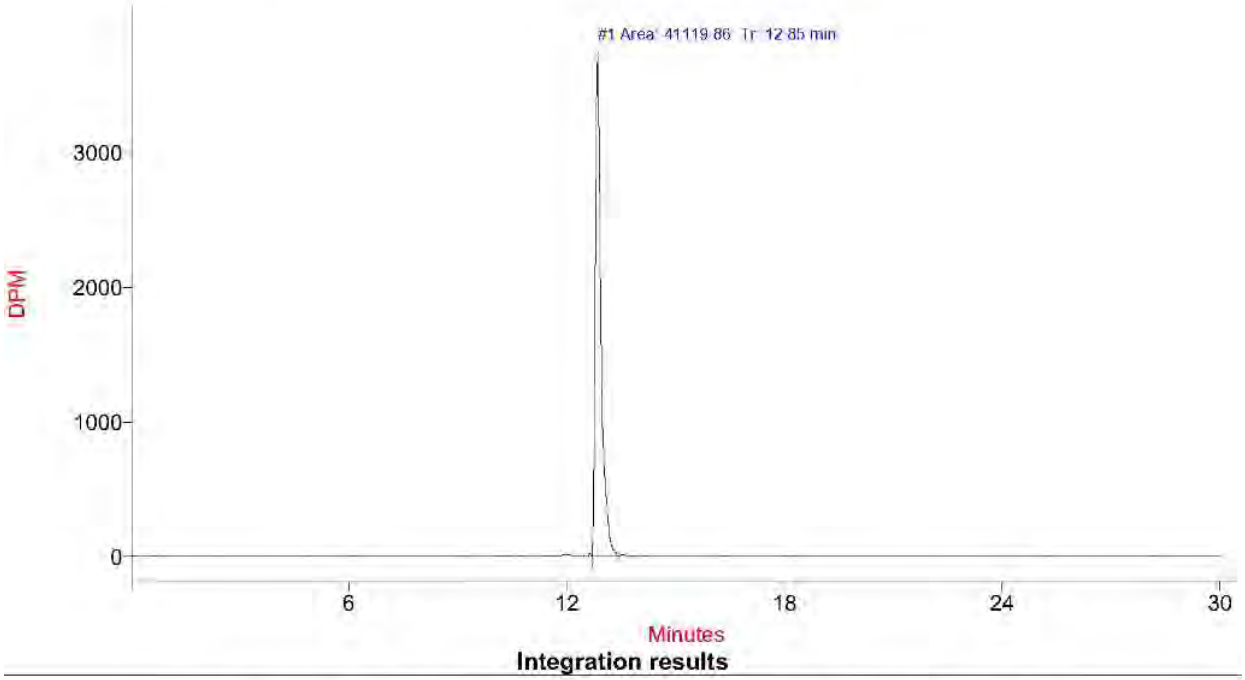
Sample information

Name	22-0148 - Diluted 3 formulation T0	Sample type	Sample
Vial #	1		
Amount	0.000000 mg	Injected volume	10.00 µl
Dilution	1	Division factor	1

(* = original value has been modified)
Information :
C18 - Grad init eau + 0.1AF 97/3 ACN + 0.1AF - up 16 to 0/100 - plat 5 - down 0.1 to 70/30 - down 1.9 to 97/3 stop 30

Acquisition information

Acquisition date	9/19/2022 12:08:22 PM (+01:00) (RadioStar 5.0.12.6)		
Acquisition source :			
	Radioflow Detector FlowstarFlowstar Channel 1		
Run length	30.00 Minutes	Nb of points	1801
Acquisition rate	1.00 points/second		



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.85	41119.86	100.00	0.00	0.00	55741.47	39948.06
SUM			**41119.86**	**100.00**	**0.00**	**0.00**		**39948.06**

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Chromatographic Method

Flowstar method

Channel 1 Nuclide :	H-3	Time Cst :	1.0
Analog Range	0	Fixed Background	0.00 CPM
Counting Efficiency	100.00 %	Spillover	0.00 %
Halflife correction	No		
Channel 2	Off		
PeakWidth Half	0.5		
Automatic background substraction	Off		
Scintillator pump	On	Flow :	2.000 ml/min
Active Cell Volume	500.000 µl	HPLC Flow	1.000 ml/mn
Flow Correction Factor	Inactive		
Fraction Collector	Off		
Waste Valve	On	Delay Time	15 s
ON-Level	4.00	OFF-Level	2.00
Cell Type	Z500-6	Serial Number	6019

Radioactivity results

Parameters

Cell type	Z	Cell Volume	500 µl
HPLC Pump Flow	1.000 ml/min	Scintillator Pump Flow	2.000 ml/min
Low energy channel	Inactive		
High energy channel	H-3	Analysis	22-0148 - Diluted 3 formu...
Signal in	DPM	Half life [(D) HH:MM:SS]	4489 - 12:00:00
Fixed background subtract	0 CPM		
Counting efficiency	100.00 %		

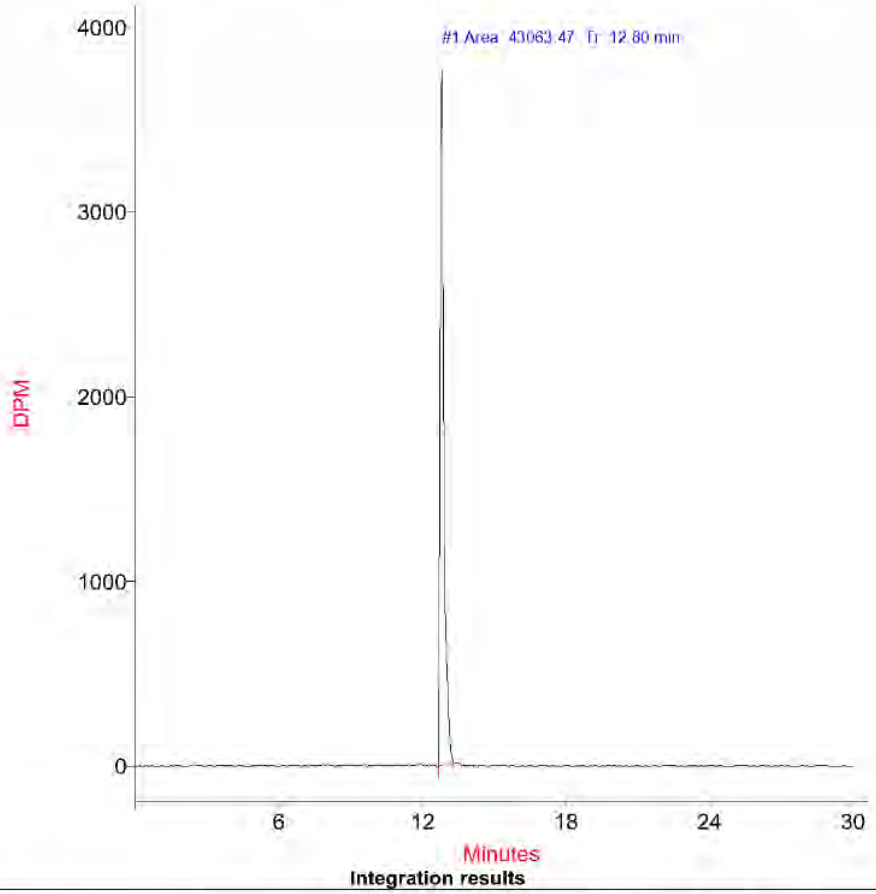
Results

Gross counts total	43500 DPM	Net counts total	43500 DPM
Gross counts of ROIs	39948 DPM	Net counts of ROIs	39948 DPM
Area of ROIs	91.83 %	Unresolved	8.17 %

Figure 13: Radiopurity of the radiolabelled preparation after 24 hours at 32°C for DDDE Dilution 0.018%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 Formulation 3 after storage at 32°C T24h



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.80	43063.47	100.00	0.00	0.00	64200.09	41730.06
SUM			**43063.47**	**100.00**	**0.00**	**0.00**		**41730.06**

Tuesday, September 20, 2022 1:38:59 PM Page 1/1

Figure 14: Radiopurity of the radiolabelled preparation at T0 for DDDE Dilution 0.012%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 - Diluted 4 formulation T0

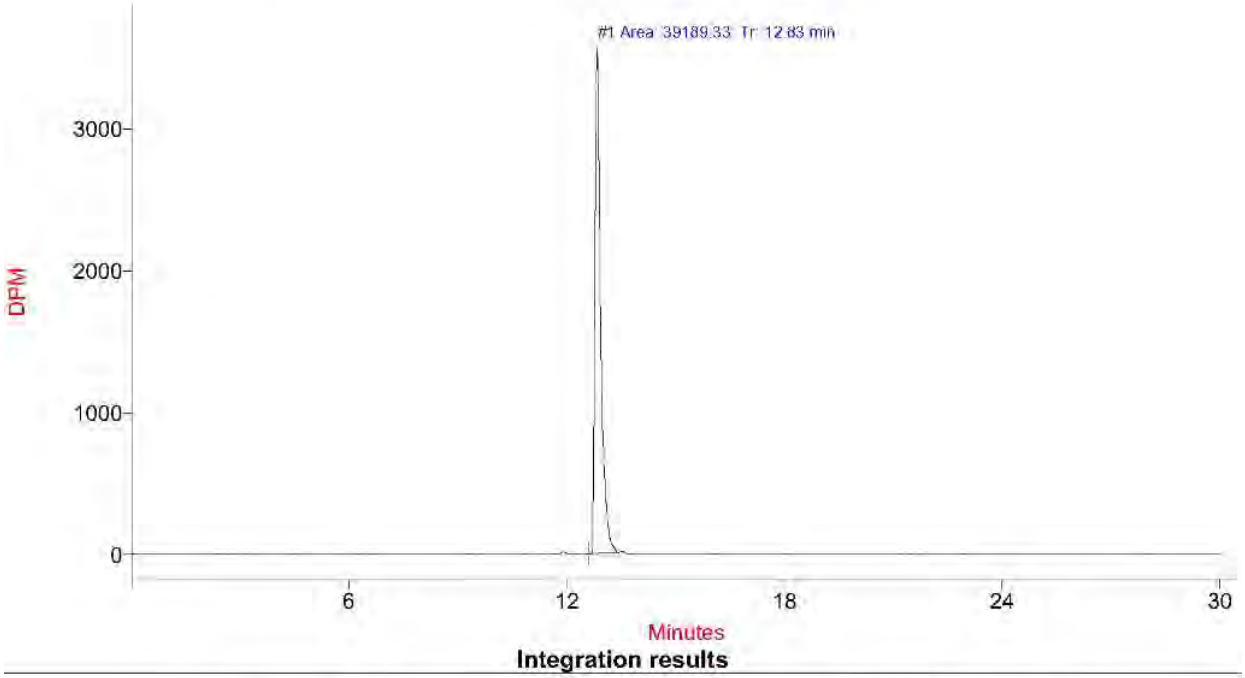
Sample information

Name	22-0148 - Diluted 4 formulation T0	Sample type	Sample
Vial #	1		
Amount	0.000000 mg	Injected volume	10.00 µl
Dilution	1	Division factor	1

(* = original value has been modified)
Information :
C18 - Grad init eau + 0.1AF 97/3 ACN + 0.1AF - up 16 to 0/100 - plat 5 - down 0.1 to 70/30 - down 1.9 to 97/3 stop 30

Acquisition information

Acquisition date	9/19/2022 12:39:39 PM (+01:00) (RadioStar 5.0.12.6)		
Acquisition source :			
	Radioflow Detector FlowstarFlowstar Channel 1		
Run length	30.00 Minutes	Nb of points	1801
Acquisition rate	1.00 points/second		



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.83	39189.33	100.00	0.00	0.00	48269.45	39420.05
SUM			**39189.33**	**100.00**	**0.00**	**0.00**		**39420.05**

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Chromatographic Method

Flowstar method

Channel 1 Nuclide :	H-3	Time Cst :	1.0
Analog Range	0	Fixed Background	0.00 CPM
Counting Efficiency	100.00 %	Spillover	0.00 %
Halflife correction	No		
Channel 2	Off		
PeakWidth Half	0.5		
Automatic background substraction	Off		
Scintillator pump	On	Flow :	2.000 ml/min
Active Cell Volume	500.000 µl	HPLC Flow	1.000 ml/mn
Flow Correction Factor	Inactive		
Fraction Collector	Off		
Waste Valve	On	Delay Time	15 s
ON-Level	4.00	OFF-Level	2.00
Cell Type	Z500-6	Serial Number	6019

Radioactivity results

Parameters

Cell type	Z	Cell Volume	500 µl
HPLC Pump Flow	1.000 ml/min	Scintillator Pump Flow	2.000 ml/min
Low energy channel	Inactive		
High energy channel	H-3	Analysis	22-0148 - Diluted 4 formu...
Signal in	DPM	Half life [(D) HH:MM:SS]	4489 - 12:00:00
Fixed background subtract	0 CPM		
Counting efficiency	100.00 %		

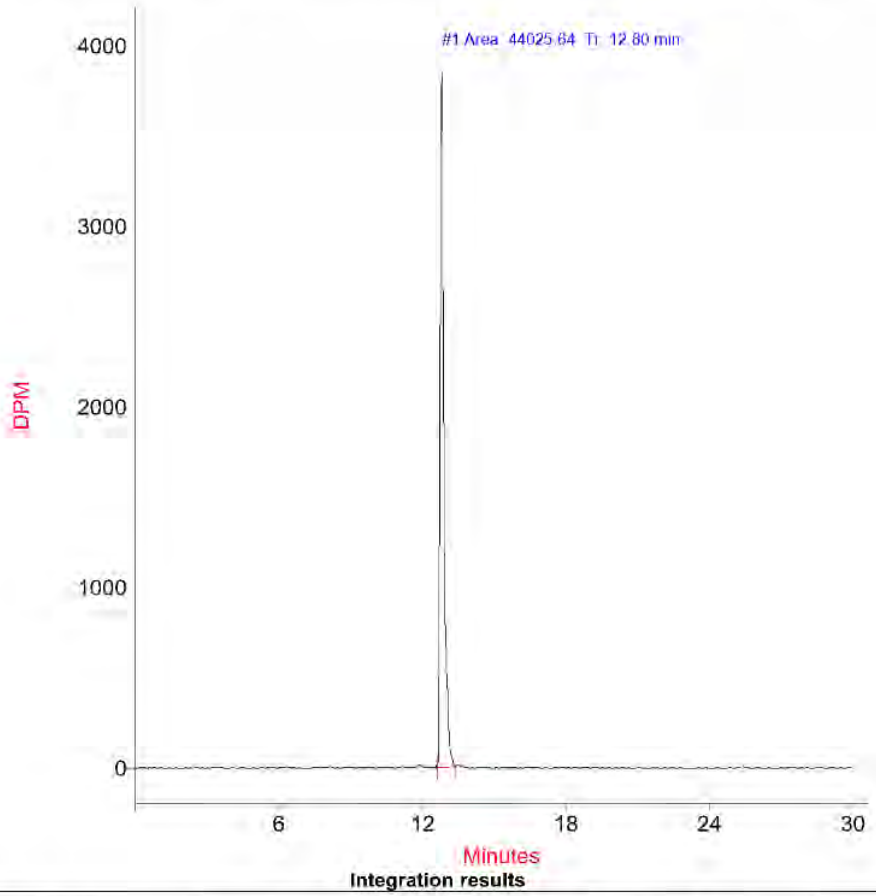
Results

Gross counts total	41886 DPM	Net counts total	41886 DPM
Gross counts of ROIs	39420 DPM	Net counts of ROIs	39420 DPM
Area of ROIs	94.11 %	Unresolved	5.89 %

Figure 15: Radiopurity of the radiolabelled preparation after 24 hours at 32°C for DDDE Dilution 0.012%

EUROFINS | ADME Bioanalyses RadioStar V 5.0.12.6

Analysis : 22-0148 Formulation 4 after storage at 32°C T24h



Integration results

#	Peak name	Rt.	Area	% Area	Results	% Results	Activity	Net DPM
1		12.80	44025.64	100.00	0.00	0.00	57215.53	41958.06
SUM			**44025.64**	**100.00**	**0.00**	**0.00**		**41958.06**

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APPENDIX C

STUDY PLAN

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STUDY PLAN

Title	*IN-VITRO* SKIN PENETRATION OF RADIOLABELLED ETHYL TAFLUPROSTAMIDE IN 4 DILUTIONS OF TEST ITEM ON HEALTHY HUMAN SKIN
Eurofins|ADME BIOANALYSES, FRANCE study code	22-0148
Name of reference item	^{3}H-Ethyl tafluprostamide (^{3}H-DDDE)
Test Facility	Eurofins|ADME BIOANALYSES 75A Avenue de Pascalet 30310 Vergèze France
Sponsor	[redacted]
Sponsor's Point of Contact	Marty Imler, Director, Business Development, Eurofins Product Testing
Document status	FINAL
Date of version	September 01st, 2022

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Confidentiality level: high

APPROVAL PAGE

This Study plan has been reviewed and approved by the Sponsor and the Test Facility Management. The following signatures document those approvals:

Test facility

Eurofins|ADME BIOANALYSES
75A Avenue de Pascalet
30310 Vergèze
France

Test Facility Management

Laurent BENAZERAF

Date and signature

Laurent BENAZERAF

Signé numériquement par : Laurent BENAZERAF
Nom DN : CN = Laurent BENAZERAF C = FR O = EUROFINS ADME BIOANALYSES OU = EUROFINS ADME BIOANALYSES, 0002 34146029300044, Laboratoire
Date : 2022.09.01 09:02:05 +02'00'
Motif : J'approuve ce document.

Study Director

Morgane DELOBEL

☎: 04 66 73 19 27
e-mail: MorganeDelobel@eurofins.com

Date and signature

Morgane DELOBEL

Signé numériquement par : Morgane DELOBEL
Nom DN : CN = Morgane DELOBEL C = FR O = EUROFINS ADME BIOANALYSES OU = EUROFINS ADME BIOANALYSES, 0002 34146029300044, Dermal unit
Date : 2022.09.01 10:39:24 +02'00'
Motif : J'approuve ce document

Deputy Study Director

Hélène MAROT
☎: 04 66 73 00 62
e-mail: HeleneMarot@eurofins.com

Date and signature

Hélène MAROT

Signé numériquement par : Hélène MAROT
Nom DN : CN = Hélène MAROT C = FR O = EUROFINS ADME BIOANALYSES OU = EUROFINS ADME BIOANALYSES, 0002 34146029300044, Dermal unit
Date : 2022.09.01 08:45:19 +02'00'
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APPROVAL PAGE (Continued)

Sponsor	[redacted]
Sponsor's Point of Contact	Eurofins Product Testing Marty IMLER ☎: +1 858 243 2464 e-mail: MartyImler@eurofins.com
Date and signature	[redacted] 9/1/22

Marty Imler, Director Business Development, 01 Sept 2022
Eurofins Product Testing US Inc
martyimler@eurofinsus.com Martha Imler

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RESPONSIBILITIES

Responsibility	Name	Contact details
Sponsor	[redacted]	[redacted]
Study Monitor	Marty IMLER MartyImler@eurofins.com Phone: +1 858 243 2464	Eurofins Product Testing 11822 North Creek Parkway, N #110 Bothell, WA 98011
Study Director	Morgane DELOBEL MorganeDelobel@eurofins.com Phone: +33 (0)4 66 73 19 27	Eurofins|ADME BIOANALYSES 75A avenue de Pascalet 30310 Vergèze France
Deputy Study Director	Hélène MAROT HeleneMarot@eurofins.com Phone: +33 (0)4 66 73 00 62	
Test Facility Management	Laurent BENAZERAF	
Head of QAU	Ophélie VAILLANT OphelieVaillant@eurofins.com Phone: +33 (0)4 66 73 17 52	

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DISTRIBUTION

Paper and/or electronic copies of the study plan and its possible amendment(s) will be distributed to the following personnel/departments:

Original and electronic copy	Study Director
Electronic copy	Quality Assurance
Papercopy	Laboratory
Electronic copy	Sponsor Monitor

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GLOSSARY

BLQ	:	Below the Limit of Quantification
C	:	Cotton-buds + tips
D	:	Dermis
E	:	Epidermis
K	:	Absorbent paper
LAV	:	Washing solution
LM	:	Scalpel blade
LR	:	Receptor fluid
SI	:	Lower seal
Max	:	Maximal value
MoS	:	Margin of Safety
Min	:	Minimal value
n	:	number of determinations or replicates
NA	:	Not applicable
QA	:	Quality Assurance
RCD	:	Cleaning of donor compartment
RCR	:	Cleaning of receptor compartment
RP	:	Remaining skin
S	:	Skin
SD	:	Standard Deviation
SOP	:	Standard Operating Procedure
SS	:	Upper seal
TEWL	:	TransEpidermal Water Loss
UHQ	:	Ultra High Quality
UST	:	Upper layers (*Stratum corneum*)

TABLE OF CONTENTS

1. Introduction

The aim of this study is to investigate the rate and extent of the *in-vitro* dermal absorption of Ethyl tafluprostamide (DDDE) with a ^{3}H-ethyl tafluprostamide (^{3}H-DDDE) radiolabelled tracer in four test items. These test items are applied to the surface of healthy human skin samples mounted on dynamic cells.

2. Contract laboratory and location

This study will be conducted at Eurofins|ADME BIOANALYSES, 75A avenue de Pascalet, 30310 Vergèze, FRANCE, under the direction of Morgane Delobel.

The study will be performed according to:

- this study plan and its possible amendment(s),
- the Standard Operating Procedures in use at Eurofins|ADME BIOANALYSES, FRANCE,
- the requirements of Good Laboratory Practices (OECD ENV/MC/CHEM (98) 17), 2004/10/EC, arrêté du 10 Août 2004 (France).

The realization of the technical part of the absorption and distribution following application on human skin will be based on:

- OECD guideline for the testing of chemicals, Test No. 428, Skin Absorption: in vitro method (13 April 2004)
- SCCS guideline (SCCS/1628/21), basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients, 30-31 March 2021. Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients. SCCS/1628/21
- OECD guidance document for the conduct of skin absorption studies, OECD series on testing and assessment. Number 28, 05-Mar-2004 (ENV/JM/MONO(2004)2).
- OECD guidance notes on dermal absorption Number 156, ENV/JM/MONO(2011)36.
- Cosmetics Europe guidelines for Percutaneous Absorption/Penetration, 1997.

3. Modification of the study plan

The study plan can be modified after agreement between Sponsor's point of contact and Eurofins|ADME BIOANALYSES, FRANCE. Each modification will be noted as an amendment by Eurofins|ADME BIOANALYSES, FRANCE and will be submitted for approval to the Sponsor's point of contact.

4. Deviation(s)

All unplanned deviation(s) from the study plan that may occur during the progression of the experiment will be noted in the paragraph "deviation(s) to the study plan" in the final report.

In case of deviation impacting the results, the Sponsor will be informed during the study.

5. Rationale

The "OECD guideline for the testing of chemicals: guideline 428, skin absorption: *in vitro* method" recommends to use a radiolabelled substance to perform this type of study.

6. Schedule of events

Expected study start date: August, 2022

Expected delivery of the pre-tests results: 1 week after the of end of the pre-tests

Expected delivery of the results: 2 weeks after the end of "*in vitro* experiment"

Expected draft report: Four weeks after the end of experiment

Any modification to this schedule will not require an amendment to the final protocol.

7. Reference items and ingredients

7.1. Reference items

Radiolabelled ^{3}H-DDDE will be provided by [redacted] informed Eurofins on 10 August 2022 that it had successfully synthesized ^{3}H-DDDE. Eurofins will notify Sponsor when the ^{3}H-DDDE is received from [redacted]

Non-radiolabelled item will be provided by the Sponsor.

	Reference items	
Name	^{3}H-Ethyl tafluprostamide*(^{3}H-DDDE)	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)
Supplier	[redacted]	[redacted]
Batch	22-0809-93	TAF-F-0122-0
Molecular Weight	437.5	437.52 g/mol
Specific activity (µCi/mg)	138.971 mCi/mg (by calculation)	-
Physical form	Liquid	Solution
Concentration	1 mCi/mL in EtOH	-
Purity by HPLC	> 98%	99.42%
Ethyl Tafluprostamide titer	-	7.5% in 2-phenoxyethanol
Expiry or Re-test date	Not relevant	03/02/2026
Storage conditions	Target temperature -80°C	Target temperature +4°C

*In the absence of stability data a radiopurity check will be performed before using.

The certificates of analysis will be included in the final report.

According to the expiry date or availability of reference item, the batch number, and/or purity could be modified.

New reference or batch of reference item could be ordered during the study, if necessary.

Note:
DDDE is a lipophilic molecule that is highly insoluble in water at 25°C: 0.09 mg/L (sccs_o_258.pdf (europa.eu)).
Molecular weight (g/mol) = 437.52
Partition Coefficient (Log Pow) = 5.03

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7.2. Blank formulation

	Blank formulation ([redacted] minus DDDE)
Name	[redacted] Blank formulation
Batch	Formula #042522-07-001
Expiry date	30/04/2025

The final product ([redacted]) is composed of 17 ingredients. In this study, the Sponsor will send the [redacted] product minus the DDDE ([redacted] blank formulation) for the radiolabelled preparation test item.

7.3. Receipt and handling

On their receipt, all relevant details and remarks relating to the condition of the products will be checked and recorded.

All test compounds will be handled with a particular care, especially to avoid any injection, swallowing and inhalation, according to Eurofins|ADME BIOANALYSES SOP.

All safety information relative to the manipulation will be sent together with the corresponding certificate of analysis (purity, water content, expiry date). Its compliance with this information will be the responsibility of the supplier.

8. Rationale for receptor fluid selection

For *in vitro* skin absorption studies, the molecule should be adequately soluble in the selected receptor fluid so that it does not act as a barrier to absorption, and the receptor fluid should maintain the skin barrier integrity. Therefore, the solubility of DDDE in the receptor fluid will be demonstrated as not being a rate limiting factor. According to the guideline, ENV/JM/MONO(2004)2, the solubility should be at least 10-fold higher than the maximal concentration expected in the receptor fluid at the end of the in vitro study.

Considering the maximal DDDE concentration in test item preparation (0.024% w/w) and the amount applied (10 µL/cm² equivalent to 10 mg/cm² by considering the density approximately equal to 1), the maximal concentration of DDDE in receptor fluid (Receptor fluid circulates at 1.5 mL/h giving a total volume of 36 mL for the volume obtained after a dismantling 24 hours after application on skin) is estimated to be 66.67 ng/mL.

DDDE solubility should be at least 666.7 ng/mL (10-fold the maximal estimated concentration) to confirm that the receptor fluid selected is suitable for the study.

Due to the low solubility in water of the DDDE, the selection of the receptor fluid will be determined by the estimation of the solubility of DDDE in the following receptor fluids:

- RF1: 5% w/w Bovine serum albumin, 0.9% NaCl in water
- RF2: 6% polyethylene glycol 20 oleyl ether in PBS 0.01 M* pH* 7.4 (*data given by the supplier)

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Solubility assessment will be undertaken as follows, n=2 for each receptor fluid:

- ✓ At least 2 µCi will be added to glass flasks (equivalent to 14.40 ng)
- ✓ A 0.2 mg/mL of DDDE solution will be prepared in ethanol and 10 µL will be added, corresponding to 2 µg (for a final concentration at 2.014 µg/mL, the amount brought by ^{3}H-DDDE is considered as negligible)
- ✓ Vortex
- ✓ The solvents will be evaporated under a stream of nitrogen gas
- ✓ 1 mL of receptor fluid will be added.
- ✓ The vials will be mixed using a magnetic stirrer for 1 hour at 32°C after which the vials will be left standing for 1 hour at 32°C.

20 µL samples will be collected from the upper, middle and lower level of each vial and will be analysed for radioactivity. Visual inspection of the vials for any precipitate will also be performed at the end of the incubation period.

Due to the low solubility in water of the DDDE, the receptor fluid presenting the highest solubility result (even if the threshold is not reached, as the worst case scenario was used for calculation) will be chosen and the sink condition at the end of the study will be checked.

9. Pre-test of the preparation of the test item (non GLP)

The pre-test will give Eurofins an opportunity to work out the procedures for preparing the test formulations containing 0.018% of DDDE using two different methods of preparation. The method yielding the best outcome, defined by homogeneity tests, will be used in the radiolabelled preparations (GLP) using for the cutaneous penetration study.

Radiolabelled preparation n°1: DDDE Dilution 0.018%

Approximately 2 g of test item will be prepared.

- Target 20 µCi of the ^{3}H-DDDE will be added in a glass flask (this amount is negligible),
- The ethanol will be evaporated under a stream of nitrogen gas.
- Around 1995.2 mg of [redacted] blank formulation ([redacted] placebo) will be added in the vial
- The mixture will be mixed
- Very slowly with adequate mixing add around 4.8 mg of non-radiolabelled DDDE into the vial and mix for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°2: DDDE Dilution 0.018%

Approximately 2 g of test item will be prepared.

- Target 20 µCi of the ^{3}H-DDDE will be added in a glass flask (this amount is negligible),
- The solvent will be evaporated under a stream of nitrogen gas.
- Around 4.8 mg of non-radiolabelled DDDE into the vial and mix for at least 2 min
- Around 1995.2 mg of [redacted] blank formulation ([redacted] placebo) will be added in the vial
- The mixture will be mixed for at least 1 hour under magnetic agitation.

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10. Preparation of the test item (radiolabelled preparation)

The radiolabelled reference item ^{3}H-DDDE will be received as liquid solution at 1 mCi/mL in EtOH.

According to the homogeneity results of the formulation tests carried out during the pre-test, one of the two preparation process described above will be chosen and described in the report.

The following test items will be prepared:

- 0.024% (w/w)
- 0.020% (w/w)
- 0.018% (w/w)
- 0.012% (w/w)

Radiolabelled preparation n°1: DDDE Dilution 0.024%

Approximately 2 g of test item will be prepared.

- Target 200 µCi of the ^{3}H-DDDE will be added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),

- The ethanol will be evaporated under a stream of nitrogen gas.

- Around 1993.6 mg of [redacted] blank formulation ([redacted] placebo) will be added in the vial

- The mixture will be mixed

- Very slowly with adequate mixing add around 6.4 mg of non-radiolabelled DDDE into the vial and mix for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°2: DDDE Dilution 0.020%

Approximately 2 g of test item will be prepared.

- Target 200 µCi of the ^{3}H-DDDE will be added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),

- The ethanol will be evaporated under a stream of nitrogen gas.

- Around 1994.6 mg of [redacted] blank formulation ([redacted] placebo) will be added in the vial

- The mixture will be mixed

- Very slowly with adequate mixing add around 5.4 mg of non-radiolabelled DDDE into the vial and mix for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°3: DDDE Dilution 0.018%

Approximately 2 g of test item will be prepared.

- Target 200 µCi of the ^{3}H-DDDEwill be added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),

- The ethanol will be evaporated under a stream of nitrogen gas.

- Around 1995.2 mg of [redacted] blank formulation ([redacted] placebo) will be added in the vial

- The mixture will be mixed

- Very slowly with adequate mixing add around 4.8 mg of non-radiolabelled DDDE into the vial and mix for at least 1 hour under magnetic agitation.

Radiolabelled preparation n°4: DDDE Dilution 0.012%

Approximately 2 g of test item will be prepared.

- Target 200 µCi of the ^{3}H-DDDE will be added in a glass flask, bringing to 1.44 µgeq (this amount is negligible),
- The ethanol will be evaporated under a stream of nitrogen gas.
- Around 1996.8 mg of [redacted] blank formulation ([redacted] placebo) will be added in the vial
- The mixture will be mixed
- Very slowly with adequate mixing add around 3.2 mg of non-radiolabelled DDDE into the vial and mix for at least 1 hour under magnetic agitation.

Approximately 1 µCi of test item will be applied to the skin surface placed in each cell (10 µL/1 cm²).

The radiolabelled preparations (RLA + ^{3}H-DDDE + DDDE) will be stored at room temperature under magnetic agitation before use.

One part of the preparations will be used for the stability test (after at least 24 hours at 32°C) and the other part for the application.

11. Design

11.1. Test item

The test item containing DDDE and the radiolabelled compound ^{3}H-DDDE will be prepared at Eurofins|ADME BIOANALYSES. Approximately 1 µCi will be applied on each cell (10 µL of test item /cell).

11.2. Cell design

Four donors will be included and three diffusion cells per donor will be measured for the test item, a total of twelve cells will be used by each formulation. 48 cells will be used for this study.

A total of 8 donors will be used with each donor will be used for 2 test items.

The test item will remain on the skin for 24 hours before removal by an appropriate washing solution.

The radioactivity will be measured in washing solution, *Stratum Corneum* by tape-stripping, epidermis, dermis and receptor fluid samples, as well as in all materials used in dosing preparation.

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Test item	Radiolabelled preparation			
Reference item	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (DDDE)			
Formulations	DDDE Dilution 0.024%	DDDE Dilution 0.02%	DDDE Dilution 0.018%	DDDE Dilution 0.012%
Theoretical concentration of reference item DDDE at 7.5% in phenoxyethanol	0.32%	0.27%	0.24%	0.16%
Theoretical concentration of active substance DDDE	0.024%	0.02%	0.018%	0.012%
Theoretical test item amount applied per cell (1 cm^2)				
Theoretical amount of reference item applied on skin (µg)	2.4 µg	2.0 µg	1.8 µg	1.2 µg
Occlusion condition	No			
Check of the test item stability at 32°C during 24 hours	Yes (n=3 (T0) and n=3 after 24 h at 32°C (T24)			
Thickness of the skin (µm)	300 - 400 µm			
Trans epidermal water loss (TEWL)	0.5 - 13 g/m^2/h			
Number of cell per donor (replicates)	3	3	3	3
Donor ID	1, 2, 3, 4	1, 2, 3, 4	5, 6, 7, 8	5, 6, 7, 8
Total number of donor	8 (each donor will be used for 2 formulations)			
Total of cells per formulation	12	12	12	12
Total cells	48			
Receptor fluid	Depending on the solubility results			
Sampling of Receptor Fluid	0.5h, 2h, 4h, 8h, 12h, 24h			
Washing	24 hours			
Washing of the test item	0.5 mL Tween 80® 5% 1 half cotton bud 3.5 mL of UHQ water (0.5 mL, 7 times) 3 dried half cotton buds			
Dismantling of the cells	24 hours			
Strips	A maximum of 20 strips will be performed*. The strips will be pooled as follows: 1-2, 3-6, 7-11, 12-15, 16-20*.			
Separation Epidermis/Dermis	Yes			
Extraction solvent for RCD and RCR, tape strips and cotton-swabs	Ethanol			

* The number of strips generated will depend of the donor. This number could be lower than 20 (between 1 and 20). If stripping induces the separation of epidermis and dermis, the stripping will be stopped.

12. Skin preparation

12.1. Preparation of human skin samples

The human skin samples used will be obtained from abdominal surgery. Just after receipt, excess subcutaneous fat will be removed if necessary. The storage of the skin at -20°C should be less than 15 months.

Information relative to the sex, age, localisation and storage condition of the human donor skins will be specified in the final report.

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Confidentiality level: high

12.2. Measurement of skin samples thickness

Skin samples will be excised and cut into pieces of 1.8 cm x 1.8 cm and the skin will be dermatomed to a thickness of 300-400 µm. The thickness will be measured using Oditest calipers according to the SOP in use in the laboratory.

12.3. Measurement of cutaneous sample integrity

Room temperature should be maintained between 20 and 25°C and the relative humidity between 30 and 70%.

Approximately 30 minutes after the set-up of the cells, *stratum corneum* integrity will be measured for each dermatomed skin sample by measuring the TEWL using evaporimeter.

There should be no water on the skin and in the cell donor compartment and the measurement will be taken away from any heating source and air stream.

The human skin will be included in the study if the TEWL is between 0.5 and 13 g/m²/h.

12.4. Measurement of temperature of cutaneous sample surface

The passive diffusion of chemicals (and therefore their dermal absorption) is affected by temperature. Therefore, the diffusion chamber and skin samples will be maintained at a constant temperature of 32 ± 1°C. The temperature will be measured for each cell just before the application.

13. Before application

13.1. Test item homogeneity

The homogeneity of the test items will be checked on 10 µL of the test item (n=6; 2 on the top, 2 on the middle and 2 on the bottom) using the same pipette than those used during the application.

10 µL will be weighed in the scintillation vial and scintillation liquid will be added.

The homogeneity of the test item will be checked before and during the application.

The homogeneity of the test item before the application will be considered acceptable if the obtained coefficient of variation (CV) on the 6 values is less or equal to 5%. If the CV is > 5%, the Sponsor will be informed.

The CV measured during application will be stated as a measure of variability. The homogeneity of the test item obtained during the application should be used to calculate the recovery.

13.2. Radiochemical purity and stability of the test item

The radiochemical purity will be tested by HPLC-radioactive analysis (Berthold Technologies: FlowStar LB 514, RadioStar 5.0.12.6).

The chromatographic conditions provided by RC-Tritec (presented in the report) or equivalent, will be used. The radiochemical purity of the test item will be checked before and after storage at 32°C for at least 24 hours (one sample injected one time in each condition) and will correspond to the stability of the reconstituted test item.

The radiochemical purity of the radiolabelled preparation will be tested by checking the absence of radiochemical peak higher than 2% of the total radioactivity.

If a degradation of more than 2% is measured, the Sponsor's point of contact will be informed immediately, and the status of the study will be decided after Sponsor's approval.

Due to the absence of stability data in the certificate of analysis of the reference item, a radiopurity check will be performed before using. If a radiochemical peak higher than 2% of the total radioactivity is observed, the results of this radiopurity will be compared with the radiopurity of the radiolabelled preparation and used to decide of the status of the study.

14. Application

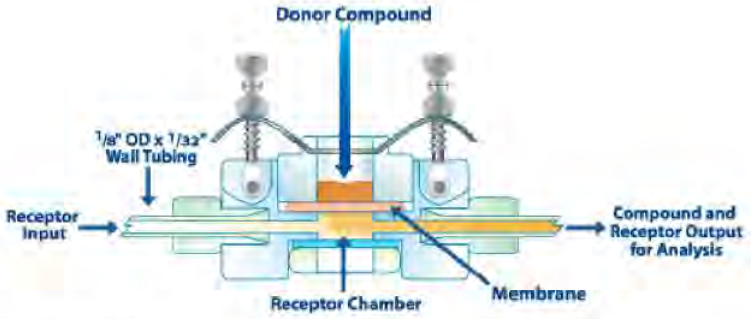
14.1. Preparation of dynamic cells

A flow-through diffusion cell system (developed by Bronaugh), also called dynamic cells or in line cells, will be used as used for the study.

Flow-through cells are characterized by a continuously replaced receptor fluid. Receptor fluid is continually replaced using tubes and a suitable pump (at a rate of about 1.5 mL/h).

The dynamic cells used will correspond to an application area equal to 1 cm^2.

Each apparatus of in-line cells is composed of seven cells.



Cells will be identified by a letter.

The skin samples will be placed on the receptor compartment. The donor compartment will be then placed onto the skin samples. A clamp will be placed to link both compartments and the cell will be tilted before being placed in the chamber in order to evacuate the presence of air bubbles in the flexible.

The skin surface temperature will be maintained at 32°C ± 1°C, with a fixed water bath integrated in the dynamic system.

14.2. Application of the test item

The preparation for the test item will be applied homogeneously at 10 µL/cm^2 (10 µL/cell), without massage, on each skin sample.

The application will be performed using a positive displacement pipette and the exact amount applied will be determined by weight then recorded and reported in the report.

Before application, the pipette containing the volume to be applied will be weighed (P1). Then the empty pipette will be weighed after application (P2). The applied amount (Px) will correspond to:

Px = P1 – P2.

14.3. Start of the experiment

The experiment will start immediately after application of the test item on the skin surface.

The experiment will be stopped 24 hours after application.

Start and end of experiment will be noted in the raw data.

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14.4. Receptor fluid sampling

The receptor fluid will be collected continuously and will pass through the dynamic system at a flow rate of 1.5 mL/h.

At each interval time, the receptor fluid will be placed into one vial (or more if necessary). If more than one vial is used, the result taken into account will be the sum of results in each vial.

The sampling time are specified in the paragraph 11.2 Cells design.

14.5. Identification of the vials

For each skin sample to be measured, vials will be identified as follows:

Washing solution (W or LAV)

Cotton buds + tip (C)

Donor Compartment Rinsing (RCD)

The *stratum corneum* (ST_X)

Epdermis (E) Dermis (D)

Remaining skin (RS)

Receptor fluid (RFx)

Receptor Compartment Rinsing (RCR)

All these identifications will be followed by the cell letter (A, B, C,...).

If several vials are used for the same compartment, number will be added (e.g.: ST1, ST2, etc...).

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14.6. Washing and Dismantling of cell and sample treatment

24 hours after application, the cells will be washed and dismantled.

The remaining formulation preparation will be washed after 24 hours with:

- 0.5 mL Tween 80® 5% with 1 half cotton bud
- 3.5 mL of UHQ water (0.5 mL, 7 times)
- 3 dried half cotton buds.

The washing solutions will be added to the skin surface (with half cotton) then removed with a pipette and will be collected for analysis. The skin surface will be carefully dried with two half cotton swabs before/during or after this washing procedure.

The washing solution (W) will be added into one vial and the half cotton-swabs and tip will be placed in another vial (or more vials if necessary). Half cotton-swabs and tip (C) will be extracted by 5 mL of appropriate solvent. Vials will be shaken at least overnight and sonicated if necessary.

The cells will be dismantled and skin samples will be treated as follows.

- Rinsing Donor compartment (RCD)

The donor compartment, the upper seal and the scalpel blade (LM) will be placed in a flask and 10 mL of ethanol will be added. The closed flask will be shaken at least overnight.

- *Stratum corneum* treatment: Upper strips (STx)

The skin will be taken with tweezers and each skin sample will be placed between seal and aluminium foil.

The stratum corneum will be taken off from the skin sample using adhesive scotch Magic 3M® by stripping. In order to standardize stripping, a pressure of 50 g/cm² from device pressure will be applied on top of the Scotch tape for 10 s before removing it.

A maximum of 20 tape strips should be performed. However, the total number of strips removed depends on the skin. Consequently, the stripping will be stopped if an epidermis/dermis separation is observed.

The strips will be pooled as follow for analysis: 1-2, 3-6, 7-10, 11-15, 16-20.

Tape strips will be extracted by 5 mL of ethanol. Vials will be shaken at least overnight and will be sonicated if necessary.

- Epidermis (E), Dermis (D) and remaining skin (RS) treatment

Using the scalpel blade, the skin corresponding to the application area will be separated from the remaining (surrounding) skin. The remaining skin is the ring of skin surrounding the application site (RS is needed to place the skin on the cell), not directly in contact with the applied formulation and with the receptor fluid. The remaining skin will be cut in four parts. After separation, epidermis and dermis will be placed in corresponding vials.

For epidermis, dermis and remaining skin, Solvable™ or equivalent (3 mL) will be added.

The E, D and RS vials will be placed in a heat chamber for the time necessary for total dissolution.

The scalpel blade and seal will be added with the donor compartment for extraction.

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Aluminium foil will be destroyed.

- Rinsing of receptor compartment (RCR)

The part of the receptor compartment in contact with the receptor fluid will be rinsing according to the process described below:

- 1 dried half cotton swab for the chamber of the receptor fluid (to absorb the remaining receptor fluid)

- 1 half cotton swab soaked with the ethanol for the entrance and the exit of the receptor fluid flow

- 1 half cotton swab soaked with the ethanol for the chamber of the receptor fluid

- 1 dried half cotton swab for entrance and the exit of the receptor fluid flow then for the chamber of the receptor fluid

The 4 half cotton swabs will be placed in a flask called RCR, then 5 mL of the ethanol will be added in the vial. The vial will be mix at least 1 night. If necessary, the vials could be sonicated.

15. Measurement of radioactivity

For receptor fluid and RCR, about 10 mL of Ultima Gold XR (or equivalent) scintillation liquid will be added.

For washing solution, half cotton-buds and tip vials and strips vial, approximately 10 mL of Ultima Gold XR (or equivalent) scintillation liquid will be added.

For the Epidermis, Dermis and remaining skin after dissolution with Solvable™ or equivalent, approximately 10 mL of Hionic Fluor (or equivalent) scintillation liquid will be added.

The RCD vial (corresponding to RCD + SS +LM), contains appropriate solvent. 1 aliquot of 1 mL will be taken. In each aliquot, approximately 10 mL of Ultima Gold XR (or equivalent) scintillation liquid will be added.

All vials can be agitated and sonicated if necessary.

Samples will be analyzed for radiolabel content by scintillation counting (Tricarb 2900TR or Tricarb 2910TR, version 2.06 Perkin). Calculations will be performed using Excel directly from the raw data obtained with the scintillation counter (QuantaSmart 2.03 or 4.00 software). Conversion of the counts per minute (cpm) to disintegrations per minute (dpm) will be performed directly by the microprocessor in the instrument using a quench curve of the appropriate scintillation cocktail stored in memory.

16. Storage of the specimens / Reference items

The radiolabelled preparation test items and the generated specimens will be analysed, stored at room temperature and destroyed after approval of the final report or 1 month after the end of experiment, due to the limited stability.

After aliquot preparation, the remaining RCD (without scintillation liquid) will be stored at a temperature set at -20°C and destroyed after approval of the final report or 1 month after the end of experiment.

The reference item (DDDE) will be destroyed after expiration date and according to the Sponsor's approval. The radiolabelled Ethyl tafluprostamide (^{3}H-DDDE) will be sent to the Sponsor or destroyed according to the Sponsor's approval.

17. Results

17.1. Calculations

Calculation will be performed using Excel. Results will be expressed as:

1. $\mu g_{eq}/cm^2$ (μg_{eq}: µg equivalent of ^{3}H-DDDE).
2. % of ^{3}H-DDDE/ applied dose.

The limit of quantitation will be 100 dpm *minus* blank value. Results below the limit of quantitation will be noted as "BLQ" in result tables and will be considered as 0 for calculation.

Results will be presented with 2 digits according to the studied compartments and/or recovered amount.

17.2. Recovery

For each experiment and each cell, a recovery balance will be calculated. The mean value should be 100 ± 15%, otherwise results will have to be justified.

- If there are some cells with inadequate recoveries, the results for low and high recovery cells (i.e. with values below or above the range 85% to 115%) can be compared to determine whether the losses are from absorbed or non-absorbed material. Losses that are considered to be from non-absorbed material will not impact the results. If missing material appears to be from the absorbed part, only values from high recovery samples will be used to determine the absorption.
- Alternatively, a "normalisation" approach could be applied in which dermal absorption is expressed as a percentage of the total amount recovered.
- If there are some replicates with adequate recoveries, then the results for low and high recovery replicates can be compared to those with adequate recovery to see if the losses are from absorbed or non-absorbed material.

Losses that are considered to be from non-absorbed material will not impact on the results.

If losses appear to be from absorbed material, the values should be corrected for the losses by considering the lost fraction as absorbed.

17.3. Absorption

The absorption will be equal to:

- Receptor fluid + Rinsing Receptor compartment (RCR) + Epidermis + Dermis (according to the SCCS guideline)
- When studies correspond to all of the basic requirements of the SCCS, the mean + 1SD will be used for the calculation of the MoS (Margin of Safety).
- In case of significant deviations from the protocol and/or very high variability, the mean + 2SD will be used as dermal absorption for the calculation of the MoS.

18. Final report

At the end of this study, Eurofins|ADME BIOANALYSES will provide to the Sponsor a preliminary report written in English describing the work carried out by Eurofins|ADME BIOANALYSES.

- Margins: right: around 1.8 cm / left: 1.8 cm / top: 2.5 cm, bottom (text): 2.5 cm
- Pagination: page number/total number of pages.
- Heading: On the top of the sheet: "Eurofins|ADME BIOANALYSES study code: 22-0148 - Type of document."
- The report will content at least:

Title page
Summary
Approval of the Report
Study Director Statement
Quality Assurance Statement
Glossary
Introduction
Results
Conclusion
Appendix A: Study plan and it(s) eventual(s) amendment(s)

Within 1 month after receipt of finalization approval, the final signed report will be sent to the Sponsor.

FileExchange secure server will be used for the transfer.

19. Quality Assurance

The study will be audited in accordance with the requirements of Good Laboratory Practice Standard (OECD ENV/MC/CHEM (98) 17) 2004/10/EC, arrêté du 10 Août 2004 (France) and with the Standard Operating Procedures in use at Eurofins|ADME BIOANALYSES, by the internal Quality Assurance Unit of Eurofins|ADME BIOANALYSES.

The study plan and its eventual amendments, the experimental part, the raw data generated and the study report will be audited.

The Quality Assurance statement will be presented in the study report.

These audit reports will be signed by the Study Director and Test Facility Management.

The Quality Assurance will include a statement in the study report specifying the dates of the audits and of transmission of audit reports to the Study Director and to the Test Facility Management.

Point of contacts of the Sponsor or regulatory authorities may conduct additional audits of the test facility and/or the raw data.

20. Archiving procedure

Within 3 months after the final report signature, all the documents relative to this study will be kept under the study code: 22-0148, in the Eurofins|ADME BIOANALYSES archives, for at least 5 years. After this period, the Sponsor will be contacted to seek his decision if the documents should remain at Eurofins|ADME BIOANALYSES archives or be sent back to the Sponsor.

Contents:
Signed study plan
All correspondence with Sponsor and letter(s) amending study plan
Copy of internal memoranda
Product specifications
Signed final report
List of staff involved
Laboratory notebooks
All raw data relevant to the study.

The content of the archives is the property of Sponsor and these archives can be made available at any time on documented request.

Electronic office data will be archived according to Eurofins|ADME BIOANALYSES SOP ARC/GEN/004P.

As the study is a short-term study, the reference item and the test item are not archived.

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Study Plan
Eurofins|ADME BIOANALYSES FRANCE study code: 22-0148
Confidentiality level: high

APPENDIX A

CERTIFICATE OF ANALYSIS

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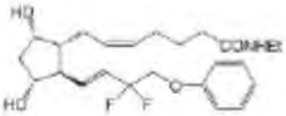
Study Plan
Eurofins|ADME BIOANALYSES FRANCE study code: 22-0148
Confidentiality level: high

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-F-022-001

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (7.5% by weight in 2-phenoxyethanol)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{37}F_2NO_4$
MW: 437.52

Manufacturing Date: January 2022
Release Date: 03-FEB-2022
Re-Test Date: 03-FEB-2026

Batch/Lot: TAF-F-0122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow solution	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomer NMT 2.5%)	NLT 99.00%	99.42%
Other Impurities	NMT 1.00%	0.58%

Result: Product Conforms to Specifications.

Quality Control: [redacted] *Date:* 03 FEB 2022

Approved: [redacted] *Date:* 07 Feb 2022

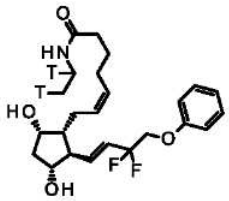
Quality Control Laboratory
[redacted]

APPENDIX D

CERTIFICATES OF ANALYSIS

Certificate of Analysis

[redacted]

Compound	[H-3]Ethyl Tafluprostamide
Molecular formula (unlabeled)	$C_{24}H_{33}F_2NO_4$
Molecular weight (unlabeled)	437.5 g/mol
Structure (unlabeled)	
Lot number	22-0809-93
Date of analysis	August 09, 2022
Radiochemical purity	>98% (HPLC)
Specific activity	60.8 Ci/mmol (2250 GBq/mmol) (determined by MS using an iterative residue correction method (^{13}C correction))
Concentration	1 mCi/ml (37 MBq/ml) in EtOH

Chromatographic data

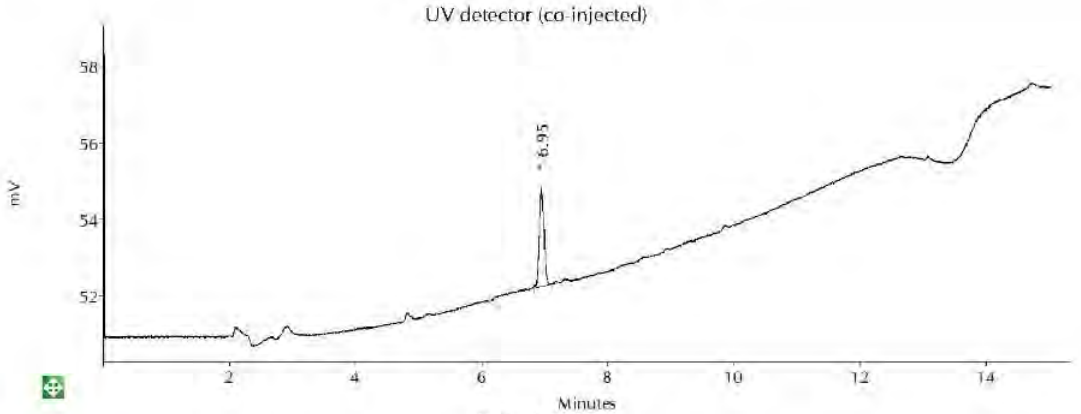
HPLC-column	Sunfire C18, 5 µm, 4.6 mm x 250 mm
Mobile phase	A: water + 0.05% TFA, B: MeCN + 0.05% TFA
Conditions	0 min 40% B; 10 min 95% B; 14.5 min 95% B; 15 min 40% B
Flow rate	1.0 ml/min
Sample	2 mCi/ml (74 MBq/ml) in EtOH
Injection	2 µl (4 µCi, 148 kBq)
UV-detection	254 nm
Temperature	30 °C
Radio detector	Berthold LB 513
Cocktail	Quicksafe Flow 2+
Flow rate	2.0 ml/min
Retention time	6.95 min (UV); 6.97 min (radio detector); The delay between the UV and the radio signal is due to the serial detection system

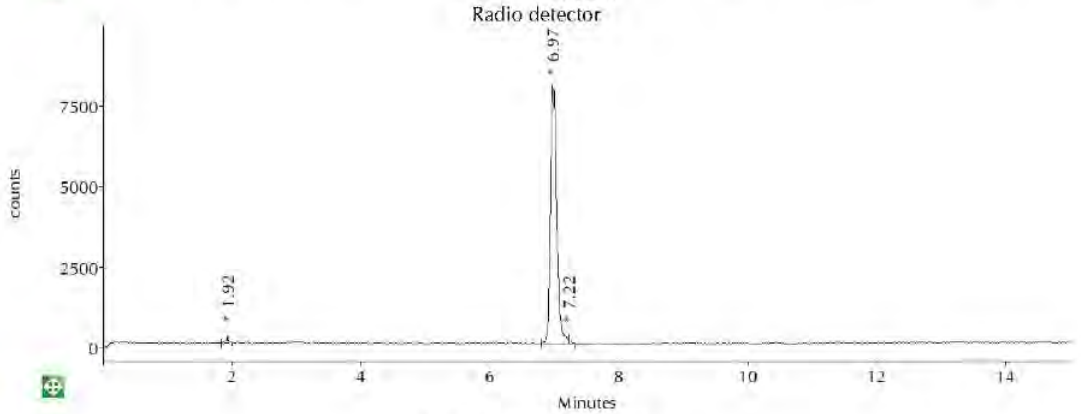
Note: The compound is dissolved in EtOH and was isolated from the HPLC eluent by solid phase extraction. The mass spectrum is consistent with the proposed structure and a non-labeled reference; the HPLC retention time is consistent with a non-labeled reference. Actual position of labels not verified. For research and development use only, not for use in humans.

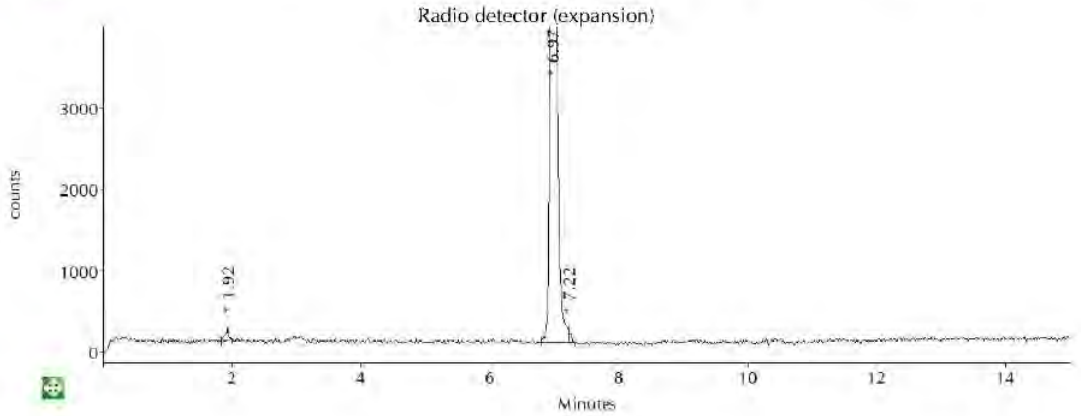
[redacted]

Certificate of Analysis

[redacted]







[redacted]

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Certificate of Analysis

[redacted]

Results radio detector

#	Peak name	Rt.	Area	% Area
1		1.92	530.00	0.95
2	**Ethyl Tafluprostamide**	**6.97**	**54667.98**	**98.39**
3		7.22	364.02	0.66
sum			55562.00	100.00

[redacted]

[redacted]

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-F-022-001

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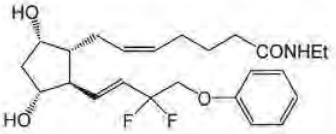

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Other Impurities	NMT 1.00%	0.58%

Result: Product Conforms to Specifications.

Quality Control: [redacted] *Date:* 03 FEB 2022

Approved: [redacted] *Date:* 07 Feb 2022

Quality Control Laboratory
[redacted]

Report

In-vitro dermal metabolism and penetration study of tafluprost ethyl amide (TPEA)

Data Requirement(s):	OECD 428 (2004) SCCS/1358/10 (2010) SCCS/1628/21 (2021)
Study Director:	FH-Prof. Priv.-Doz. Dr. Julian Weghuber
Study Completion Date:	4.10.2022
Test Facility:	University of Applied Sciences Upper Austria, Center of Excellence Food Technology and Nutrition Stelzhamerstraße 23, 4600 Wels Austria
Laboratory Project ID:	Report ID: [redacted]_01 Study ID: [redacted]
Sponsor:	[redacted]

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The University of Applied Sciences Upper Austria conducts research in accordance with its internal guidelines entitled “Ethikkodex der FH OÖ zur Sicherung guter wissenschaftlicher Praxis” (2010). These guidelines are consistent with the “Safeguarding Good Scientific Practice” issued by the Deutsche Forschungsgemeinschaft (DFG; 1998, amended 2013).

I, the undersigned, declare that the objectives of the study were achieved and that the data generated are valid. The report fully and accurately reflects the procedures used and the raw data generated in this study.

FH-Prof. Priv.-Doz. Dr. Julian Weghuber
Head Center of Excellence
Food Technology and Nutrition
Stelzhamerstraße 23, 4600 Wels/Austria

FH-Prof. Priv.-Doz. Dr. Julian Weghuber
(Study Director)

4.10.2022
Date

FH-Prof. Priv.-Doz. Dr. Julian Weghuber
(Study Director)

Date

Test Facility:

University of Applied Sciences
Upper Austria,
Center of Excellence Food
Technology and Nutrition
Stelzhamerstraße 23
4600 Wels
Austria

GENERAL INFORMATION

Contributors

The following at University of Applied Sciences Upper Austria, Center of Excellence Food Technology and Nutrition contributed to this report in the capacities indicated:

Name	Title
FH-Prof. Priv.-Doz. Dr. Julian Weghuber	Study Director
Priv.-Doz. Mag. Clemens Röhrl, PhD	Senior Researcher
Melanie Wallner, MSc	Researcher
Katja Essl, MSc	Researcher

Study dates

Study initiation date:	24.01.2022
Experimental start date:	01.02.2022
Experimental completion date:	25.05.2022

Deviations from the guidelines/guidance

Full thickness skin instead of half thickness utilized due to limited availability. Exposed skin area was 0.5 cm² instead of 0.64 cm².

Skin temperature was not assessed throughout experiments.

Separation of *stratum corneum* from epidermis was not compatible with compound extraction and analytical methods.

Overall, the deviations were considered as acceptable and without a relevant impact on the study rational and obtained results. Further arguments are provided in the respective sections below.

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1.0 SUMMARY

1.1 Study design

The purpose of this study was to determine the *in vitro* percutaneous absorption, distribution and metabolism of Tafluprost ethyl amide (TPEA) in its pure form as well as of TPEA from a leave-on PRODUCT [redacted] through viable human skin for up to 24 hours of exposure. These application conditions were designed to simulate potential human dermal exposure to the product during normal use.

Methodologies for analytical detection as well as for skin penetration assays using fresh human skin were established. Compounds of interest were applied for 2, 4 or 24 hours to human skin on a static transwell system using a physiological buffer as receptor fluid. Afterwards, content of compounds of interest were analyzed at the skin surface, in individual skin compartments and the receptor fluid.

Moreover, the metabolism of TPEA into Tafluprost free acid (TPFA) in human skin was focus of this study

1.2 Results

Identification and quantitation of TPEA and TPFA accomplished by HPLC-MS was sensitive and reproducible. Human skin models were viable and penetration of the marker compound caffeine was comparable with data from the literature.

Recovery rates of compounds of interests (mass balances) met the SCCS/1358/10 guidance criteria (≥ 85% for pure TPEA as well as for [redacted]n applied for 24 hours). Mass balance of TPEA applied for 4 hours was close (83.5%) to recommended recovery values.

TPEA was extensively metabolized into TPFA (68.5 +/- 2.7%) after 24 hours.

Bioavailabilities of TPEA in the human *in vitro* skin model were 12.3 +/- 2.2 % and 42.2 +/- 23.1% after 4 and 24 hours, respectively. TPEA from [redacted] was bioavailable at a rate of 24.4 +/-16.5% after 24 hours.

1.3 Conclusion

In summary, reliable and robust methodologies for the quantification of TPEA and TPEA as well as suitable viable human skin models were established. This study shows that TPEA undergoes extensive metabolism to its main metabolite TPFA and that TPEA is considerably bioavailable (42.2% after 24 hrs) under the selected test conditions using viable human skin samples. Moreover, TPEA permeation through the skin layers was characterized by considerable interindividual differences between individual donors.

2.0 INTRODUCTION

2.1 Purpose

The purpose of this study was to determine the *in vitro* percutaneous absorption, distribution and metabolism of Tafluprost ethyl amide (TPEA) in its pure form as well as of TPEA from a leave-on PRODUCT[redacted] through viable human skin for up to 24 hours of exposure. The skin surface was left unoccluded for the duration of the experiment. These application conditions were designed to simulate potential human dermal exposure to TPEA in the product during normal use.

2.2 Dose level selection

The application rates and exposure conditions used in this study were designed to simulate normal human exposure to the test substance when used appropriately. Applied dose of tafluprost ethyl amide deviates from the intended dose due to technical reasons (see. 4.6.1).

2.3 Regulatory guidelines and guidance documents

1) OECD Test Guideline 428 (2004). Skin Absorption: *In Vitro* Method.
2) Scientific Committee on Consumer Safety, SCCS/1358/10: Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients (2010)
3) Scientific Committee on Consumer Safety, SCCS/1628/21, SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation, 11th revision (2021)

2.4 Justification for selection of the test system

A valid static perfusion system using fresh (unfrozen) human abdominal skin was chosen based on the recommendation of the guideline SCCS/1358/10. Full thickness skin was used instead of half thickness skin due to a lack of availability of the later.

2.5 Data storage

Experimental raw data and data analyses will be retained at the University of Applied Sciences Upper Austria, Center of Excellence Food Technology and Nutrition for 10 years according to our good scientific practice guidelines. Remaining test materials will be retained until expiration.

3.0 TEST SUBSTANCES

3.1 Tafluprost ethyl amide (TPEA)

TPEA (CAS Number: 1185851-52-8) was provided in solution (10% in ethanol) by [redacted] without further documentation. TPEA was stored at -20°C upon arrival. TPEA was used as both reference standard and treatment compound.

3.2 Tafluprost free acid (TPFA)

TPFA (CAS Number: 209860-88-8; purity 100.0%, **see section 8.0 Extended material for CoA**) was purchased from [redacted]). TPFA was stored at -20°C upon arrival and used within the expiry date. TPEA was used as reference standard only.

3.3 [redacted] (Product)

[redacted] containing [redacted] TPEA was provided by [redacted] and stored at room temperature upon arrival. [redacted] was used as treatment compound mixture. Reported TPEA concentration was successfully verified by HPLC-MS analysis.

3.4 Caffeine

Caffeine (CAS Number: 58-08-2; purity ≥ 99%) was purchased from Sigma-Aldrich (St. Louis, MO, US). Caffeine was stored at -20°C upon arrival and used within the expiry date. Caffeine was used as both reference standard and positive treatment control compound.

4.0 EXPERIMENTAL PROCEDURES

4.1 Analytical instrumentation

Identification and quantification of TPEA, its metabolite TPFA and the reference substance caffeine was carried out using a Vanquish UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a solvent degasser, binary pump, autosampler, thermostated column compartment coupled to a diode array detector (DAD) and a single-quadrupole mass spectrometer equipped with a heated electrospray ionization source (Vanquish ISQ EC, Thermo Fisher Scientific, Waltham,

MA, USA). For instrument control and data processing Chromeleon 7.2.10 software (Thermo Fisher Scientific) was used.

4.2 Analytical procedure

Chromatographic separation was performed on a reverse-phase C18 column (Accucore C18, 150 x 2.1 mm, 2.6 µm particle size, Thermo Fisher Scientific) equipped with a guard column (Accucore C18, 10 x 2.1 mm, 2.6 µm). The flow rate was set to 0.7 mL/min and the column was kept at 30°C. The composition of the mobile phases was water for eluent A and 90% ACN for eluent B, both containing 5 mM ammonium formate buffer adjusted to pH 3.65. After an initial equilibration time of 3.5 min the gradient profile started from 10% B to 25% B within 2 min followed by a linear gradient to 80% B after 5 min and a hold time for 0.5 min at 80% B before returning to initial conditions. The total run time was 9.1 min (incl. equilibration time). Injection volume was 5 µL.

The mass spectrometer was operated in single ion mode in positive polarity. The electrospray voltage was set to 4 kV, capillary temperature was 350°C and ion transfer tube temperature was 300°C. Sheath gas pressure was set at 45 psig, aux gas at 15 psig and sweep gas at 2 psig.

4.3 Compound identification, quantitation and method validation

For the quantification of TPEA and the metabolite TPFA a six-point, weighted (1/x), external calibration in the concentration range from 0.05 µg/mL to 2.5 µg/mL was applied. The analytes were identified according to their specific masses in individual mass traces. The retention time of TPEA was 4.28 min with a mass of 438.2 m/z, showing the protonated molecular ion. TPFA eluted at 4.30 min and the formation of a sodium adduct was observed at 433.2 m/z. For the detection of caffeine, the DAD was set to 273 nm.

The level of quantification (LOQ) defined as the signal-to noise ratio equal to 10 (S/N=10) and the level of detection (LOD) (S/N=3) were determined using the lowest standard of the calibration curve for TPEA and TPFA.

Caffeine was identified at 1.39 min by comparing the retention times in samples and reference standards. Quantification was carried out using a five-point calibration in the range from 1 µg/mL to 40 µg/mL.

4.4 Receptor fluid

Receptor fluid consisted of Hanks′ Balanced Salt solution (HBSS; Sigma-Aldrich) containing glucose [1 g/L], supplemented with 5 g/L albumin (BSA; Sigma-Aldrich). The solubility of TPEA in the receptor fluid was ≥ 15 mg/L.

4.5 Skin samples

Fresh (unfrozen), abdominal skin samples ("NativeSkin access) were obtained from Genoskin (Toulouse, France). Samples were prepared as full thickness skin (4 mm) on transwell inserts and embedded into a fibrin-containing matrix in order to maintain skin viability and metabolic activity upon shipping. Skin samples were 0,95 cm^2 in size, whereof 0,5 cm^2 were accessible. In total, samples from four female donors (32 – 40 years, Caucasian, Fitzpatrick classification 2) were obtained. Upon receipt, samples were handled according to the manufacturer's instructions: Provided medium was added as the dermal site and renewed daily. Samples were placed in an incubator (37 °C, 5 % CO2, 95 % humidity) and used for experiments not later than three days after receipt.

4.6 Skin integrity

Transepidermal electrical resistance (TEER) was measured using the Millicell ERS-2 unit with an STX chopstick electrode (Merck, Darmstadt, Germany). This method was considered not suitable in the course of the study and was dismissed. Instead, caffeine as validated positive control was used as an established skin penetration marker substance and included in every set of subsequent experiments.

4.7 Penetration assay

Skin penetration assays were performed in a static cell system. Therefore, skin samples provided on transwell inserts where placed into standard 12-well tissue culture plates (Greiner Bio One, Kremsmünster, Austria). Compounds of interest were applied at the epidermal side and the dermal side was in contact with receptor fluid. According to the skin sample manufacturer, samples were incubated at 37°C, 5 % CO_2, 95 % humidity and gently agitated using a tide 2D rocker throughout experiments. Experiments were terminated 2, 4 or 24 hrs after application followed by sampling of skin compartments and receptor fluid.

4.7.1 Compound application

For TPEA, 3.0 µg (6.0 µg/cm^2; diluted in 50% ethanol) were applied. Initially, 1.5 µg (equivalent to a single, typical application of 10 µg of product) were tested. One point five µg is equivalent to a typical application of the final product [redacted]. This concentration was increased to 3.0 µg after initial tests, allowing for more reliable quantitation in skin compartments and receptor fluid by the analytical method.

For [redacted] 10 µl (20 µl/cm^2) were applied in its original (undiluted) form.

Caffeine as reference substance was applied at a dose 50 µg (100 µg/cm^2; diluted in 50% ethanol).

Overall, the slight differences to guideline/guidance requirements in respect to the selected application volumes were considered as acceptable with regards to the rational of the study and obtained results.

Exact dosage in the final treatment solution was measured by HPLC-MS before each individual experiment.

4.7.2 Sample collection and processing

Analyte sampling from the skin surface was done via two cycles of wiping with cotton swabs. One cycle consisted of an ethanol moistened swab followed by a dry swab. The swabs from the same cycle were combined for extraction as described below. Initial tests applying tape stripping to remove the stratum corneum failed because tape-bound compounds could not be extracted from the tapes. No homogenous solutions suitable for HPLC-MS analyses could be obtained.

To investigate the analyte content in the individual compartments of the skin insert (stratum corneum + epidermis, dermis, matrix), the skin model was removed from the trans-well and dissected to separate compartments using a scalpel. Epidermis and dermis were likewise dissected using a scalpel. The individual tissues were dissected into smaller pieces for the extraction process, which was carried out at room temperature while shaking on a thermo-shaker in 1 mL of extraction solvent (acetonitrile with 1% formic acid). After the removal of cotton swabs and tissues, the liquid was evaporated under vacuum to complete dryness. Samples were reconstituted in 0.5 mL 40% acetonitrile with 0.1% formic acid and syringe-filtered (0.2 µm filter size) prior to LC-MS analysis.

To determine the amount of analyte passed through the skin and the matrix, receptor fluid was collected and analyzed directly without further processing.

4.8 Data evaluation

Date were evaluated via Microsoft Excel and GraphPad Prism.

Mass balance was calculated by dividing combined concentrations of detected TPEA including its metabolite across all skin compartments including surface and receptor fluid per actually applied dosage of TPEA.

Bioavailability was calculated as concentrations of TPEA including its metabolite in (epidermis including stratum corneum + dermis + receptor fluid) per applied TPEA.

5.0 RESULTS AND DISCUSSION

5.1 Preliminary investigations

5.1.1 Analytical methodologies

An HPLC-MS method for the detection of TPEA as well as TPFA was successfully developed. For TPEA, the LOQ and LOD were 3 µg/L and 0.9 µg/L, respectively. For TPFA, the LOQ and LOD were 50 µg/L and 15 µg/L, respectively. Detector response was linear for concentrations of at least up 2.5 mg/L and all samples measured fell into this linear range.

5.1.2 Receptor fluid and compound solubility

The acceptor buffer was chosen based on the requirements for physiological osmolarity and pH, the presence of albumin as a physiological carrier for lipophilic compounds and the presence of glucose to maintain skin sample viability. Solubility of TPEA in receptor fluid was > 15 mg/L and therefore >~10-fold higher than concentrations observed in the final skin penetration assays. Therefore, quantification of TPEA in the receptor fluid is not limited by the solubility of TPEA in the receptor fluid in the subsequent experiments.

5.1.3 Skin integrity

TEER measurements to evaluate skin integrity yielded low values (≤ 200 Ω), which were considered implausible according to relevant literature [Guth et al., Toxicology *In Vitro* 2015; 29(1):113-23]. These TEER measurements, which yield consistent and reliable results for cell culture monolayer in our lab, are putatively not applicable for skin tissues in the current configuration. Therefore, caffeine was used as validated and accepted marker compound in all subsequent experiments. Application of caffeine for 24 hrs resulted in a relative transport rate of 29% to the receptor fluid (**Fig. 1**). Noteworthy, combined data from 9 laboratories show that 24,5 % +/- 11,6% of caffeine applied is transported to the receptor fluid after 24 hrs [van de Sandt et al., Regulatory Toxicology and Pharmacology 2004; 39: 271–281]. This indicates that skin model integrity and transport capability are comparable with data from relevant literature and confirmed the suitability and reliability of the test system.

5.2 Metabolism of TPEA

In-vivo, it is known that TPEA is hydrolyzed to form TPFA, the main metabolite and biologically active compound. To gain insights into the metabolic activity of skin samples, the conversion of TPEA into TPFA was followed over time. **Figure 2** shows that after 24 hours, the majority (68.5 +/- 2.7%) of TPEA was converted into its free acid. These data indicate that utilized human skin samples were metabolically active and that TPEA was converted into its active compound in relevant amounts.

5.3 Transport kinetics of TPEA

TPEA was applied to viable human skin samples followed by separation of skin compartments and receptor fluid after 2, 4 and 24 hours in order to gain insight into relative transport and permeation rates over time. TPEA including its metabolite TPFA were quantitated across skin compartments and receptor fluid (**Fig. 3**). After 2 hours 82.4 +/- 5.2% of applied TPEA and TPFA were localized at the skin surface. After 4 hours, still no apparent transport to the receptor fluid was detectable. After 24 hours, 9.3 +/- 2.4% were detected in the receptor fluid. The differentiation between TPEA and TPFA is given in **Tables 1 and 2**.

5.4 Mass balances (recovery) of TPEA

In order to test the efficiency and reliability of compound recovery, mass balances were calculated. Therefore, TPEA was applied for 4 and 24 hours and [redacted] was applied for 24 hours to human skin samples (**Table 3**). Recovery of TPEA including its metabolite was ≥ 85% for TPEA after 24 hours as well as for [redacted] and therefore met recommendations according to SCCS/1358/10 guidelines. Mass balance of TPEA applied for 4 hours was close (83.5% +/- 12% SD) to recommended recovery values. Noteworthy, mass balances, especially for TPEA applied for 24 hours, displayed considerable standard variations due to large interindividual differences. Especially, 3 samples showed mass balances > 100%, for which no reason could be identified.

5.5 Bioavailability of TPEA

Bioavailability of TPEA was assessed in human skin models for isolated TPEA after 4 and 24 hours (**Tables 4-9**).

After 4 hours, 12.3 +/- 2.2 % of TPEA are bioavailable (**Table 4**). **Tables 5 and 6** show the differentiation between TPEA and its metabolite TPEA. After 4 hours, the majority of TPEA is present in its initial form.

After application of TPEA for 24 hours 42.2 +/- 23.1% are bioavailable (**Table 7**). TPEA is extensively hydrolyzed to TPFA, which es especially found in the dermis and the receptor fluid, while TPEA is only converted to a limited amount at the surface (**Tables 8 and 9**).

After application of [redacted] for 24 hours, bioavailability was 24.4 +/-16.5 (**Table 10**). Again, extensive metabolism was observed, especially in the bioavailable compartments (**Tables 11 and 12**).

Of note, besides considerable variances between individual samples, also skewed distribution of data was observed.

6.0 CONCLUSIONS

This study shows that TPEA undergoes extensive metabolism to TPFA and that TPEA is bioavailable (42.2% after 24 hrs). Moreover, TPEA transport rate is characterized by considerable interindividual differences between individual donors.

7.0 TABLES AND FIGURES

Table 1. Transport kinetics of TPEA and TPFA in viable human skin samples. TPEA was applied to human skin models for the indicated time points followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid. All values represent means and are given as µg/cm^2 (n=3).

	TPEA				**TPFA**		
	2 hrs	4 hrs	24 hrs		2 hrs	4 hrs	24 hrs
skin surface	2.948	2.091	0.725		0.102	0.122	0.083
stratum corneum + epidermis	0.346	0.612	0.614		0.054	0.044	0.690
dermis	0.037	0.075	0.181		0.062	0.042	0.369
matrix	0.050	0.061	0.103		0.063	0.061	0.506
receptor fluid	0.000	0.000	0.058		0.035	0.000	0.382
bioavailable	**0.383**	**0.687**	**0.853**		**0.151**	**0.086**	**1.441**

Table 2. Transport kinetics of TPEA and TPFA in viable human skin samples. TPEA was applied to human skin models for the indicated time points followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid. This table shows the very same data as Table 1. Values here represent means and are given as % of totally detected TPEA plus TPFA (n=3).

	TPEA				**TPFA**		
	2 hrs	4 hrs	24 hrs		2 hrs	4 hrs	24 hrs
skin surface	79.74	67.28	19.54		2.76	3.93	2.24
stratum corneum + epidermis	9.36	19.69	16.55		1.46	1.42	18.59
dermis	1.00	2.41	4.88		1.68	1.35	9.94
matrix	1.35	1.96	2.78		1.70	1.96	13.64
receptor fluid	0.00	0.00	1.56		0.95	0.00	10.29
bioavailable	**10.36**	**22.10**	**22.99**		**4.08**	**2.77**	**38.83**

Table 3. Mass balance (recovery) of applied test compounds in viable human skin samples. TPEA including TPFA concentrations were summarized across all skin compartments and receptor fluid. Samples were derived from 3 individual donors

	mean [%]	SD [%]	number of samples
tafluprost ethyl amid, 4 hrs	83.5	12.0	9
tafluprost ethyl amid, 24 hrs	94.8	50.6	11
[redacted] **24 hrs**	85.9	32.0	8

Table 4. Distribution and bioavailability of TPEA after 4 hours. TPEA was applied to viable human skin samples for 4 hours followed by quantitation of total TPEA (i.e. TPEA plus TPFA) across all skin compartments including receptor fluid and skin surface (n=9 samples from 3 individual donors).

	mean [µg/cm^2]	SD [µg/cm^2]		mean [%]	SD [%]
skin surface	2.591	0.727		43.2	12.1
stratum corneum + epidermis	0.644	0.141		10.7	2.4
dermis	0.087	0.036		1.5	0.6
matrix	0.121	0.125		2.0	2.1
receptor fluid	0.007	0.017		0.1	0.3
bioavailable	**0.738**	**0.134**		**12.3**	**2.2**

Table 5. Distribution and bioavailability of TPEA after 4 hours. TPEA was applied to viable human skin samples for 4 hours followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid and skin surface (n=9 samples from 3 individual donors). This table show the same data as table 3, but differentiated for TPEA and its metabolite.

	TPEA			**TPFA**	
	mean [µg/cm^2]	SD [µg/cm^2]		mean [µg/cm^2]	SD [µg/cm^2]
skin surface	2.541	0.742		0.051	0.065
stratum corneum + epidermis	0.595	0.140		0.121	0.089
dermis	0.073	0.033		0.032	0.039
matrix	0.085	0.086		0.044	0.049
receptor fluid	0.001	0.002		0.006	0.018
bioavailable	**0.670**	**0.155**		**0.159**	**0.105**

Table 6. Distribution and bioavailability of TPEA after 4 hours. TPEA was applied to viable human skin samples for 4 hours followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid and skin surface (n=9 samples from 3 individual donors). This table show the same data as table 5. However, values here are given as % of totally detected TPEA plus TPFA.

	TPEA			**TPFA**	
	mean [%]	SD [%]		mean [%]	SD [%]
skin surface	71.6	20.9		1.4	1.8
stratum corneum + epidermis	16.8	3.9		3.4	2.5
dermis	2.1	0.9		0.9	1.1
matrix	2.4	2.4		1.2	1.4
receptor fluid	0.0	0.1		0.2	0.5
bioavailable	**18.9**	**4.4**		**4.5**	**3.0**

Table 7. Distribution and bioavailability of TPEA after 24 hours. TPEA was applied to viable human skin samples for 24 hours followed by quantitation of total TPEA (i.e. TPEA plus TPFA) across all skin compartments including receptor fluid and skin surface (n=11 samples from 3 individual donors).

	mean [µg/cm^2]	SD [µg/cm^2]		mean [%]	SD [%]
skin surface	0.874	0.376		14.6	6.3
stratum corneum + epidermis	1.556	0.644		25.9	10.7
dermis	0.57	0.418		9.5	7.0
matrix	0.822	0.754		13.7	12.6
receptor fluid	0.404	0.448		6.7	7.5
bioavailable	**2.53**	**1.386**		**42.2**	**23.1**

Table 8. Distribution and bioavailability of TPEA after 24 hours. TPEA was applied to viable human skin samples for 24 hours followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid and skin surface (n=11 samples from 3 individual donors). This table show the same data as table 7, but differentiated for TPEA and its metabolite.

	TPEA			**TPFA**	
	mean [µg/cm^2]	SD [µg/cm^2]		mean [µg/cm2]	SD [µg/cm2]
skin surface	0.769	0.386		0.105	0.089
stratum corneum + epidermis	0.721	0.297		0.835	0.713
dermis	0.101	0.111		0.468	0.423
matrix	0.099	0.054		0.723	0.752
receptor fluid	0.064	0.092		0.340	0.372
bioavailable	0.886	0.203		1.643	1.474

Table 9. Distribution and bioavailability of TPEA after 24 hours. TPEA was applied to viable human skin samples for 24 hours followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid and skin surface (n=11 samples from 3 individual donors). This table show the same data as table 8. However, values here are given as % of totally detected TPEA plus TPFA.

	TPEA			**TPFA**	
	mean [%]	SD [%]		mean [%]	SD [%]
skin surface	18.2	9.1		2.5	2.1
stratum corneum + epidermis	17.1	7.0		19.8	16.9
dermis	2.4	2.6		11.1	10.0
matrix	2.3	1.3		17.1	17.8
receptor fluid	1.5	2.2		8.0	8.8
bioavailable	**21.0**	**4.8**		**38.9**	**34.9**

Table 10. Distribution and bioavailability of TPEA from [redacted] after 24 hours. [redacted] was applied to viable human skin samples for 24 hours followed by quantitation of total TPEA (i.e. TPEA plus TPFA) across all skin compartments including receptor fluid and skin surface (n=8 samples from 3 individual donors).

	mean [µg/cm^2]	SD [µg/cm^2]		mean [%]	SD [%]
skin surface	0.700	0.374		23.3	12.5
stratum corneum + epidermis	0.408	0.222		16.6	7.4
dermis	0.126	0.074		4.2	2.5
matrix	0.460	0.350		15.3	11.7
receptor fluid	0.208	0.274		6.9	9.1
bioavailable	**0.742**	**0.494**		**24.4**	**16.5**

Table 11. Distribution and bioavailability of TPEA from [redacted] after 24 hours. [redacted] was applied to viable human skin samples for 24 hours followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid and skin surface (n=8 samples from 3 individual donors). This table show the same data as table 10, but differentiated for TPEA and its metabolite.

	TPEA			**TPFA**	
	mean [µg/cm^2]	SD [µg/cm^2]		mean [µg/cm2]	SD [µg/cm2]
skin surface	0.590	0.410		0.109	0.080
stratum corneum + epidermis	0.107	0.034		0.301	0.210
dermis	0.009	0.007		0.117	0.076
matrix	0.062	0.099		0.398	0.295
receptor fluid	0.030	0.086		0.178	0.197
bioavailable	**0.145**	**0.103**		**0.596**	**0.436**

Table 12. Distribution and bioavailability of TPEA from [redacted] fter 24 hours. [redacted] was applied to viable human skin sam [redacted] urs followed by quantitation of TPEA and its metabolite TPFA across all skin compartments including receptor fluid and skin surface (n=8 samples from 3 individual donors). This table show the same data as table 11. However, values here are given as % of totally detected TPEA plus TPFA.

	TPEA			**TPFA**	
	mean [%]	SD [%]		mean [%]	SD [%
skin surface	31.0	21.6		5.7	4.2
stratum corneum + epidermis	5.6	1.8		15.8	11.0
dermis	0.5	0.4		6.2	4.0
matrix	3.3	5.2		20.9	15.5
receptor fluid	1.6	4.5		9.4	10.4
bioavailable	**7.6**	**5.4**		**31.4**	**22.9**

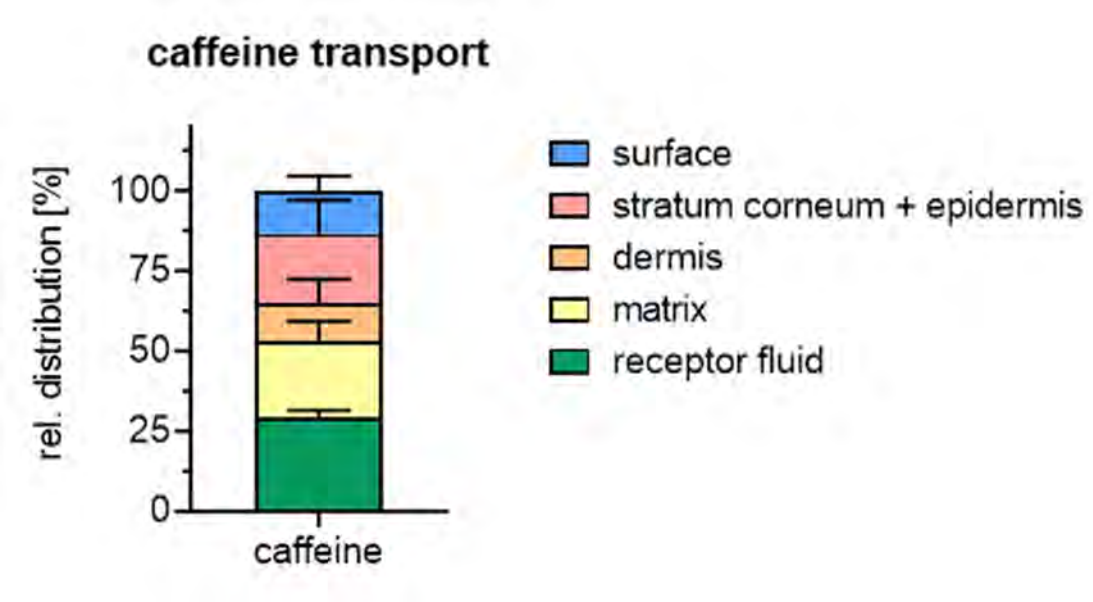

Figure 1. Caffeine transport through viable human skin samples. Caffeine was applied to human skin models for 24 hrs. Relative distribution across skin compartments is depicted (n=3).

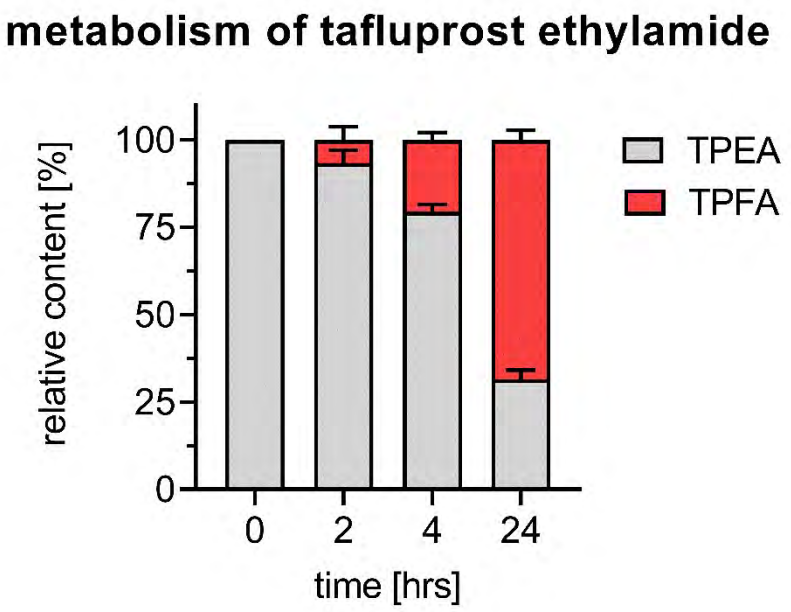

Figure 2. Metabolism of TPEA in viable human skin samples. TPEA was applied to human skin models for the indicated time points followed by quantitation of TPEA and its metabolite TPFA. Concentrations across all skin compartments including receptor fluid and skin surface were summarized (n=3).

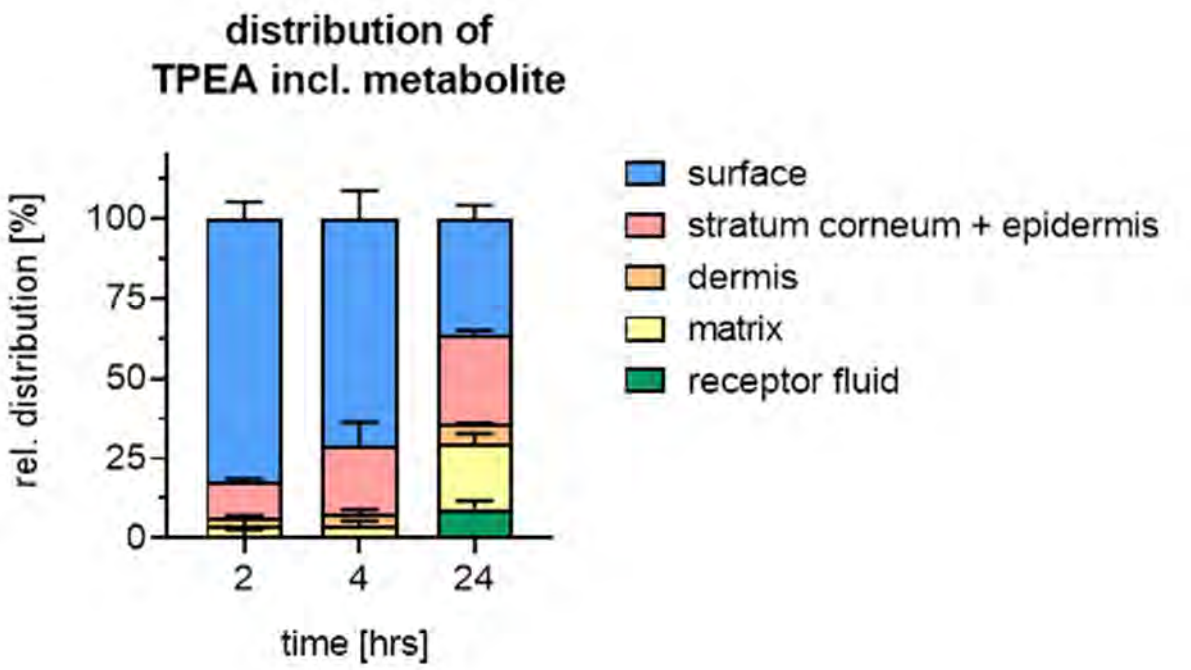

Figure 3. Transport kinetics of TPEA in viable human skin samples. TPEA was applied to human skin models for the indicated time points followed by quantitation of total TPEA (TPEA plus its metabolite TPFA) across all skin compartments including receptor fluid (n=3).

8.0 EXTENDED MATERIAL

[redacted]

CERTIFICATE of ANALYSIS

Tafluprost (free acid)

9α,11α-dihydroxy-15,15-difluoro-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-oic acid

Item No. 10005439 • Batch No. 0442914

Purity Specification: >98%
Molecular Formula : C22H28F2O5
CAS Number: 209860-88-8 **Formula Weight : 410.5** **Expiry date: 17FEB2023**

Overview

Tests	Results
HPLC	Purity: 100.0 %
IR	Conforms
Mass spec	M-H+: 409.8
TLC	Purity: 100 %
UV	λ max: 269, 276 nm

Reviewed and approved by: [redacted]

[redacted]

Test Report No. 202301137
Report Date June 26, 2023

Client

[redacted]

Approval Date	September 27, 2022	**Delivery Conditions**	Satisfactory, samples tested as received
Date of Receipt	October 5, 2022	**Testing Date Range**	01-17-2023 to 04-17-2023
Test Request Form #	TRF221010266		

Eurofins ID	Sample Name	Lot/Batch Number/Other	Supplier/Manufacturer	Country of Origin
202301137-1	Dechloro Dihydroxy DIfluoro Ethylcloprostenolamide	214	N/A	US

The following test item(s) was/were performed on submitted sample(s) and/or component(s) confirmed by applicant

TEST REQUESTED	RESULT
Bacterial Reverse Mutation Test (Ames-Test) OECD 471, OPPTS 870.5100, GLP	See Attachment
Bacterial Reverse Mutation Test (Ames-Test) (2nd experiment/confirmatory assay)	See Attachment

Analysis completed by Eurofins Subcontract Laboratory

Signed for and on behalf of
Eurofins MTS Consumer Product Testing US, Inc.

Alexis Klock / Project Coordinator

06-26-2023

Eurofins MTS Consumer Product Testing US, Inc.
11822 North Creek Parkway N, Suite 110 Bothell WA 98011
Phone: (425) 686-3575/Fax: (425) 686-3096 Web: www.EurofinsUS.com

eurofins | BioPharma Product Testing

Reverse Mutation Assay using Bacteria

(Salmonella typhimurium and *Escherichia coli*)

with

Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)

Report

Version: Final

Eurofins Munich Study No.: STUGC22AA2158-2

Sponsor:

[redacted]

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany

Tel | +49 (0)89 899 650-0
Fax | +49 (0)89 899 650-11

GLP / GMP FDA approved

1. Copy of the GLP Certificate

Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance

(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Eurofins BioPharma Product Testing Munich GmbH
Behringstraße 6/8
82152 Planegg
(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

Prüfkategorie 2: Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**Category 2:** toxicity studies
Prüfkategorie 3: Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**Category 3:** mutagenicity studies
Prüfkategorie 8: Analytische Prüfungen an biologischen Materialien	**Category 8:** analytical and clinical chemistry testing
Prüfkategorie 9: Sonstige Prüfungen: *biologische und mikrobiologische Sicherheitsprüfungen an Medi-zinprodukten und Arzneimitteln; Auftragsarchivierung*	**Category 9:** other tests: *biological and microbiological safety evaluation on medical devices and pharmaceuticals; contract archiving*

Datum der Inspektion/Date of Inspection:
(Tag.Monat.Jahr/day.month.year)

29.06.2021

Die/Der genannte Prüfeinrichtung/ Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.
Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/ diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP

Datum, Unterschrift/Date, Signature:

Schwabach, 14.02.2022

Dr. Joachim Strobel
Leiter der GLP-Leitstelle Bayern/
Head of the GLP Monitoring Authority

(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

GLP-Leitstelle Bayern, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit,
Rathausgasse 4, 91126 Schwabach
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority)

2. Contents

3. List of Tables

page

4. Preface

4.1. Abbreviations

2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
A. dest.	Aqua destillata *(purified water)*
Art.	Artikel *(article)*
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
bio	biotin
cf.	confer
chl	“Chlorate resistant” *E. coli* mutants, lack a functional nitrate reductase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EC	European Commission
e.g.	exempli gratia *(for example*)
EPA	Environmental Protection Agency
Eurofins Munich	Eurofins BioPharma Product Testing Munich GmbH
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
his	histidine
mg/kg bw	milligram/kilogram body weight
MMS	methylmethanesulfonate
NADP	nicotinamide adenine dinucleotide phosphate
OECD	Organisation for Economic Cooperation and Development
OCSPP	Office of Chemical Safety and Pollution Prevention
QAU	Quality Assurance Unit
rfa	deep rough factor
RSD	relative standard deviation
S9	microsomal fraction of rat liver homogenate
SD	standard deviation
SOPs	Standard Operating Procedures
uvrB	repair mutant, UV light sensitive
v/v	volume per volume

4.2. General

Sponsor:	[redacted]
Study Monitors:	Dr. Thomas Petry (technical matters) ToxMinds Avenue de Broqueville 116 1200 Brussels - Belgium Thomas.Petry@toxminds.com
	[redacted] Alexis Klock Eurofins Product Testing US Inc 11822 North Creek Pkwy Suite 110 Bothell, WA 98011
Test Facility:	Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany
Eurofins Munich Study No.:	STUGC22AA2158-2
Test Item:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)
Title:	Reverse Mutation Assay using Bacteria *(Salmonella typhimurium* and *Escherichia coli)* with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)

4.3. Project Staff

Study Director:	Kathleen Burns
Team Leader Operational QA GLP/GCP/ISO:	Uwe Hamann

4.4. Schedule

Arrival of the Test Item:	05 December 2022
Study Initiation Date:	16 January 2023
Experimental Starting Date:	17 January 2023
Experimental Completion Date:	17 April 2023
Study Completion Date:	Date of the study director’s signature

5. Quality Assurance

5.1. GLP Compliance

This study was conducted to comply with:

Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436) [1],

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis (“Consensus Document of the National and Länder Working Party on Good Laboratory Practice“) on the archiving and storage of records and materials, 5 May 1998 [2].

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998 [3].

The OECD Principles of Good Laboratory Practice are accepted by regulatory authorities throughout the European Community, USA and Japan.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of Eurofins Munich. The study and/or the test facility are inspected periodically by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

5.2. Guidelines

This study followed the procedures indicated by internal Eurofins Munich SOPs and the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted 21 July 1997, corrected 26 June 2020 [4].

Commission Regulation (EC) No. 440/2008 B.13/14: ”Mutagenicity – Reverse Mutation Test using Bacteria“, dated May 30, 2008 [5].

EPA Health Effects Test Guidelines, OCSPP 870.5100 "Bacterial Reverse Mutation Test" EPA 712-C-98-247, August 1998 [6].

5.3. Archiving

For a period of 15 years (or shorter if in compliance with the GLP regulations) Eurofins Munich will store the records, materials and specimens in their scientific archives according to the GLP regulations.

The following records have to be stored according to the GLP regulations:

The final report, the study plan and documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study. Any document relating to the study will be discarded only with the prior consent of the sponsor.

The following materials and samples have to be stored according to the period of time specified in the GLP regulations:

A retained sample of the test item will be archived according to the GLP regulations, if possible, and will be discarded without the sponsor’s prior consent.

Other materials and specimens have to be stored according to the GLP regulations and disposed of after the respective archiving period with the sponsor’s prior consent.

Unless otherwise agreed in writing, the remaining test item will be discarded three months after the release of the report.

6. Statement of Compliance

Eurofins Munich Study No.:	STUGC22AA2158-2
Test Item:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)
Study Director:	Kathleen Burns

This study performed in the test facility Eurofins Munich was conducted in compliance with Good Laboratory Practice Regulations:

Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436) [1],

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998 [3].

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Kathleen Burns

.....KBurns.....

09 JUN 2023

Date: .....

7. Statement of the Quality Assurance Unit

Eurofins Munich Study No.:	STUGC22AA2158-2
Test Item:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)
Study Director:	Kathleen Burns

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phase of QAU Inspection	Date of QAU Inspection	Date of Reporting to the Study Director and Management
Audit Final Study Plan:	11 January 2023	11 January 2023
Audit Experimental Phase (process-based):	10 February 2023	10 February 2023
Audit Final Report:	09 JUN 2023	09 JUN 2023

This report reflects the raw data.

Member of the
Quality Assurance Unit:

.....
Print Name: Nehal Omar

Date: 09 JUN 2023

8. Summary

8.1. Summary Results

A study was conducted to evaluate the potential to induce gene mutations of the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil), according to OECD Guideline 471, in compliance with GLP. In this assay, the substance was tested according to the plate incorporation method (experiment I) and the pre-incubation method (experiment II) using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and tester strain *E. coli* WP2 uvrA (pKM101).

Two independent experiments were performed using several concentrations of the test item. Each assay was conducted **with** and **without** metabolic activation. The concentrations, including the controls, were tested in triplicate. The following concentrations of the test item were prepared and used in the experiments:

Experiment I:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA100)

31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA98, TA1535, TA1537, E. coli WP2 uvrA (pKM101))

Experiment II:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA100, TA1535 [without metabolic activation])

31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA98, TA1535 [with metabolic activation], TA1537, E. coli WP2 uvrA (pKM101))

No precipitation of the test item was observed in any tester strain used in experiment I and II (**with** and **without** metabolic activation).

In experiment I toxic effects of the test item were observed at concentrations of 2500 µg/plate and higher (**with** and **without** metabolic activation), depending on the particular tester strain.

In experiment II toxic effects of the test item were noted at concentrations of 316 µg/plate and higher (**with** and **without** metabolic activation), depending on the particular tester strain.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil) at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II.

All criteria of validity were met (see section 10.8).

8.2. Conclusion

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil) did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil) is considered to be non-mutagenic in this bacterial reverse mutation assay.

9. Introduction

9.1. Aim of the Study

Bacterial reverse mutation assays use amino-acid requiring strains of *Salmonella typhimurium (S. typhimurium)* and *Escherichia coli (E. coli)* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of these bacterial reversion assays is that they detect mutations which functionally reverse mutations present in the tester strains and restore the capability to synthesize an essential amino acid [7], [9], [12].

The purpose of this study is to establish the potential of the test item to induce gene mutations in bacteria by means of a *S. typhimurium* and *E. coli* reverse mutation assay. There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentrations spacing and / or the method of treatment (pre-incubation method). In case of severe toxicity of the test item or the use of e.g., ethanol, acetone or tetrahydrofuran as the most appropriate solvent, the confirmatory experiment is carried out according to the plate incorporation method with a different spacing between dose levels.

The *S. typhimurium* histidine (his) reversion system and the *E. coli* tryptophan (trp) reversion system measures $his^- \rightarrow his^+$ reversions and $trp^- \rightarrow trp^+$. The *S. typhimurium* strains are constructed to differentiate between base pair (TA100, TA1535) and frameshift (TA98, TA1537) mutations [12]. The *E. coli* strain detects only base substitution mutagens.

These assays directly measure heritable DNA mutations of a type which is associated with adverse effects [13], [14], [16], [17]. Point mutations are the cause of many human genetic diseases and there is substantial evidence that somatic cell point mutations in oncogenes and tumor suppressor genes are involved in cancer in humans and experimental systems [8].

The tester strains have several features that make them more sensitive for the detection of mutations. The specificity of the strains can provide useful information on the types of mutations that are induced by mutagenic agents.

According to the direct plate incorporation or the pre-incubation method the bacteria are exposed to the test item with and without metabolic activation and plated on selective medium. After a suitable period of incubation, revertant colonies are counted [12].

At least five different concentrations of the test item are tested with approximately half log (i.e. $\sqrt{10}$) intervals between test points for an initial test. Narrower spacing between dose levels may be appropriate when a dose response is investigated. For soluble, non-toxic test compounds the recommended maximum test concentration is 5 mg/plate or 5 µL/plate.

To validate the test, reference mutagens are tested in parallel to the test item [10].

9.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals, Section 4, No. 471 – Bacterial Reverse Mutation Test - recommends using a combination of *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101).

10. Materials and Methods

10.1. Characterisation of the Test Item

The identity of the test item was inspected upon delivery at the test facility (e.g. test item name, batch no. and additional data were compared with the label) based on the following specifications provided by the sponsor. The following listed information applies to the sample as received.

Name:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil)
CAS No.:	1185851-52-8
EC No.:	867-521-0
Batch No.:	TAF-10-1122-01
Molecular Weight:	437.52 g/mol
Physical State:	liquid, oil
Colour:	colourless to pale yellow
Purity:	99.78%
Expiry Date:	23 November 2026
Storage Conditions:	2-8°C in a well-closed container
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

A copy of the Certificate of Analysis is reported in 16.2 Appendix 2: Certificate of Analysis.

10.2. Preparation of the Test Item

The test item was dissolved in DMSO, processed by ultrasound for 10 min at 37 °C and diluted prior to treatment. The solvent was compatible with the survival of the bacteria and the S9 activity.

10.3. Controls

Negative and solvent, as well as positive controls were included in each experiment. Strain specific positive controls were included in the assay, which demonstrated the effective performance of the test.

Negative and Solvent Controls

Negative controls (A. dest., Eurofins Munich, Lot No. 20230111, 20230130, 230223, 20230320, 230313) and solvent controls (DMSO, AppliChem Lot No. 0002204737) were treated in the same way as all dose groups.

Positive Controls

Without metabolic activation

Tester Strains:	*S. typhimurium*: TA100, TA1535
Name:	NaN_3; sodium azide
CAS No.:	26628-22-8
Supplier:	Sigma
Batch No.:	STBF8665V
Dissolved in:	A. dest.
Concentration:	10 µg/plate

Tester Strains:	*S. typhimurium*: TA98, TA1537
Name:	4-NOPD; 4-nitro-o-phenylene-diamine
CAS No.:	99-56-9
Supplier:	Sigma
Batch No.:	MKCF1418, MKCK4760
Dissolved in:	DMSO
Concentrations:	10 µg/plate for TA98; 40 µg/plate for TA1537

Tester Strain:	*E. coli* WP2 uvrA (pKM101)
Name:	MMS; methylmethanesulfonate
CAS No.:	66-27-3
Supplier:	Sigma
Batch No.:	MKCG1346
Dissolved in:	A. dest.
Concentration:	1 µL/plate

With metabolic activation

Tester Strains:	*S. typhimurium*: TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101)
Name:	2-AA; 2-aminoanthracene
CAS No.:	613-13-8
Supplier:	Alfa Aesar
Batch No.:	10218135
Dissolved in:	DMSO
Concentrations:	2.5 µg/plate for TA98, TA100, TA1535 and TA1537; 10 µg/plate for *E. coli* WP2 uvrA (pKM101)

The stability of the positive control substances in solution is unknown but a mutagenic response in the expected range is sufficient evidence of biological stability.

10.4. Test System

10.4.1. Bacteria

Four strains of *S. typhimurium* and one strain of *E. coli* WP2 uvrA (pKM101) with the following characteristics were used:

TA98:
his D 3052; *rfa*$^-$; *uvrB*$^-$; R-factor: frame shift mutations

TA100:
his G 46; *rfa*$^-$; *uvrB*$^-$; R-factor: base-pair substitutions

TA1535:
his G 46; *rfa*$^-$; *uvrB*$^-$: base-pair substitutions

TA1537:
his C 3076; *rfa*$^-$; *uvrB*$^-$: frame shift mutations

E. coli:
WP2 uvrA (pKM101): *trp*$^-$; *uvrA*$^-$: base-pair substitutions

Tester strains TA98, TA1535 and *E. coli* were obtained from MOLTOX, INC., NC 28607, USA. Tester strains TA100 and TA1537 were obtained from Xenometrix AG, Switzerland. They were stored as

stock cultures in ampoules with nutrient broth (OXOID) supplemented with DMSO (approx. 8% v/v) over liquid nitrogen.

All *Salmonella* strains contain mutations in the histidine operon, thereby imposing a requirement for histidine in the growth medium. They contain the deep rough (*rfa*) mutation, which deletes the polysaccharide side chain of the lipopolysaccharides of the bacterial cell surface. This increases cell permeability of larger substances. The other mutation is a deletion of the *uvrB* gene coding for a protein of the DNA nucleotide excision repair system resulting in an increased sensitivity in detecting many mutagens. This deletion also includes the nitrate reductase (*chl*) and biotin (*bio*) genes (bacteria require biotin for growth).

The tester strains TA98, TA100 and *E. coli* contain the R-factor plasmid, pKM101. These strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains. pKM101 increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in these organisms [12], [15].

The tester strain *E. coli* WP2 uvrA (pKM101) carries the defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent mutants (revertants) can arise either by a base change at the site of the original alteration or by a base change elsewhere in the chromosome so that the original defect is suppressed. This second possibility can occur in several different ways so that the system seems capable of detecting all types of mutagens which substitute one base for another. Additionally, the strain is deficient in the DNA nucleotide excision repair system.

The properties of the *S. typhimurium* and *E. coli* strains with regard to membrane permeability, ampicillin- and tetracycline-resistance as well as normal spontaneous mutation rates are checked regularly according to Ames *et al.* [7]. In this way it is ensured that the experimental conditions set up by Ames are fulfilled.

10.4.2. Preparation of Bacteria

Samples of each tester strain were grown by culturing for 12 h at 37 °C in *S. typhimurium* medium (Nutrient Broth) and *E. coli* medium (Luria Bertani), respectively, to the late exponential or early stationary phase of growth (approx. 10^9 cells/mL).

The *S. typhimurium* medium (Nutrient Broth) contains per litre of purified water:

8	g	Nutrient Broth
5	g	NaCl

The *E. coli* medium (Luria Bertani) contains per litre of purified water:

10	g	tryptone
10	g	NaCl
5	g	yeast extract

A solution of 125 µL ampicillin (10 mg/mL) (TA98, TA100, *E. coli* WP2 uvrA (pKM101)) was added in order to retain the phenotypic characteristics of the strain.

10.4.3. Agar Plates

The Vogel-Bonner Medium E agar plates with 2% glucose used in the Ames Test were prepared by Eurofins Munich or provided by an appropriate supplier. Quality controls were performed.

Vogel-Bonner-salts contain per litre of purified water:

10 g	$MgSO_4$ x 7 H_2O
100 g	citric acid
175 g	$NaNH_4HPO_4$ x 4 H_2O
500 g	K_2HPO_4

Sterilisation was performed for 20 min at 121 °C in an autoclave.

Vogel-Bonner Medium E agar plates contain per litre of purified water:

15	g	Agar Agar
20	mL	Vogel-Bonner salts
50	mL	glucose-solution (40%)

Sterilisation was performed for 20 min at 121 °C in an autoclave.

10.4.4. Overlay Agar

The overlay agar contains per litre of purified water:

S. typhimurium:

7.0	g	Agar Agar
6.0	g	NaCl
10.5	mg	L-histidine x HCl x H_2O
12.2	mg	biotin

E. coli:

7.0	g	Agar Agar
6.0	g	NaCl
10.2	mg	tryptophan

Sterilisation was performed for 20 min at 121 °C in an autoclave.

10.4.5. Mammalian Microsomal Fraction S9 Mix

The bacteria most commonly used in these reverse mutation assays do not possess the enzyme system which, in mammals, is known to convert promutagens into active DNA damaging metabolites. In order to overcome this major drawback an exogenous metabolic system is added in the form of mammalian microsome enzyme activation mixture.

10.4.6. S9 Homogenate

The S9 liver microsomal fraction was prepared at Eurofins Munich and obtained from Trinova Biochem GmbH, Gießen, Germany. Male Wistar rats were induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route (Eurofins Munich) and male Sprague Dawley rats were induced with phenobarbital/β-naphthoflavone (Trinova).

The following quality control determinations were performed:

- Eurofins Munich-prepared S9 Homogenate:
  Quality control determinations performed by Eurofins Munich:

a) Biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene

b) Sterility Test

- Trinova Biochem GmbH-prepared S9 Homogenate:

  Quality control determinations performed by Trinova Biochem GmbH:

a) Alkoxyresorufin-O-dealkylase activities

b) Test for the presence of adventitious agents

c) Promutagen activation (including biological activity in the Salmonella typhimurium assay using 2-aminoanthracene and benzo[*a*]pyrene)

A stock of the supernatant containing the microsomes was frozen in aliquots of 2 and 4 mL (Eurofins Munich) and 5 mL (Trinova) and stored at ≤ -75 °C.

The protein concentration in the S9 preparation of Eurofins Munich was 39.0 mg/mL (Lot: 251122), and in the S9 preparation of Trinova, 36.9 mg/mL (Lot: 4654). The protein concentrations were adjusted to 30 mg/mL.

10.4.7. Preparation of S9 Mix

The S9 mix preparation was performed according to Ames *et al.* [7].

100 mM of ice-cold sodium-ortho-phosphate-buffer, pH 7.4, was added to the following pre-weighed sterilised reagents to give final concentrations in the S9 mix of:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	glucose-6-phosphate
4	mM	NADP

This solution was mixed with the liver 9000 x g supernatant fluid in the following proportion:

co-factor solution	9.5 parts
liver preparation	0.5 parts

During the experiment the S9 mix was stored on ice.

10.4.8. S9 Mix Substitution Buffer

The S9 mix substitution buffer was used in the study as a replacement for S9 mix, without metabolic activation (-S9).

Phosphate-buffer (0.2 M) contains per litre of purified water:

0.2 M NaH_2PO_4 x H_2O	120 mL
0.2 M Na_2HPO_4	880 mL

The two solutions were mixed and the pH was adjusted to 7.4. Sterilisation was performed for 20 min at 121 °C in an autoclave.

This 0.2 M phosphate-buffer was mixed with 0.15 M KCl solution (sterile) in the following proportion:

0.2 M phosphate-buffer	9.5 parts
0.15 M KCl solution	0.5 parts

This S9 mix substitution buffer was stored at 4 °C.

10.5. Experimental Design

10.5.1. Pre-Experiment for Toxicity

The toxicity of the test item was determined with tester strains TA98 and TA100 in a pre-experiment. Eight concentrations were tested for toxicity and induction of mutations with three plates each. The experimental conditions in this pre-experiment were the same as described below for the main experiment I (plate incorporation test).

Toxicity may be detected by a clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control.

The test item was tested in the pre-experiment at the following concentrations:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate

10.5.2. Exposure Concentrations

The test item concentrations to be applied in the main experiments were chosen according to the results of the pre-experiment (see chapter 12.1.1 Pre-Experiment). 5000 µg/plate was selected as the maximum concentration. The concentration range covered two logarithmic decades. Two independent experiments were performed at the following concentrations:

Experiment I:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA100)

31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA98, TA1535, TA1537, E. coli WP2 uvrA (pKM101))

Experiment II:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA100, TA1535 [without metabolic activation])

31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA98, TA1535 [with metabolic activation], TA1537, E. coli WP2 uvrA (pKM101))

As the results of the pre-experiment were in accordance with the criteria of validity (10.8), these were additionally reported as a part of the main experiment I.

10.5.3. Experimental Performance

For the plate incorporation method, the following materials were mixed in a test tube and poured over the surface of a minimal agar plate:

100	µL	Test solution at each dose level, solvent or negative control or reference mutagen solution (positive control),
500	µL	S9 mix (for testing with metabolic activation) or S9 mix substitution buffer (for testing without metabolic activation),
100	µL	Bacteria suspension (cf. Preparation of Bacteria, pre-culture of the strain),
2000	µL	Overlay agar.

For the pre-incubation method 100 µL of the test item-preparation is pre-incubated with the tester strains (100 µL) and sterile buffer or the metabolic activation system (500 µL) for 60 min at 37 °C prior to adding the overlay agar (2000 µL) and pouring onto the surface of a minimal agar plate.

For each strain and dose level, including the controls, three plates were used (in a few cases two plates were evaluated, see results 12.1 Results).

After solidification the plates were inverted and incubated at 37 °C for at least 48 h in the dark.

10.6. Data Recording

The colonies were counted using a Sorcerer Colony Counter (Perceptive Instruments). If precipitation of the test item precluded automatic counting the revertant colonies were counted by hand.

10.7. Evaluation of Cytotoxicity

Cytotoxicity can be detected by a clearing or rather diminution of the background lawn (indicated as "N" or “B”, respectively in the result tables) or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control.

10.8. Criteria of Validity

A test is considered acceptable if for each strain:

- the bacteria demonstrate their typical responses to ampicillin (TA98, TA100, *E. coli* WP2 uvrA (pKM101))
- the negative control plates (A. dest.) with and without S9 mix are within the following ranges (mean values of the spontaneous reversion frequency are within the historical control data range (January – December 2020 for all tester strains)):

	- S9		+ S9	
	min	max	min	max
TA98	13	71	26	68
TA100	55	155	53	176
TA1535	5	34	4	37
TA1537	7	32	7	46
E. coli WP2 uvrA (pKM101)	108	327	122	355

- corresponding background growth on both negative control and test plates is observed.
- the positive controls show a distinct enhancement of revertant rates over the control plate
- at least five different concentrations of each tester strain are analysable.

10.9. Evaluation of Mutagenicity

The Mutation Factor is calculated by dividing the mean value of the revertant counts by the mean values of the solvent control (the exact and not the rounded values are used for calculation).

A test item is considered as mutagenic if:

- a clear and dose-related increase in the number of revertants occurs and/or
- a biologically relevant positive response for at least one of the dose groups occurs

in at least one tester strain with or without metabolic activation.

A biologically relevant increase is described as follows:

- if in tester strains TA98, TA100 and *E. coli* WP2 uvrA (pKM101) the number of reversions is at least twice as high
- if in tester strains TA1535 and TA1537 the number of reversions is at least three times higher

as compared to the reversion rate of the solvent control [11].

According to the OECD guidelines, the biological relevance of the results is the criterion for the interpretation of results, a statistical evaluation of the results is not regarded as necessary.

A test item producing neither a dose related increase in the number of revertants nor a reproducible biologically relevant positive response at any of the dose groups is considered to be non-mutagenic in this system.

11. Deviations from the Study Plan

There were no deviations from the study plan.

12. Results and Discussion

12.1. Results

12.1.1. Pre-Experiment

In the pre-experiment assay for toxicity, the substance was tested up to concentrations of 5000 µg/plate in the presence and absence of metabolic activation in tester strains strains TA98 and TA100. The results are reported in Table 1. Toxicity may be detected by a clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control. No precipitation of the test item was observed in either tester strain used (**with** and **without** metabolic activation).

Table 1: Results Pre-Experiment

Substance	Dose (µg/plate)	TA98 Mutation Factor [toxicity / precipitation]*		TA100 Mutation Factor [toxicity / precipitation]*	
		without S9	with S9	without S9	with S9
Solvent Control (DMSO)		1.0	1.0	1.0	1.0
4-NOPD	10.0	14.1	-	-	-
NaN_3	10.0	-	-	8.9	-
2-AA	2.50	-	85.8	-	13.8
Test Item	3.16	0.8	1.1	1.4	0.9
	10.0	1.0	1.1	1.3	1.0
	31.6	0.7	1.1	1.2	1.0
	100	1.0	1.2	1.2	0.9
	316	1.1	1.5	1.1	1.1
	1000	1.2	1.1	1.0	0.9
	2500	0.7 [B]	0.9 [B]	0.6 [B]	0.0 [B]
	5000	0.2 [B]	0.9 [B]	0.1 [B]	0.0 [B]

* [toxicity / precipitation parameter]: B = Background lawn reduced; N = No background lawn; P = Precipitation

12.1.2. Experiment I *(Plate-incorporation Test)*

The results of the bacterial reverse mutation assay conducted according to the plate incorporation test (experiment I) are reported in Table 2.

Table 2: Results Experiment I

Tester Strain: TA98 Experiment: 1

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		43 50 40	**44**	4.2	36 42 27	**35**	6.2	**1.5**	**1.1**
DMSO		28 34 28	**30**	2.8	30 31 33	**31**	1.2	**1.0**	**1.0**
Test Item	31.6 µg	24 28 14	**22**	5.9	32 30 39	**34**	3.9	**0.7**	**1.1**
Test Item	100 µg	35 24 34	**31**	5.0	32 41 36	**36**	3.7	**1.0**	**1.2**
Test Item	316 µg	29 31 35	**32**	2.5	48 49 42	**46**	3.1	**1.1**	**1.5**
Test Item	1000 µg	37 27 41	**35**	5.9	31 36 33	**33**	2.1	**1.2**	**1.1**
Test Item	2500 µg	18 B 20 B 24 B	**21**	2.5	30 B 25 B 28 B	**28**	2.1	**0.7**	**0.9**
Test Item	5000 µg	18 B 0 B 0 B	**6**	8.5	30 B 23 B 31 B	**28**	3.6	**0.2**	**0.9**
4-NOPD	10 µg	415 428 425	**423**	5.6	/ / /	**/**	/	**14.1**	**/**
2-AA	2.5 µg	/ / /	**/**	/	2679 2382 3007	**2689**	255.3	**/**	**85.8**

SD: Standard-deviation P: Precipitation
B: Background lawn reduced C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: TA100 Experiment: 1

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		114 133 131	**126**	8.5	141 138 139	**139**	1.2	**1.1**	**1.3**
DMSO		110 118 119	**116**	4.0	120 105 97	**107**	9.5	**1.0**	**1.0**
Test Item	3.16 µg	155 166 165	**162**	5.0	96 113 88	**99**	10.4	**1.4**	**0.9**
Test Item	10 µg	158 137 149	**148**	8.6	120 97 100	**106**	10.2	**1.3**	**1.0**
Test Item	31.6 µg	137 148 142	**142**	4.5	115 108 111	**111**	2.9	**1.2**	**1.0**
Test Item	100 µg	135 153 136	**141**	8.3	86 124 90	**100**	17.0	**1.2**	**0.9**
Test Item	316 µg	117 122 133	**124**	6.7	135 101 129	**122**	14.8	**1.1**	**1.1**
Test Item	1000 µg	121 111 115	**116**	4.1	90 104 100	**98**	5.9	**1.0**	**0.9**
Test Item	2500 µg	82 B 65 B 53 B	**67**	11.9	0 B 4 B 5 B	**3**	2.2	**0.6**	**0.0**
Test Item	5000 µg	17 B 14 B 0 B	**10**	7.4	0 B 0 B 0 B	**0**	0.0	**0.1**	**0.0**
NaN_3	10 µg	976 968 1139	**1028**	78.8	/ / /	**/**	/	**8.9**	**/**
2-AA	2.5 µg	/ / /	**/**	/	1424 1662 1363	**1483**	129.0	**/**	**13.8**

SD: Standard-deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: TA1535 Experiment: 1

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		8 13 12	**11**	2.2	12 11 5	**9**	3.1	**1.1**	**1.8**
DMSO		9 13 7	**10**	2.5	6 7 3	**5**	1.7	**1.0**	**1.0**
Test Item	31.6 µg	4 5 8	**6**	1.7	13 8 7	**9**	2.6	**0.6**	**1.8**
Test Item	100 µg	6 11 12	**10**	2.6	12 9 13	**11**	1.7	**1.0**	**2.1**
Test Item	316 µg	6 4 9	**6**	2.1	8 10 11	**10**	1.2	**0.7**	**1.8**
Test Item	1000 µg	5 12 7	**8**	2.9	11 12 7	**10**	2.2	**0.8**	**1.9**
Test Item	2500 µg	6 B 2 B 3 B	**4**	1.7	5 B 8 B 10 B	**8**	2.1	**0.4**	**1.4**
Test Item	5000 µg	2 B 5 B / nA	**4**	1.5	10 B 7 B 8 B	**8**	1.2	**0.4**	**1.6**
NaN_3	10 µg	1026 998 1265	**1096**	119.8	/ / /	**/**	/	**113.4**	**/**
2-AA	2.5 µg	/ / /	**/**	/	236 271 187	**231**	34.5	**/**	**43.4**

SD: Standard-deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination
nA: not analysable

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: TA1537 Experiment: 1

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		24 19 18	**20**	2.6	12 14 18	**15**	2.5	**1.2**	**0.8**
DMSO		17 19 14	**17**	2.1	14 22 21	**19**	3.6	**1.0**	**1.0**
Test Item	31.6 µg	21 18 16	**18**	2.1	22 21 23	**22**	0.8	**1.1**	**1.2**
Test Item	100 µg	22 11 11	**15**	5.2	24 31 24	**26**	3.3	**0.9**	**1.4**
Test Item	316 µg	14 23 10	**16**	5.4	17 17 26	**20**	4.2	**0.9**	**1.1**
Test Item	1000 µg	16 13 10	**13**	2.4	16 20 16	**17**	1.9	**0.8**	**0.9**
Test Item	2500 µg	17 7 14	**13**	4.2	19 14 8	**14**	4.5	**0.8**	**0.7**
Test Item	5000 µg	9 15 14	**13**	2.6	11 13 8	**11**	2.1	**0.8**	**0.6**
4-NOPD	40 µg	143 144 130	**139**	6.4	/ / /	**/**	/	**8.3**	**/**
2-AA	2.5 µg	/ / /	**/**	/	246 216 180	**214**	27.0	**/**	**11.3**

SD: Standard-deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: WP2 uvrA (pKM101)

Experiment: 1

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		246 281 259	**262**	14.4	122 120 155	**132**	16.0	**1.1**	**1.1**
DMSO		246 255 246	**249**	4.2	91 120 136	**116**	18.6	**1.0**	**1.0**
Test Item	31.6 µg	235 199 220	**218**	14.8	121 104 104	**110**	8.0	**0.9**	**0.9**
Test Item	100 µg	243 246 252	**247**	3.7	127 116 113	**119**	6.0	**1.0**	**1.0**
Test Item	316 µg	218 243 254	**238**	15.1	132 126 143	**134**	7.0	**1.0**	**1.2**
Test Item	1000 µg	219 207 226	**217**	7.8	125 133 151	**136**	10.9	**0.9**	**1.2**
Test Item	2500 µg	145 B 142 B 140 B	**142**	2.1	125 127 109	**120**	8.1	**0.6**	**1.0**
Test Item	5000 µg	207 B 201 B 156 B	**188**	22.8	101 85 86	**91**	7.3	**0.8**	**0.8**
MMS	1.0 µL	2031 1966 2025 C	**2007**	29.3	/ / /	**/**	/	**8.1**	**/**
2-AA	10 µg	/ / /	**/**	/	726 796 949	**824**	93.1	**/**	**7.1**

SD: Standard-deviation
P: Precipitation
B: Background lawn reduced
C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

12.1.3. Experiment II *(Pre-incubation Test)*

The results of the bacterial reverse mutation assay conducted according to the pre-incubation test (experiment II) are reported in Table 3.

Table 3: Results Experiment II

Tester Strain: TA98 Experiment: 2

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		46 34 29	**36**	7.1	22 36 23	**27**	6.4	**1.5**	**1.0**
DMSO		33 25 16	**25**	6.9	37 21 22	**27**	7.3	**1.0**	**1.0**
Test Item	31.6 µg	28 26 22	**25**	2.5	26 32 23	**27**	3.7	**1.0**	**1.0**
Test Item	100 µg	25 28 37	**30**	5.1	27 26 22	**25**	2.2	**1.2**	**0.9**
Test Item	316 µg	25 30 22	**26**	3.3	34 25 25	**28**	4.2	**1.0**	**1.1**
Test Item	1000 µg	24 B 17 B 20 B	**20**	2.9	19 23 32	**25**	5.4	**0.8**	**0.9**
Test Item	2500 µg	3 B 0 B 4 B	**2**	1.7	21 B 18 B 17 B	**19**	1.7	**0.1**	**0.7**
Test Item	5000 µg	8 B 0 B 13 B	**7**	5.4	17 B 24 B 26 B	**22**	3.9	**0.3**	**0.8**
4-NOPD	10 µg	458 387 492	**446**	43.7	/ / /	***/***	/	**18.1**	***/***
2-AA	2.5 µg	/ / /	***/***	/	254 298 1791	**781**	714.4	***/***	**29.3**

SD: Standard-deviation P: Precipitation
B: Background lawn reduced C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: TA100 Experiment: 2

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		105 135 132	**124**	13.5	128 136 149	**138**	8.7	**1.3**	**1.1**
DMSO		87 113 76	**92**	15.5	119 145 127	**130**	10.9	**1.0**	**1.0**
Test Item	3.16 µg	72 71 88	**77**	7.8	106 106 101	**104**	2.4	**0.8**	**0.8**
Test Item	10 µg	89 95 102	**95**	5.3	120 117 121	**119**	1.7	**1.0**	**0.9**
Test Item	31.6 µg	56 77 83	**72**	11.6	138 155 106	**133**	20.3	**0.8**	**1.0**
Test Item	100 µg	112 97 80	**96**	13.1	138 137 127	**134**	5.0	**1.0**	**1.0**
Test Item	316 µg	9 B 78 B 32 B	**40**	28.7	150 129 132	**137**	9.3	**0.4**	**1.1**
Test Item	1000 µg	0 B 66 B 0 B	**22**	31.1	123 B 131 B 101 B	**118**	12.7	**0.2**	**0.9**
Test Item	2500 µg	0 N 0 N 0 B	**0**	0.0	0 B 0 B 0 B	**0**	0.0	**0.0**	**0.0**
Test Item	5000 µg	0 N 0 N 0 B	**0**	0.0	0 B 0 B 0 B	**0**	0.0	**0.0**	**0.0**
NaN_3	10 µg	695 828 936	**820**	98.6	/ / /	**/**	/	**8.9**	**/**
2-AA	2.5 µg	/ / /	**/**	/	521 589 504	**538**	36.7	**/**	**4.1**

SD: Standard-deviation P: Precipitation
B: Background lawn reduced C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: TA1535 Experiment: 2

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		5 7 10	**7**	2.1	9 6 5	**7**	1.7	**1.8**	**1.3**
DMSO		3 8 1	**4**	2.9	6 6 3	**5**	1.4	**1.0**	**1.0**
Test Item	3.16 µg	5 2 13	**7**	4.6	/ / /	**/**	/	**1.7**	**/**
Test Item	10 µg	6 10 9	**8**	1.7	/ / /	**/**	/	**2.1**	**/**
Test Item	31.6 µg	6 4 6	**5**	0.9	10 4 8	**7**	2.5	**1.7**	**1.5**
Test Item	100 µg	8 5 6	**6**	1.2	11 5 6	**7**	2.6	**2.1**	**1.5**
Test Item	316 µg	5 B 5 B 0 B	**3**	2.4	2 B 5 B 6 B	**4**	1.7	**1.3**	**0.9**
Test Item	1000 µg	5 B 0 B 0 B	**2**	2.4	0 B 3 B 4 B	**2**	1.7	**1.6**	**0.5**
Test Item	2500 µg	1 N 1 N 0 B	**1**	0.5	5 B 2 B 0 B	**2**	2.1	**0.8**	**0.5**
Test Item	5000 µg	1 N 0 N 2 B	**1**	0.8	0 B 0 B 0 B	**0**	0.0	**0.4**	**0.0**
NaN_3	10 µg	1039 692 1405	**1045**	291.1	/ / /	**/**	/	**261.3**	**/**
2-AA	2.5 µg	/ / /	**/**	/	23 26 49	**33**	11.6	**/**	**6.5**

SD: Standard-deviation P: Precipitation
B: Background lawn reduced C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: TA1537 Experiment: 2

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		23 9 10	**14**	6.4	16 17 15	**16**	0.8	**1.5**	**1.7**
DMSO		10 4 14	**9**	4.1	9 10 10	**10**	0.5	**1.0**	**1.0**
Test Item	31.6 µg	10 6 8	**8**	1.6	12 17 16	**15**	2.2	**0.9**	**1.6**
Test Item	100 µg	9 13 10	**11**	1.7	12 15 22	**16**	4.2	**1.1**	**1.7**
Test Item	316 µg	13 9 4	**9**	3.7	10 13 11	**11**	1.2	**0.9**	**1.2**
Test Item	1000 µg	15 7 10	**11**	3.3	20 13 11	**15**	3.9	**1.1**	**1.5**
Test Item	2500 µg	0 B 3 B 5 B	**3**	2.1	5 B 14 B 10 B	**10**	3.7	**0.3**	**1.0**
Test Item	5000 µg	7 B 2 B 3 B	**4**	2.2	9 B 9 B 13 B	**10**	1.9	**0.4**	**1.1**
4-NOPD	40 µg	94 104 91	**96**	5.6	/ / /	**/**	/	**10.3**	**/**
2-AA	2.5 µg	/ / /	**/**	/	18 30 61	**36**	18.1	**/**	**3.8**

SD: Standard-deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

Tester Strain: WP2 uvrA (pKM101)

Experiment: 2

Treatment	Dose/Plate	Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		254 270 271	**265**	7.8	141 152 169	**154**	11.5	**1.1**	**1.3**
DMSO		253 237 257	**249**	8.6	109 107 132	**116**	11.3	**1.0**	**1.0**
Test Item	31.6 µg	157 212 202	**190**	23.9	84 102 100	**95**	8.1	**0.8**	**0.8**
Test Item	100 µg	242 230 263	**245**	13.6	114 108 111	**111**	2.4	**1.0**	**1.0**
Test Item	316 µg	226 208 204	**213**	9.6	105 108 126	**113**	9.3	**0.9**	**1.0**
Test Item	1000 µg	212 213 217	**214**	2.2	81 107 121	**103**	16.6	**0.9**	**0.9**
Test Item	2500 µg	196 B 179 B 197 B	**191**	8.3	80 83 86	**83**	2.4	**0.8**	**0.7**
Test Item	5000 µg	202 B 188 B 213 B	**201**	10.2	83 88 79	**83**	3.7	**0.8**	**0.7**
MMS	1.0 µL	1009 1284 1646	**1313**	260.9	/ / /	**/**	/	**5.3**	**/**
2-AA	10 µg	/ / /	**/**	/	336 268 503	**369**	98.7	**/**	**3.2**

SD: Standard-deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (vehicle control)}}$$

12.2. Discussion

The test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil) was investigated for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and tester strain *E. coli* WP2 uvrA (pKM101).

In two independent experiments several concentrations of the test item were used. Each assay was conducted **with** and **without** metabolic activation. The concentrations, including the controls, were tested in triplicate. The following concentrations of the test item were prepared and used in the experiments:

Experiment I:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA100)

31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA98, TA1535, TA1537, E. coli WP2 uvrA (pKM101))

Experiment II:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA100, TA1535 [without metabolic activation])

31.6, 100, 316, 1000, 2500 and 5000 µg/plate
(TA98, TA1535 [with metabolic activation], TA1537, E. coli WP2 uvrA (pKM101))

No precipitation of the test item was observed in any tester strain used in experiment I and II (**with** and **without** metabolic activation).

The microbial contamination observed in one plate of the positive control in tester strain E. coli WP2 uvrA (pKM101), (experiment I, **without** metabolic activation) did not affect the quality or integrity of the results as the microbial contamination could be clearly distinguished from the *E. coli* revertants and thus did not affect the evaluation.

In experiment I toxic effects of the test item were observed in tester strains TA98, TA100 and TA1535 (**with** and **without** metabolic activation) and in tester strain *E. coli* WP2 uvrA (pKM101) (**without** metabolic activation) at concentrations of 2500 µg/plate and higher.

In experiment II toxic effects of the test item were noted in each particular tester strain:

- In tester strain TA98, toxic effects of the test item were observed at concentrations of 1000 µg/plate and higher (**without** metabolic activation) and at concentrations of 2500 µg/plate and higher (**with** metabolic activation).
- In tester strain TA100, toxic effects of the test item were observed at concentrations of 316 µg/plate and higher (**without** metabolic activation) and at concentrations of 1000 µg/plate and higher (**with** metabolic activation).
- In tester strain TA1535, toxic effects of the test item were observed at concentrations of 316 µg/plate and higher (**with** and **without** metabolic activation).
- In tester strain TA1537, toxic effects of the test item were observed at concentrations of 2500 µg/plate and higher (**with** and **without** metabolic activation).
- In tester strain *E. coli* WP2 uvrA (pKM101) toxic effects of the test item were observed at concentrations of 2500 µg/plate and higher (**without** metabolic activation).

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil) at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II.

All criteria of validity were met (see section 10.8).

13. Conclusion

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil) did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil) is considered to be non-mutagenic in this bacterial reverse mutation assay.

14. Distribution of the Report

Original:	Eurofins Munich
Copy:	sponsor

15. References

15.1. Guidelines

[1] Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436)

[2] Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis (“Consensus Document of the National and Länder Working Party on Good Laboratory Practice“) on the archiving and storage of records and materials, 5 May 1998.

[3] OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

[4] OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted 21 July 1997, corrected 26 June 2020.

[5] Commission Regulation (EC) No. 440/2008 B.13/14: "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008.

[6] EPA Health Effects Test Guidelines, OCSPP 870.5100 "Bacterial Reverse Mutation Test" EPA 712-C-98-247, August 1998.

15.2. Literature

[7] Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. (1973), Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection, Proc. Natl. Acad. Sci. (USA) 70, 2281-2285

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[17] Zeiger, E., Anderson, B., Haworth, S., Lawlor, T. and Mortelmans, K. (1992). Salmonella mutagenicity tests V. Results from the testing of 311 Chemicals. Environ. Mol. Mutagen. 19 (Suppl. 21), 2-141

15.3. Internal Eurofins Munich SOPs

Standard Operating Procedures (SOPs), No. 15-1-1, No. 15-1-2, No. 15-2-2, No. 15-2-3, No. 4-6-6, No. 4-6-7

16. Appendix

16.1. Appendix 1: Historical Laboratory Control Data

Table 4: Historical Laboratory Control Data of the Negative Control without S9 (-S9)

(Data from January – December 2020 for all tester strains)

	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)
Mean	39.0	106.4	11.0	18.1	204.0
SD	9.1	14.9	4.1	5.4	46.4
Min	13	55	5	7	108
Max	71	155	34	32	327
RSD [%]	23.3	14.0	37.4	29.6	22.7
n	297	297	281	281	267

S9: metabolic activation
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table 5: Historical Laboratory Control Data of the Positive Control without S9 (-S9)

(Data from January – December 2020 for all tester strains)

	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)
Substance	4-NOPD	NaN_3	NaN_3	4-NOPD	MMS
Conc./plate	10 µg	10 µg	10 µg	40 µg	1 µL
Mean	508.6	554.9	1033.3	94.7	1640.1
SD	158.3	142.2	248.4	22.6	574.4
Min	85	220	408	23	239
Max	1069	923	1936	181	4123
RSD [%]	31.1	25.6	24.0	23.8	35.0
n	297	297	281	280	268

S9: metabolic activation
Conc.: concentration
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table 6: Historical Laboratory Control Data of the Negative Control with S9 (+S9)

(Data from January – December 2020 for all tester strains)

	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)
Mean	41.1	104.0	11.6	21.2	232.4
SD	7.5	14.5	3.9	6.5	46.0
Min	26	53	4	7	122
Max	68	176	37	46	355
RSD [%]	18.3	14.0	34.0	30.7	19.8
n	297	297	281	281	267

S9: metabolic activation
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table 7: Historical Laboratory Control Data of the Positive Control with S9 (+S9)

(Data from January – December 2020 for all tester strains)

	TA98	TA100	TA1535	TA1537	WP2 uvrA (pKM101)
Substance	2-AA	2-AA	2-AA	2-AA	2-AA
Conc./plate	2.5 µg	2.5 µg	2.5 µg	2.5 µg	10 µg
Mean	1515.9	1027.0	139.0	150.0	804.6
SD	550.3	400.0	57.2	71.8	268.8
Min	212	148	23	25	372
Max	3061	2251	314	826	3272
RSD [%]	36.3	38.9	41.1	47.8	33.4
n	297	297	281	280	268

S9: metabolic activation
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

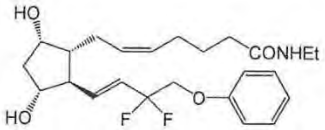
16.2. Appendix 2: Certificate of Analysis

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{33}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications.

Quality Control: [redacted] *Date:* 23NOV 2022

Approved: [redacted] *Date:* 23 Nov 2022

Quality Control Laboratory: [redacted]

Qualitätssicherung
11. Jan. 2023
Kürzel: BTE

16.3. Appendix 3: Quality Control & Production Certificate S9-Homogenate

TRINOVABIOCHEM

MOLTOX®
Molecular Toxicology, Inc.

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information	**Part Number Information**	**PREP:** October 18, 2022
SPECIES: Rat	**LOT NO.:** 4654	**EXPIRY:** October 18, 2024
STRAIN: Sprague Dawley	**PART NO.:** 11-105	**INDUCING AGENT:**
SEX: Male	**VOLUME:** 5 mL	Phenobarbital-5,6
AGE: 5 – 6 weeks	**BUFFER:** 0.15 M KCl	Benzoflavone
WEIGHT: 175 – 199 g	**STORAGE:** At or below -70°C	
TISSUE: Liver		

REFERENCE: Matsushima, et al., *In Vitro Metabolic Activation in Mutagenesis Testing* (F.J. de Serres, ed.), Elsevier, 1976, p 85 **For Research Purposes Only**

BIOCHEMISTRY:

- PROTEIN: 36.9 mg/ml — Assayed according to the method of Lowry et al., *JBC* **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
BROD	2B1, 2B2	88.3
EROD	1A1, 1A2	181.5
MROD	1A1, 1A2	28.4
PROD	2B1, 2B2	45.2

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & PROD, MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/ mg protein) were 38.6, 24.7, 12.9, & 13.7 for BROD, EROD, MROD and PROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microorganisms by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants

TA98	TA1535
48.4	558

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**: 299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 – 10% in S9 mix, were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to metabolites mutagenic to TA100. Assays were conducted as described by Maron & Ames, (*Mutat Res* **113**: 173, 1983.)

	µl S9 per plate/number *his*$^+$ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	91	172	196	269	397	493
AA (2.5 µg)	122	446	653	1569	1716	2173

Approved: 10/24/2022

MOLECULAR TOXICOLOGY, INC. www.moltox.com (828) 264-9099

TRINOVA BIOCHEM GmbH
Rathenaustr. 2
35394 Giessen
GERMANY
fon: +49 641 94390-0
fax: +49 641 94390-22
info@trinova.de
www.trinova.de

eurofins | BioPharma Product Testing

Quality Control & Production Certificate
S9-Homogenate (Eurofins Charge No. 251122)

Species, Strain, Sex, Tissue:	rat, Wistar, male, liver
Supplier:	Charles River
Weight at Delivery:	approx. 200-250 g
Inducing Agents:	Phenobarbital (Na-Salt) [Sigma, SLCD9096; 80 mg/kg bw β-Naphthoflavone [Sigma, SLCF5489]; 100 mg/kg bw
Vehicle:	Cotton Seed Oil [Sigma, MKCR3879]
Application:	per oral route on 3 consecutive days
Date of Preparation:	25 November 2022
Expiry Date:	25 November 2024
Determination of Protein Content:	BCA-Assay (BSA-calibration curve), 12 January 2023
Protein Content:	39.0 mg/mL
Sterility Test:	28 November 2022 / passed

Metabolic Activation Tests:
a) in Bacteria: Ames Test (2.5 µg 2-AA/plate):

Volume S9-	TA 98			TA 100		
Homogenate [µL]	Revertants	Range[a] (Min - Max)	passed/ failed	Revertants	Range[a] (Min - Max)	passed/ failed
0	43 47 30	15 - 140	passed passed passed	100 120 131	83 - 335	passed passed passed
20	1425 1749 1517	338 - 3065	passed passed passed	2358 2327 2824	994 - 2814	passed passed passed[b]
50	1015 1805 1627	526 - 3252	passed passed passed	2790 1773 1828	856 - 2311	passed[b] passed passed

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D 82152 Planegg/Munich
Germany
Tel. +49 (0)89 899 650-0
Fax +49 (0)89 899 650-11

GLP/GMP FDA approved

b) in Bacteria: Ames Test (5 µg B[*a*]P/plate):

Volume S9-Homogenate [µL]	TA 98 Revertants	TA 98 Range[a] (Min - Max)	TA 98 passed/failed	TA 100 Revertants	TA 100 Range[a] (Min - Max)	TA 100 passed/failed
0	36 47 30	10 - 62	passed passed passed	93 98 86	40 - 160	passed passed passed
20	117 139 127	69 - 201	passed passed passed	241 254 271	153 - 479	passed passed passed
50	143 188 155	83 - 264	passed passed passed	367 349 315	211 - 711	passed passed passed

a: Range of historical control data from 2011 – June 2022 for Ames Test.
b: Considered as acceptable for inclusion in the historical control database.

C. Freitag

Dr. Christine Freitag
Deputy Head of *in vitro* Pharmacology/Toxicology

16.4. Appendix 4: Final Study Plan

eurofins | BioPharma Product Testing

Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)

Study Plan

Version: Final

Eurofins Munich Study No.: STUGC22AA2158-2

Sponsor:

[redacted]

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany

Tel | +49 (0)89 899 650-0
Fax | +49 (0)89 899 650-11

GLP / GMP FDA approved

1. Project Staff Signatures

Member of the
Quality Assurance Unit

F.B

Print Name: Felix Bunert

Date: 11 JAN 2023

Study Director

Kathleen Burns

K Burns

Date: 16 January 2023

2. Contents

3. Preface

3.1. Abbreviations

2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
A. dest.	Aqua destillata (*purified water*)
Art.	Artikel *(article)*
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
bio	biotin
cf.	confer
chl	"Chlorate resistant" *E. coli* mutants, lack a functional nitrate reductase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EC	European Commission
e.g.	exempli gratia *(for example)*
EPA	Environmental Protection Agency
Eurofins Munich	Eurofins BioPharma Product Testing Munich GmbH
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
his	histidine
mg/kg bw	milligram/kilogram body weight
MMS	methylmethanesulfonate
NADP	nicotinamide adenine dinucleotide phosphate
OECD	Organisation for Economic Cooperation and Development
OCSPP	Office of Chemical Safety and Pollution Prevention
QAU	Quality Assurance Unit
rfa	deep rough factor
RSD	relative standard deviation
S9	microsomal fraction of rat liver homogenate
SD	standard deviation
SOPs	Standard Operating Procedures
uvrB	repair mutant, UV light sensitive

3.2. General

Sponsor:	[redacted]
Study Monitors:	Dr. Thomas Petry (technical matters) ToxMinds Avenue de Broqueville 116 1200 Brussels - Belgium Thomas.Petry@toxminds.com
	[redacted]
	Alexis Klock Eurofins Product Testing US Inc 11822 North Creek Pkwy Suite 110 Bothell, WA 98011
Test Facility:	Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany
Eurofins Munich Study No.:	STUGC22AA2158-2
Test Item:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)

3.3. Project Staff

Study Director:	Kathleen Burns
Team Leader Operational QA GLP/GCP/ISO:	Uwe Hamann

3.4. Schedule

Arrival of the Test Item:	05 December 2022
Study Initiation Date:	Date of the study director's signature
Proposed Experimental Starting Date:	January 2023
Proposed Experimental Completion Date:	January / February 2023
Proposed Study Completion Date and Draft Report:	February 2023

4. Quality Assurance

4.1. GLP Compliance

This study will be conducted to comply with:

Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436) [1],

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998 [3].

The OECD Principles of Good Laboratory Practice are accepted by regulatory authorities throughout the European Community, USA and Japan.

This study will be assessed for compliance with the study plan and the Standard Operating Procedures of Eurofins Munich The study and/or the test facility are inspected periodically by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

4.2. Guidelines

This study will follow the procedures indicated by internal Eurofins Munich SOPs and the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted 21 July 1997, corrected 26 June 2020 [4].

Commission Regulation (EC) No. 440/2008 B.13/14: "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008 [5].

EPA Health Effects Test Guidelines, OCSPP 870.5100 "Bacterial Reverse Mutation Test" EPA 712-C-98-247, August 1998 [6].

4.3. Amendment Procedures

This study plan can be amended at the discretion of the study director and in consultation with the Study Monitor. Changes or revisions of this study plan will be documented for stated reasons, signed and dated by the study director. All such amendments will be sent to the sponsor for approval and will be retained with the original study plan.

4.4. Deviation Procedures

Unplanned changes to the study plan and/or SOPs will be documented in the raw data. The chapter "Deviations from the Study Plan" in the report will reflect every deviation and its anticipated effect on the outcome of the study.

4.5. Archiving

For a period of 15 years (or shorter if in compliance with the GLP regulations) Eurofins Munich will store the records, materials and specimens in their scientific archives according to the GLP regulations.

The following records have to be stored according to the GLP regulations:

The final report, the study plan and documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study. Any document relating to the study will be discarded only with the prior consent of the sponsor.

The following materials and samples have to be stored according to the period of time specified in the GLP regulations:

A retained sample of the test item will be archived according to the GLP regulations, if possible, and will be discarded without the sponsor's prior consent.

Other materials and specimens have to be stored according to the GLP regulations and disposed of after the respective archiving period with the sponsor's prior consent.

Unless otherwise agreed in writing, the remaining test item will be discarded three months after the release of the report.

5. Introduction

5.1. Aim of the Study

Bacterial reverse mutation assays use amino acid requiring strains of *Salmonella typhimurium (S. typhimurium)* and *Escherichia coli (E. coli)* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of these bacterial reversion assays is that they detect mutations which functionally reverse mutations present in the tester strains and restore the capability to synthesize an essential amino acid [7], [9], [12].

The purpose of this study is to establish the potential of the test item to induce gene mutations in bacteria by means of a *S. typhimurium* and *E. coli* reverse mutation assay. There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentrations spacing and / or the method of treatment (pre-incubation method). In case of severe toxicity of the test item or the use of e.g. ethanol, acetone or tetrahydrofuran as the most appropriate solvent, the confirmatory experiment is carried out according to the plate incorporation method with a different spacing between dose levels.

The *S. typhimurium* histidine (his) reversion system and the *E. coli* tryptophan (trp) reversion systems measure $his^- \rightarrow his^+$ reversions and $trp^- \rightarrow trp^+$. The *S. typhimurium* strains are constructed to differentiate between base pair (TA100, TA1535) and frameshift (TA98, TA1537) mutations [12]. The *E. coli* strain detects only base substitution mutagens.

These assays directly measure heritable DNA mutations of a type which is associated with adverse effects [13], [14], [16], [17]. Point mutations are the cause of many human genetic diseases and there is substantial evidence that somatic cell point mutations in oncogenes and tumor suppressor genes are involved in cancer in humans and experimental systems [8].

The tester strains have several features that make them more sensitive for the detection of mutations. The specificity of the strains can provide useful information on the types of mutations that are induced by mutagenic agents.

According to the direct plate incorporation or the pre-incubation method the bacteria are exposed to the test item with and without metabolic activation and plated on selective medium. After a suitable period of incubation, revertant colonies are counted [12].

At least five different concentrations of the test item are tested with approximately half log (i.e. $\sqrt{10}$) intervals between test points for an initial test. Narrower spacing between dose levels may be appropriate when a dose response is investigated. For soluble, non-toxic test compounds the recommended maximum test concentration is 5 mg/plate or 5 µL/plate.

To validate the test, reference mutagens are tested in parallel to the test item [10].

5.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals, Section 4, No. 471 – Bacterial Reverse Mutation Test - recommends using a combination of *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101).

6. Materials and Methods

6.1. Characterisation of the Test Item

The identity of the test item was inspected upon delivery at the test facility (e.g. test item name, batch no. and additional data were compared with the label) based on the following specifications provided by the sponsor. The following listed information applies to the sample as received.

Name:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
CAS No.:	1185851-52-8
EC No.:	867-521-0
Batch No.:	TAF-10-1122-01
Molecular Weight:	437.52 g/mol
Physical State:	liquid, oil
Colour:	colourless to pale yellow
Purity:	99.78%
Expiry Date:	23 November 2026
Storage Conditions:	2-8°C in a well-closed container
Safety Precautions:	The routine hygienic procedures will be sufficient to assure personnel health and safety.

If the sponsor provides any additional specifications concerning the test item these will be added in the report and will not require an amendment to the study plan.

6.2. Preparation of the Test Item

The test item is prepared in an appropriate solvent (e.g., A. dest, DMSO, ethanol, acetone or THF) and diluted freshly prior to each treatment. The solvent is compatible with the survival of the bacteria and the S9 activity. It is recommended that wherever possible, the use of an aqueous solvent is considered first. If necessary, the test item will be processed by ultrasound.

6.3. Controls

Solvent and/or negative as well as positive controls are included in each experiment. Strain specific positive controls are included in the assay, which demonstrate the effective performance of the test.

Negative/Solvent Controls

Negative controls (A. dest.) are treated in the same way as all dose groups. If A. dest. is used as appropriate solvent no additional solvent control is necessary. In case A. dest. is not used, extra solvent controls are added.

Positive Controls

Without metabolic activation

Tester Strains:	*S. typhimurium*: TA100, TA1535
Name:	NaN_3; sodium azide
CAS No.:	26628-22-8
Supplier:	Sigma
Batch No.:	will be added in the report
Dissolved in:	A. dest.
Concentration:	10 µg/plate
Tester Strains:	*S. typhimurium*: TA98, TA1537
Name:	4-NOPD; 4-nitro-o-phenylene-diamine
CAS No.:	99-56-9
Supplier:	Sigma
Batch No.:	will be added in the report
Dissolved in:	DMSO
Concentration:	10 µg/plate for TA98, 40 µg/plate for TA1537
Tester Strain:	*E. coli* WP2 uvrA (pKM101)
Name:	MMS; methylmethanesulfonate
CAS No.:	66-27-3
Supplier:	Sigma
Batch No.:	will be added in the report
Dissolved in:	A. dest.
Concentration:	1 µL/plate

With metabolic activation

Tester Strains:	*S. typhimurium*: TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA (pKM101)
Name:	2-AA; 2-aminoanthracene
CAS No.:	613-13-8
Supplier:	Alfa Aesar
Batch No.:	will be added in the report
Dissolved in:	DMSO
Concentrations:	2.5 µg/plate; 10 µg/plate for *E. coli* WP2 uvrA (pKM101)

The stability of the positive control substances in solution is unknown but a mutagenic response in the expected range is sufficient evidence of biological stability.

6.4. Test System

6.4.1. Bacteria

Four strains of *S. typhimurium* and one strain of *E. coli* with the following characteristics are used:

TA98: his D 3052; *rfa*⁻; *uvrB*⁻; R-factor:	frame shift mutations
TA100: his G 46; *rfa*⁻; *uvrB*⁻; R-factor:	base-pair substitutions
TA1535: his G 46; *rfa*⁻; *uvrB*⁻:	base-pair substitutions
TA1537: his C 3076; *rfa*⁻; *uvrB*⁻:	frame shift mutations
E. coli: WP2 uvrA (pKM101): *trp*⁻; *uvrA*⁻:	base-pair substitutions

Tester strains TA98, TA1535 and *E. coli* are obtained from MOLTOX, INC., NC 28607, USA. Tester strains TA100 and TA1537 are obtained from Xenometrix AG, Switzerland. They are stored as stock cultures in ampoules with nutrient broth (OXOID) supplemented with DMSO (approx. 8% v/v) over liquid nitrogen.

All *Salmonella* strains contain mutations in the histidine operon, thereby imposing a requirement for histidine in the growth medium. They contain the deep rough (*rfa*) mutation, which deletes the polysaccharide side chain of the lipopolysaccharides of the bacterial cell surface. This increases cell permeability of larger substances. The other mutation is a deletion of the *uvrB* gene coding for a protein of the DNA nucleotide excision repair system resulting in an increased sensitivity in detecting many mutagens. This deletion also includes the nitrate reductase (*chl*) and biotin (*bio*) genes (bacteria require biotin for growth).

The tester strains TA98, TA100 and *E. coli* contain the R-factor plasmid, pkM101. These strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains. pkM101 increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in these organisms [12], [15].

The tester strain *E. coli* WP2 uvrA (pKM101) carries the defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent mutants (revertants) can arise either by a base change at the site of the original alteration or by a base change elsewhere in the chromosome so that the original defect is suppressed. This second possibility can occur in several different ways so that the system seems capable of detecting all types of mutagens which substitute one base for another. Additionally, the strain is deficient in the DNA nucleotide excision repair system.

The properties of the *S. typhimurium* and *E. coli* strains with regard to membrane permeability, ampicillin- and tetracycline-resistance as well as normal spontaneous mutation rates are checked during the preparation of the mother and working stocks according to Ames *et al.* [7]. In this way it is ensured that the experimental conditions set up by Ames are fulfilled.

6.4.2. Preparation of Bacteria

Samples of each tester strain are grown by culturing for 12 h at 37 °C in *S. typhimurium* medium (Nutrient Broth) and *E. coli* medium (Lysogeny broth), respectively, to the late exponential or early stationary phase of growth (approx. 10^9 cells/mL).

The *S. typhimurium* medium (Nutrient Broth) contains per litre of purified water:

8 g	Nutrient Broth
5 g	NaCl

The *E. coli* medium (Lysogeny broth) contains per litre of purified water:

10	g	tryptone
10	g	NaCl
5	g	yeast extract

A solution of 125 µL ampicillin (10 mg/mL) (TA98, TA100, *E. coli* WP2 uvrA (pKM101)) is added in order to retain the phenotypic characteristics of the strain.

6.4.3. Agar Plates

The Vogel-Bonner Medium E agar plates with 2% glucose used in the Ames Test are prepared by Eurofins Munich or provided by an appropriate supplier. Quality controls are performed.

Vogel-Bonner-salts contain per litre of purified water:

10	g	$MgSO_4$ x 7 H_2O
100	g	citric acid
175	g	$NaNH_4HPO_4$ x 4 H_2O
500	g	K_2HPO_4

Sterilisation is performed for 20 min at 121 °C in an autoclave.

Vogel-Bonner Medium E agar plates contain per litre of purified water:

15	g	Agar Agar
20	mL	Vogel-Bonner salts
50	mL	glucose solution (40%)

Sterilisation is performed for 20 min at 121 °C in an autoclave.

6.4.4. Overlay Agar

The overlay agar contains per litre of purified water:

S. typhimurium:

7.0	g	Agar Agar
6.0	g	NaCl
10.5	mg	L-histidine x HCl x H_2O
12.2	mg	biotin

E. coli:

7.0	g	Agar Agar
6.0	g	NaCl
10.2	mg	tryptophan

Sterilisation is performed for 20 min at 121 °C in an autoclave.

6.4.5. Mammalian Microsomal Fraction S9 Mix

The bacteria most commonly used in these assays do not possess the enzyme system which, in mammals, is known to convert promutagens into active DNA damaging metabolites. In order to overcome this major drawback an exogenous metabolic system is added in form of mammalian microsome enzyme activation mixture.

6.4.6. S9 Homogenate

The S9 liver microsomal fraction is prepared at Eurofins Munich or obtained from Trinova Biochem GmbH, Gießen, Germany. Male Wistar rats are induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route (Eurofins) or male Sprague Dawley rats are induced with phenobarbital/ β-naphthoflavone (Trinova).

The following quality control determinations are performed by Eurofins Munich:

a) Biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene
b) Sterility Test

The following quality control determinations are performed by Trinova Biochem GmbH:

a) Alkoxyresorufin-O-dealkylase activities
b) Test for the presence of adventitious agents
c) Promutagen activation (including biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene)

A stock of the supernatant containing the microsomes is frozen in aliquots between 2 and 5 mL and stored at ≤ -75 °C.

The protein concentration in the S9 preparation is usually between 20 and 45 mg/mL.

6.4.7. Preparation of S9 Mix

The S9 mix preparation is performed according to Ames *et al.* [7].

100 mM sodium-ortho-phosphate-buffer, pH 7.4, is ice-cold added to the following pre-weighed sterilised reagents to give final concentrations in the S9 mix of:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	glucose-6-phosphate
4	mM	NADP

This solution is mixed with the liver 9000 x g supernatant fluid in the following proportion:

co-factor solution	9.5 parts
liver preparation	0.5 parts

During the experiment the S9 mix is stored on ice.

6.4.8. S9 Mix Substitution Buffer

The S9 mix substitution buffer is used in the study as a replacement for S9 mix, without metabolic activation (-S9).

Phosphate-buffer (0.2 M) contains per litre of purified water:

0.2 M $NaH_2PO_4 \times H_2O$	120 mL
0.2 M Na_2HPO_4	880 mL

The two solutions are mixed and the pH is adjusted to 7.4. Sterilisation is performed for 20 min at 121 °C in an autoclave.

This 0.2 M phosphate-buffer is mixed with 0.15 M KCl solution (sterile) in the following proportion:

0.2 M phosphate-buffer	9.5 parts
0.15 M KCl solution	0.5 parts

This S9 mix substitution buffer is stored at 4 °C.

6.5. Experimental Design

6.5.1. Pre-Experiment for Toxicity

The toxicity of the test item is determined with tester strains TA98 and TA100 in a pre-experiment. Eight concentrations (3.16, 10, 31.6, 100, 316, 1000, 2500, 5000 µg/plate) are tested for toxicity and mutation induction with each three plates. The experimental conditions in this pre-experiment are the same as described below for the main experiment I (plate incorporation test).

In this study the highest concentration tested will be 5 µL/plate, respectively 5 mg/plate, if it was found to be too viscous to handle.

Toxicity may be detected by a clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control.

6.5.2. Exposure Concentrations

At least five concentrations with and without metabolic activation will be tested.

The concentrations to be applied in the main experiments are chosen according to the results of the pre-experiment.

In case the results of the pre-experiment are in accordance with the criteria of validity, these are reported as a part of the main experiment I.

For soluble, non-toxic test compounds the recommended maximum test concentration is 5 mg/plate or 5 µL/plate. For test compounds that are not soluble at 5 mg/plate or 5 µL/plate and that are not toxic at levels lower than an insoluble level, the highest dose tested is at least one insoluble concentration in the final treatment mixture under the actual conditions of the test at the start of the experiment. Alternatively, if it is demonstrated that toxicity occurs only at higher than the lowest insoluble concentration and in addition, there are positive responses in other mutagenicity test systems then the highest dose is based on toxicity irrespective of solubility.

Testing above the concentrations indicated has to be considered in case of substantial or potentially mutagenic impurities. At least five different concentrations of the test item are tested with approximately half log intervals between test points for an initial test. Narrower spacing between dose levels is appropriate when a dose response is investigated.

6.5.3. Experimental Performance

For the plate incorporation method, the following materials are mixed in a test tube and poured over the surface of a minimal agar plate:

- 100 µL test solution at each dose level, solvent control, negative control or reference mutagen solution (positive control),
- 500 µL S9 mix (for testing with metabolic activation) or S9 mix substitution buffer (for testing without metabolic activation),
- 100 µL bacteria suspension (cf. Preparation of Bacteria, pre-culture of the strain),
- 2000 µL overlay agar.

For the pre-incubation method 100 µL of the test item-preparation is pre-incubated with the tester strains (100 µL) and sterile buffer or the metabolic activation system (500 µL) for 60 min at 37 °C prior to adding the overlay agar (2000 µL) and pouring onto the surface of a minimal agar plate.

The exact method or methods used will be specified in the report.

For each strain and dose level, including the controls, a minimum of three plates is used.

After solidification the plates are inverted and incubated at 37 °C for at least 48 h in the dark.

Experiment I

Experiment I is carried out according to the plate incorporation method.

Experiment II

There is no requirement for verification of a clear positive response. If negative or equivocal results are obtained, they should be confirmed using a modification of experimental conditions. Study parameters that might be modified include the concentrations spacing and / or the method of treatment (pre-incubation method).

6.6. Data Recording

The colonies are counted using a Sorcerer Colony Counter (Perceptive Instruments). If precipitation of the test item precludes automatic counting the revertant colonies are counted by hand. In addition, tester strains with a low spontaneous mutation frequency like TA1535 and TA1537 may be counted manually.

6.7. Evaluation of Cytotoxicity

Cytotoxicity can be detected by a clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control.

6.8. Criteria of Validity

A test is considered acceptable if for each strain:

- the bacteria demonstrate their typical responses to ampicillin (TA98, TA100, *E. coli* WP2 uvrA (pKM101))
- the control plates (e. g. DMSO, A. dest.) with and without S9 mix are within the historical control data range (mean values of the spontaneous reversion frequency)
- corresponding background growth on solvent control, negative control and test plates is observed
- the positive controls show a distinct enhancement of revertant rates over the control plate
- at least five different concentrations of each tester strain are analysable

6.9. Evaluation of Mutagenicity

The mutation factor is calculated by dividing the mean value of the revertant counts by the mean values of the solvent control (the exact and not the rounded values are used for calculation).

A test item is considered as mutagenic if:

- a clear and dose-related increase in the number of revertants occurs and/or
- a biologically relevant positive response for at least one of the dose groups occurs

in at least one tester strain with or without metabolic activation.

A biologically relevant increase is described as follows:

- if in tester strains TA98, TA100 and *E. coli* the number of reversions is at least twice as high
- if in tester strains TA1535 and TA1537 the number of reversions is at least three times higher

as compared to the reversion rate of the solvent control [11].

According to the OECD guidelines, the biological relevance of the results is the criterion for the interpretation of results, a statistical evaluation of the results is not regarded as necessary. A test item producing neither a dose related increase in the number of revertants nor a reproducible biologically relevant positive response at any of the dose groups is considered to be non-mutagenic in this system.

7. Reporting

The results of the study will be reported in a detailed report. The report will include the following:

- a copy of the GLP certificate of the test facility
- the name and address of the sponsor, the study monitor and the test facility
- the study schedule
- the names of the study director, other scientists and supervisory personnel involved in the study
- the dates of all study plan amendments
- a list of all deviations from the study plan
- the Quality Assurance Statement, signed by the Quality Assurance Unit
- the statement of compliance, signed by the study director
- information about the storage locations of the final report and related documents, specimens and samples
- the test item identification, either by name or by code number
- the preparation of the test item
- the concentration, purity, stability, composition and other appropriate characteristics of the test item, if data are provided by the sponsor
- a description of the test system, including the biological materials used
- a detailed description of application and treatment, dose levels of the test item, toxicity data, negative and positive controls, historical laboratory data
- data in tabular form
- a description of the methods with references
- a description, discussion and interpretation of all results
- a copy of the study plan
- a copy of the certificate of analysis
- a copy of the quality control and production certificate of the S9-Homogenate.

8. Distribution of the Study Plan

Original:	Eurofins Munich
Copy:	sponsor, study director, QAU

9. References

9.1. Guidelines

[1] Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436)

[2] Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998.

[3] OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

[4] OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted 21 July 1997, corrected 26 June 2020.

[5] Commission Regulation (EC) No. 440/2008 B.13/14: "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008.

[6] EPA Health Effects Test Guidelines, OCSPP 870.5100 "Bacterial Reverse Mutation Test" EPA 712-C-98-247, August 1998.

9.2. Literature

[7] Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. (1973). Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. (USA) 70, 2281-2285

[8] Ames, B.N., McCann, J. and Yamasaki, E. (1977). Methods of detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. In: B.J. Kilbey et al. (Eds.) "Handbook of Mutagenicity Test Procedures" Elsevier, Amsterdam, 1-17

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[11] Kier, L.E., Brusick, D.J., Auletta, A.E., von Halle, E.S., Brown, M.M., Simmon, V.F., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T.K. and Ray, V. (1986). The *Salmonella typhimurium*/mammalian microsomal assay. A report of the U.S. Environmental Protection Agency Gene-Tox-Program. Mutat. Res. 168, 69-240

[12] Maron, D.E. and Ames, B.N. (1983). Revised methods for the Salmonella mutagenicity test. Mutat. Res. 113, 173-215.

[13] McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 Chemicals. Proc. Natl. Acad. Sci. (USA) 72, 5135-5139

[14] McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 Chemicals: Discussion. Proc. Natl. Acad. Sci. (USA) 73, 950-954

[15] Mortelmans, K. and Zeiger, E. (2000). The Ames Salmonella/microsome mutagenicity assay. Mutat. Res. 455, 29-60

[16] Zeiger, E., Haseman, J., Shelby, M., Margolin, B. and Tennant, R. (1988). Salmonella mutagenicity tests IV. Results from testing of 300 Chemicals. Environ. Mol. Mutagen. 11, 1-158

[17] Zeiger, E., Anderson, B., Haworth, S., Lawlor, T. and Mortelmans, K. (1992). Salmonella mutagenicity tests V. Results from the testing of 311 Chemicals. Environ. Mol.Mutagen. 19 (Suppl. 21), 2-141

9.3. Internal Eurofins Munich SOPs

Standard Operating Procedures (SOPs), No. 15-1-1, No. 15-1-2, No. 15-2-2, No. 15-2-3, No. 4-6-6, No. 4-6-7

Test Report No. 202301138
Report Date September 8, 2023

Client

[redacted]

Approval Date	October 7, 2022	**Delivery Conditions**	Satisfactory, samples tested as received
Date of Receipt	October 5, 2022	**Testing Date Range**	01-30-2023 to 07-20-2023
Test Request Form #	TRF221010265		

Eurofins ID	Sample Name	Lot/Batch Number	Supplier/Manufacturer	Country of Origin
202301138-1	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide	214	N/A	US

The following test item(s) was/were performed on submitted sample(s) and/or component(s) confirmed by applicant

TEST REQUESTED	RESULT
Micronucleus Test in Mammalian Cells (in vitro) - human lymphocytes - OECD 487 MNT, GLP	See Attachment

Analysis completed by Eurofins Subcontract Laboratory

Signed for and on behalf of
Eurofins MTS Consumer Product Testing US, Inc.

Alexis Klock / Project Coordinator

09-08-2023

Eurofins MTS Consumer Product Testing US, Inc.
11822 North Creek Parkway N, Suite 110 Bothell WA 98011
Phone: (425) 686-3575/Fax: (425) 686-3096 Web: www.EurofinsUS.com

eurofins | BioPharma Product Testing

In vitro Mammalian Micronucleus Assay in Human Lymphocytes with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)

Report

Version: Final

Eurofins Munich Study No.: STUGC22AA2158-3

Sponsor:

[redacted]

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany

Tel | +49 (0)89 899 650-0
Fax | +49 (0)89 899 650-11

GLP / GMP FDA approved

1. Copy of the GLP Certificate

Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance

(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Eurofins BioPharma Product Testing Munich GmbH
Behringstraße 6/8
82152 Planegg
(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

Prüfkategorie 2: Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**Category 2:** toxicity studies
Prüfkategorie 3: Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**Category 3:** mutagenicity studies
Prüfkategorie 8: Analytische Prüfungen an biologischen Materialien	**Category 8:** analytical and clinical chemistry testing
Prüfkategorie 9: Sonstige Prüfungen: *biologische und mikrobiologische Sicherheitsprüfungen an Medi-zinprodukten und Arzneimitteln; Auftragsarchivierung*	**Category 9:** other tests: *biological and microbiological safety evaluation on medical devices and pharmaceuticals; contract archiving*

Datum der Inspektion/Date of Inspection:
(Tag.Monat.Jahr/day.month.year)

29.06.2021

Die/Der genannte Prüfeinrichtung/ Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.
Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/ diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP

Datum, Unterschrift/Date, Signature:

Schwabach, 14.02.2022

Dr. Joachim Strobel
Leiter der GLP-Leitstelle Bayern/
Head of the GLP Monitoring Authority

(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

GLP-Leitstelle Bayern, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit,
Rathausgasse 4, 91126 Schwabach
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority)

2. Contents

3. List of Tables and Figures

3.1. List of Tables

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4. Preface

4.1. Abbreviations

Art.	Artikel *(article)*
ATCC	American Type Culture Collection
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
CBPI	cytokinesis block proliferation index
CPA	cyclophosphamide
DNA	desoxyribonucleic acid
e.g.	exempli gratia *(for example)*
EC	European Commission
EPA	Environmental Protection Agency
Eurofins Munich	Eurofins BioPharma Product Testing Munich GmbH
FBS	fetal bovine serum
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
i.e.	id est *(that is)*
KCl	potassium chloride
MMS	methylmethanesulfonate
MNvit	*in vitro* micronuclei
NADP	nicotinamide adenine di-phosphate
No	number
OECD	Organisation for Economic Cooperation and Development
PBS	phosphate buffered saline
PHA	phytohemagglutinin
QA	Quality Assurance
QAU	Quality Assurance Unit
RPMI	Roswell Park Memorial Institute medium
S9	microsomal fraction of rat liver homogenate
SOPs	Standard Operating Procedures
v/v	volume per volume

4.2. General

Sponsor:	[redacted]
Study Monitors:	Dr. Thomas Petry (technical matters) ToxMinds Avenue de Broqueville 116 1200 Brussels - Belgium Thomas.Petry@toxminds.com
	[redacted]
	Alexis Klock Eurofins Product Testing US Inc 11822 North Creek Pkwy Suite 110 Bothell, WA 98011
Test Facility:	Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany
Eurofins Munich Study No.:	STUGC22AA2158-3
Test Item:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Title:	*In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)

4.3. Project Staff

Study Director:	Dr. Stephanie Lacmanski
Team Leader Operational QA GLP/GCP/ISO:	Uwe Hamann

4.4. Schedule

Arrival of the Test Item:	05 December 2022
Study Initiation Date:	30 January 2023
Date of 1st Amendment to Study Plan:	16 February 2023
Date of 2nd Amendment to Study Plan:	25 August 2023
Experimental Starting Date:	31 January 2023
Experimental Completion Date:	20 July 2023
Study Completion Date:	Date of the study director's signature

5. Quality Assurance

5.1. GLP Compliance

This study was conducted to comply with:

Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998 [3].

The OECD Principles of Good Laboratory Practice are accepted by regulatory authorities throughout the European Community, USA and Japan.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of Eurofins Munich. The study and/or the test facility are inspected periodically by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

5.2. Guidelines

This study followed the procedures indicated by internal Eurofins Munich SOPs and the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, Section 4, No. 487, "*In Vitro* Mammalian Cell Micronucleus Test", adopted 29 July, 2016 [4].

Commission regulation (EU) 2017/735 B.49 "*In Vitro* Mammalian Cell Micronucleus Test", dated February 14, 2017 [5]

5.3. Archiving

For a period of 15 years (or shorter if in compliance with the GLP regulations) Eurofins Munich will store the records, materials and specimens in their scientific archives according to the GLP regulations.

The following records have to be stored according to the GLP regulations:

The final report, the study plan and documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study. Any document relating to the study will be discarded only with the prior consent of the sponsor.

The following materials and samples have to be stored according to the period of time specified in the GLP regulations:

A retained sample of the test item will be archived according to the GLP regulations, if possible, and will be discarded without the sponsor's prior consent.

Other materials and specimens have to be stored according to the GLP regulations and disposed of after the respective archiving period with the sponsor's prior consent.

Unless otherwise agreed in writing, the remaining test item will be discarded three months after the release of the report.

6. Statement of Compliance

Eurofins Munich Study No.:	STUGC22AA2158-3
Test Item:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Title:	*In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Study Director:	Dr. Stephanie Lacmanski

This study performed in the test facility Eurofins Munich was conducted in compliance with Good Laboratory Practice Regulations:

Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998 [3].

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dr. Stephanie Lacmanski

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Date: 06 SEP 2023

7. Statement of the Quality Assurance Unit

Eurofins Munich Study No.:	STUGC22AA2158-3
Test Item:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Title:	*In vitro* Mammalian Micronucleus Assay in Human Lymphocytes with Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Study Director:	Dr. Stephanie Lacmanski

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phase of QAU Inspection	Date of QAU Inspection	Date of Reporting to the Study Director and Management
Audit Final Study Plan:	26 January 2023	26 January 2023
Audit 1st Amendment to Study Plan:	16 February 2023	16 February 2023
Audit 2nd Amendment to Study Plan:	25 August 2023	25 August 2023
Audit Experimental Phase (process-based):	17 May 2023	17 May 2023
Audit Final Report:	05 SEP 2023	05 SEP 2023

Facilities relevant for study performance were included in the annual facility-inspection program.

This report reflects the raw data.

Member of the Quality Assurance Unit:

Anna Gebauer

Print Name: Anna Gebauer

Date: 05 SEP 2023

8. Summary

8.1. Summary Results

In order to investigate a possible potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) to induce micronuclei in human lymphocytes an *in vitro* micronucleus assay was carried out. The following study design was performed:

	Without S9		With S9
	Exp. I	Exp. II	Exp. I
Exposure period	4 h	44 h	4 h
Cytochalasin B exposure	40 h	43 h	40 h
Preparation interval	44 h	44 h	44 h
Total culture period*	92 h	92 h	92 h

*Exposure started 48 h after culture initiation

The selection of the concentrations was based on data from the pre-experiment. In the main experiment **without** and **with** metabolic activation 350 µg/mL and 300 µg/mL, respectively, was selected as highest concentration for the microscopic analysis of micronuclei. In experiment **II without** metabolic activation 100 µg/mL was selected as highest concentration for the microscopic analysis of micronuclei.

The following concentrations were evaluated for micronuclei frequencies:

Experiment I with short-term exposure (4 h):

without metabolic activation: 250, 325 and 350 µg/mL

with metabolic activation: 100, 250 and 300 µg/mL

Experiment II with long-term exposure (44 h):

without metabolic activation: 25, 50 and 100 µg/mL

No precipitate of the test item was noted in the cultures at the end of treatment in any concentration evaluated in experiment I and II.

Table 1: Summary: Experiment I and II, without metabolic activation

	Dose Group	Concentration [µg/mL]	Number of cells evaluated	Cytostasis [%]	Relative Cell Growth [%]	Micro-nucleated Cells Frequency [%]	Historical Control Limits Solvent Control	P	Statistical Significant Increase[a]
Exp. I 4 h treatment, 44 h fixation interval	**C**	0	2000	0*	102	**0.35**	0.02% - 1.38%	/	/
	S	0	2000	0	100	**0.35**		/	/
	2	250	2000	21	79	**0.50**		-	-
	4	325	2000	37	63	**0.30**		-	-
	5	350	2000	55	45	**0.45**		-	-
	MMS	65	2000	33	67	**3.75**		-	+
	Colc	0.4	1349	66	34	**1.66**		-	+
Exp. II 44 h treatment, 44 h fixation interval	**C**	0	2000	0*	120	**0.40**	0.06% - 1.10%	/	/
	S	0	2000	0	100	**0.55**		/	/
	1	25	2000	21	79	**0.60**		-	-
	2	50	2000	14	86	**0.25**		-	-
	3	100	2000	66	34	**0.65**		-	-
	MMS	50	1864	42	58	**3.35**		-	+
	Colc	0.02	1242	81	19	**2.06**		-	+

Table 2: Summary: Experiment I, with metabolic activation

	Dose Group	Concentration [µg/mL]	Number of cells evaluated	Cytostasis [%]	Relative Cell Growth [%]	Micro-Nucleated Cells Frequency [%]	Historical Control Limits Solvent Control	P	Statistical Significant Increase[a]
Exp. I 4 h treatment, 44 h fixation interval	**C**	0	2000	20	**80**	**0.45**	0.13% - 1.22%	/	/
	S	0	2000	0	**100**	**0.35**		/	/
	1	100	2000	1	**99**	**0.35**		-	-
	2	250	2000	44	**56**	**0.40**		-	-
	3	300	2000	59	**41**	**0.30**		-	-
	CPA	15 µg/mL	2000	43	**57**	**3.75**		-	+

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
P: Precipitation (+: precipitation, -: no precipitation)
a: statistically significant increase compared to solvent control (χ^2 test , $p<0.05$).
+: significant; -: not significant
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colc.: Colchicine, Positive Control (without metabolic activation)
CPA: Cyclophosphamide, Positive Control (with metabolic activation)

Relative Cell Growth: $100 \times ((CBPI_{Test\ conc} - 1) / (CBPI_{control} - 1))$

Cytostasis [%] = 100- Relative Cell Growth [%]
*: the cytostasis is defined 0, when the relative cell growth exceeds 100%

If cytotoxicity is observed the highest concentration evaluated should not exceed the limit of 55% ± 5% cytotoxicity according to the OECD Guideline 487 [4]. Higher levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity. The other concentrations evaluated should exhibit intermediate and little or no toxicity. However, OECD 487 does not define the limit for discriminating between cytotoxic and non-cytotoxic effects. According to laboratory experience this limit is a value of the relative cell growth of 70% compared to the negative/solvent control which corresponds to 30% of cytostasis.

In experiment I **without** metabolic activation no increase of the cytostasis above 30% was noted up to 250 µg/mL. At 325 µg/mL a cytostasis of 37% and at 350 µg/mL a cytostasis of 55% was noted.
In experiment I **with** metabolic activation no increase of the cytostasis above 30% was noted up to 100 µg/mL. At 250 µg/mL a cytostasis of 44% and at 300 µg/mL a cytostasis of 59% was observed.
In experiment II **without** metabolic activation no increase of the cytostasis above 30% was noted up to 50 µg/mL. At 100 µg/mL a cytostasis of 66% was observed.

In experiment I and II **without** and **with** metabolic activation no biologically relevant increase of the micronucleus frequency was noted after treatment with the test item.

The nonparametric χ^2 Test was performed to verify the results in both experiments. No statistically significant increase ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I and II.

The χ^2 Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions. No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II.

Methylmethanesulfonate (MMS, 50 and 65 µg/mL) and cyclophosphamide (CPA, 15 µg/mL) were used as clastogenic controls. Colchicine (Colc, 0.02 and 0.4 µg/mL) was used as aneugenic control. All induced distinct and statistically significant increases of the micronucleus frequency. This demonstrates the validity of the assay.

8.2. Conclusion

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) did not induce structural and/or numerical chromosomal damage in human lymphocytes.

Therefore, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus Test.

9. Introduction

9.1. Aim of the Study

The *in vitro* micronucleus assay detects the activity of clastogenic and aneugenic chemicals in cells that have undergone cell division during or after exposure to the test substance. The micronuclei may originate from acentric fragments (chromosome fragments lacking a centromer) or whole chromosomes which are unable to migrate with the rest of the chromosomes during the anaphase of the cell division. Micronuclei formation may also result at the level of proteins directly or indirectly involved in chromosome segregation (e.g. tubulin). The addition of the actin polymerisation inhibitor Cytochalasin B prior to the targeted mitosis, allows the identification and analysis of micronucleus frequency only in those cells, which have completed mitosis. These cells are binucleate [7], [8]. The *in vitro* micronucleus test is an *in vitro* test which is able to detect both numerical and structural chromosomal aberrations, two mechanisms involved in genetic and carcinogenic risk [6], [7]. Clastogenic or aneugenic incidents are critical lesions for the cell that could cause alterations in protooncogens and tumor suppressor genes. These alterations could result in oncogenesis. However, the *in vitro* micronucleus assay does not allow the identification of polyploidic cells.

Human peripheral blood lymphocytes should be obtained from healthy, non-smoking donors. The most efficient approach is to test human lymphocytes 44 - 48 h after PHA stimulation, when the cell cycle synchronisation will have declined. It is recommended to perform the first experiment with a 3 - 6 h (short-term) treatment in presence and absence of metabolic activation, with sampling occurring at a time equivalent to about two normal cell cycle lengths after the beginning of treatment. If the first experiment gives negative or equivocal results a second experiment with modified conditions should be done. In absence of metabolic activation the cells should be exposed continuously to the test substance (long-term treatment) [4].

At least three analysable concentrations of the test item with concentration intervals of approximately 2 to 3 fold should be tested. For soluble, non-toxic test items the highest concentration should correspond to 10 mM, 2 µL/mL or 2 mg/mL, whichever is the lowest. When the test chemical is not of defined composition, e.g. substance of unknown or variable composition, the top concentration may need to be higher (e.g. 5 mg/mL) in the absence of sufficient cytotoxicity. For poorly soluble compounds, one concentration with precipitate visible by the unaided eye at the end of treatment should be used as highest concentration. The precipitate should not interfere with scoring. If toxicity is found, the highest concentration evaluated should induce approximately 55 ± 5% toxicity [4], [9], [10]. If possible, the concentration tested should exhibit substantial toxicity, intermediate toxicity and/or no toxicity [4], [10].

Assessment of cytotoxicity should be performed by determining cell proliferation in both treated and control cultures. The proliferation rate is determined by calculation of the cytokinesis-block proliferation index (CBPI) and the cytostasis [10].

As validity criterion for the test, reference mutagens are tested in parallel to the test item. It is recommended to use clastogenic and aneugenic positive controls.

9.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals Section 4, No 487 – "*In Vitro* Mammalian Cell Micronucleus Test, adopted 29 July, 2016" – recommends using a variety of cell lines or primary cell cultures (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes) in the presence or absence of cytochalasin B.

10. Materials and Methods

10.1. Characterisation of the Test Item

The identity of the test item was inspected upon delivery at the test facility (e.g. test item name, batch no. and additional data were compared with the label) based on the following specifications provided by the sponsor. The following listed information applies to the sample as received.

Name:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
CAS No.:	1185851-52-8
EC No.:	867-521-0
Batch No.:	TAF-10-1122-01
Molecular Weight:	437.52 g/mol
Physical State:	liquid, oil
Colour:	colourless to pale yellow
Purity:	99.78%
Expiry Date:	23 November 2026
Storage Conditions:	2-8°C in a well-closed container
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

A certificate of analysis was provided by the sponsor, accepted by Eurofins Munich and can be found in the Appendix 3: Certificate of Analysis.

10.2. Preparation of the Test Item

A solubility test was performed with different solvents and vehicles up to the maximum recommended concentration of 2000 µg/mL.

Due to the nature of the test item it was not possible to prepare a solution of the test item with cell culture medium (RPMI). Therefore, the test item was dissolved in dimethylsulfoxide (DMSO) and diluted in cell culture medium to reach a final concentration of 1% v/v DMSO in the samples.

The pH value detected with the test item was within the physiological range. The solvent was compatible with the survival of the cells and the S9 activity.

For the maximum concentration without metabolic activation the osmolality (in comparison to solvent control) and pH value were determined:

		Concentration (µg/mL)	Osmolality (mOsm/kg)	pH value
Exp. I	Solvent control	-	427	-
	Test item	500	433	7.4
Exp. II	Solvent control	-	435	-
	Test item	350	445	7.0

10.3. Controls

Negative/solvent and positive controls were included in each experiment.

Negative Controls

Negative controls (cell culture medium) were treated in the same way as all dose groups.

Solvent Controls

Solvent controls (cell culture medium with 1% DMSO; AppliChem Germany; pre-experiment, main experiment I: lot 0002204737, expiry date July 2026; main experiment II: lot 0002289707, expiry date March 2027) were treated in the same way as all dose groups.

Positive Controls

Positive controls should employ a known inducer of micronuclei formation at exposure levels expected to give a reproducible and detectable increase over background, which demonstrates the sensitivity of the test system.

Clastogenic Controls

Without metabolic activation

Name	MMS, methylmethanesulfonate
CAS No.	66-27-3
Supplier	Sigma Aldrich, Germany
Catalogue No.	129925
Batch No.	MKCL6261 (experiment I), MKCM5645 (experiment II)
Dissolved in	RPMI
Final concentrations	50 and 65 µg/mL
Expiry Date	batch MKCL6261: December 2023

With metabolic activation

Name	CPA, cyclophosphamide monohydrate
CAS No.	6055-19-2
Supplier	Sigma Aldrich, Germany
Catalogue No.	C0768
Batch No.	MKCS5505
Dissolved in	RPMI
Final concentrations	15 µg/mL
Expiry Date	January 2025

CPA displays a good stability at room temperature. At 25 °C only 3.5% of its potency is lost after 24 h [14]. The solution was aliquoted and stored at ≤ -15 °C. Additionally, the stability of CPA in solution was proven by the clastogenic response in the expected range.

Aneugenic Control

Without metabolic activation

Name	Colchicine
CAS No.	64-86-8
Supplier	Sigma Aldrich, Germany
Catalogue No.	C 9754
Batch No.	SLCM9637
Dissolved in	RPMI
Final concentrations	0.02 and 0.4 µg/mL
Expiry Date	March 2025

The dilutions of the stock solutions of the positive controls were prepared on the day of the experiment and used immediately.

The stability of both positive control substances in solution is proven by the mutagenic response in the expected range.

10.4. Test System

10.4.1. Blood Collection

Human peripheral blood lymphocytes from young (approximately 18-35 years of age), healthy and non-smoking donors with no known recent exposure to genotoxic chemicals and radiation were used to examine the ability of chemicals to induce cytogenetic damage and thus to identify potential carcinogens or mutagens *in vitro*.

For the pre-experiment blood was collected from a single female donor. For the main experiment I and II blood was used from a single male donor.

Blood samples were drawn by venous puncture and collected in heparinized tubes. Before use the blood was stored under sterile conditions at 4 °C for a maximum of 4 h. Whole blood samples treated with an anti-coagulant (e. g. heparin) were pre-cultured in the presence of mitogen (phyto-hemagglutinin, PHA).

10.4.2. Mammalian Microsomal Fraction S9 Homogenate

An advantage of using *in vitro* cell cultures is the accurate control of the concentration and exposure time of cells to the test item under study. However, due to the limited capacity of cells growing *in vitro* for metabolic activation of potential mutagens, an exogenous metabolic activation system is necessary. Many substances only develop mutagenic potential when they are metabolized by the mammalian organism. Metabolic activation of substances can be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix).

The S9 liver microsomal fraction was prepared at Eurofins Munich GmbH. Male Wistar rats were induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route [11], [12]. The preparation was performed according to Ames et al. [13].

The following quality control determinations were performed:

a) Biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene

b) Sterility Test

A stock of the supernatant containing the microsomes was frozen in aliquots of 2 and 4 mL and stored at ≤ -75°C.

The protein concentration in the S9 preparation (Lot: 251122) was 39 mg/mL.

10.4.3. S9 Mix

An appropriate quantity of the S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the concentrations below:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	Glucose-6-phosphate
5	mM	NADP

in 100 mM sodium-phosphate-buffer pH 7.4. During the experiment the S9 mix was stored on ice. The final concentration of S9 mix in the cultures was 5%.

10.4.4. Culture Medium

Complete Culture Medium

RPMI 1640 medium supplemented with:

15	%	fetal bovine serum (FBS)
100 U/100	µg/mL	penicillin/streptomycin solution
2.4	µg/mL	phytohemagglutinin (PHA)

Treatment Medium (short-term exposure)

Complete culture medium without FBS.

After Treatment Medium / Treatment Medium (long-term exposure)

Complete culture medium with 15 % FBS and 6 µg/mL cytochalasin B.

10.5. Experimental Design

10.5.1. Pre-Experiment for Toxicity

A pre-experiment was conducted under identical conditions as described for the main experiment I (4 h incubation) in order to determine the toxicity of the test item. The CBPI was used for the quantification of cytotoxicity. The following concentrations were tested **without** and **with** S9 mix:

7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 1500, 2000 µg/mL

The concentration of 2000 µg/mL was considered to be the highest test concentration to be used in this test system following the recommendation of the corresponding OECD testing guideline 487 [4].

10.5.2. Exposure Concentrations

Duplicate cultures were treated at each concentration. The selection of the concentrations used in experiment I and II based on data from the pre-experiment. The following concentrations were used in the main experiments:

Experiment I:

without and **with** metabolic activation:100, 250, 300, 325, 350, 375, 400, 425, 450 and 500 µg/mL

Experiment II:

without metabolic activation: 25, 50, 100, 150, 200, 250, 300 and 350 µg/mL

The following concentrations were selected in the main experiment for the microscopic analyses. The selection of the maximum concentration was based on cytotoxicity for all experimental conditions.

Experiment I with short-term exposure (4 h):

without metabolic activation: 250, 325 and 350 µg/mL

with metabolic activation: 100, 250 and 300 µg/mL

Experiment II with long-term exposure (44 h):

without metabolic activation: 25, 50 and 100 µg/mL

10.5.3. Experimental Performance

Experiment I

Whole blood samples were treated with anti-coagulant (Heparin) and were pre-cultured (44 to 48 h) in presence of PHA prior to exposure to the test item. It is recommended to test human lymphocytes 44 to 48 h after PHA stimulation, when the cell cycle synchronisation is disappeared. The lymphocytes were incubated with the test item for 4 h in presence or absence of metabolic activation. At the end of the incubation, the treatment medium was removed and the cells were washed twice with PBS + 10% FBS. Subsequently the cells were incubated in complete culture medium + 6 µg/mL cytochalasin B for 40 h to 42 h at 37°C and 5% CO_2 [9].

Experiment II

If negative or equivocal results are obtained, they should be confirmed using continuous treatment (long-term treatment) **without** metabolic activation. The whole blood cultures were pre-cultured in the presence of PHA for 44 to 48 h prior to exposure to the test item. Then the test item was added in complete culture medium. 1 h later 6 µg/mL cytochalasin B were added and the cells were incubated for further 43 h at 37 °C. At the end of the treatment the cell culture medium was removed and the cells were prepared for microscopic analysis.

10.5.4. Number of Cultures

Duplicate cultures were performed at each dose level except for the pre-experiment.

10.5.5. Preparation of the Cultures

At the end of the cultivation, the complete culture medium was removed. Subsequently, the cells were treated with cold hypotonic solution (0.075 M KCl) for some minutes at room temperature and immediately centrifuged. The pellet was resuspended with a solution consisted of fixation solution + NaCl 0.9% (1+1) and centrifuged. After that the cells were fixed with methanol + glacial acetic acid (3+1). The cells were resuspended gently and the suspension was dropped onto clean glass slides. Consecutively, the cells were dried on a heating plate. The cells were stained with acridine orange solution.

10.5.6. Analysis of Micronuclei

All slides, including those of positive and negative controls were independently coded before microscopic analysis. For each dose group at least 2000 binucleated cells (if possible) per concentration (1000 binucleated cells per slide) were analysed for micronuclei according to the criteria of Fenech [7], i.e. clearly surrounded by a nuclear membrane, having an area of less than one-third of that of the main nucleus, being located within the cytoplasm of the cell and not linked to the main nucleus via nucleoplasmic bridges. Mononucleated and multinucleated cells and cells with more than six micronuclei were not considered [8].

10.5.7. Cytokinesis Block Proliferation Index

As an assessment of the cytotoxicity, a cytokinesis block proliferation index (CBPI) was determined from 500 cells according to the following formula:

$$CBPI = \frac{(c_1 \times 1) + (c_2 \times 2) + (c_x \times 3)}{n}$$

c_1: mononucleate cells

c_2: binucleate cells

c_x: multinucleate cells

n: total number of cells

The CBPI was used to calculate the % cytostasis, which indicates the inhibition of cell growth of treated cultures in comparison to control cultures:

$$\% \text{ Cytostasis} = 100 - 100 \times ((CBPI_T - 1) / (CBPI_C - 1))$$

$CBPI_T$: Cytokinesis Block proliferation index of treated cultures

$CBPI_C$: Cytokinesis Block proliferation index of control cultures

10.6. Data Recording

The data generated were recorded in the raw data. The results were presented in tables, including experimental groups with the test item, negative and positive controls. The experimental unit was the cell and therefore, the percentage of cells with micronuclei was evaluated. A concurrent measurement of cytotoxicity was also recorded.

10.7. Acceptability of the Assay

A mutation assay is considered acceptable if it meets the following criteria:

- The concurrent negative/solvent control is considered acceptable for addition to the laboratory historical negative/solvent control database.
- Concurrent positive controls should induce responses that are compatible with those generated in the laboratory's historical positive control data base and produce a statistically significant increase compared with the concurrent negative/solvent control.
- Cell proliferation criteria in the negative/solvent control should be fulfilled.
- All experimental conditions are tested unless one resulted in positive results.
- Adequate number of cells and concentrations are analysable.
- Criteria for the selection of top concentration are fulfilled.

10.8. Evaluation of Results

A test item is considered to be clearly positive if, in any of the experimental conditions examined:

- at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative/solvent control
- the increase is concentration-related in at least one experimental condition when evaluated with an appropriate trend test
- any of the results are outside the distribution of the historical negative/solvent control data (e.g. Poisson-based 95% control limits).

When all of these criteria are met, the test item is considered able to induce chromosome breaks and/or gain or loss in this test system.

A test item is considered to be clearly negative if in all experimental conditions examined none of the criteria mentioned above are met.

10.9. Statistics

An appropriate statistical analysis was performed. The proportion of cells containing micronuclei was calculated for each test group. A comparison of the number of micronucleated cells of each test group with the concurrent vehicle control group was evaluated by the non-parametric χ^2 test at a statistical significance level of 5% ($p < 0.05$, two-sided).

If the results of this test were statistically significant compared with the respective vehicle control ($p < 0.05$), labels (*) were printed in the figures.

The χ^2 test for trend (Cochran-Armitage test for trend) at a statistical significance level of 5% ($p < 0.05$, two-sided) was used to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions.

Statistical methods were performed using the software GraphPad Prism version 6.

11. Deviations from the Study Plan

There was the following deviation from the study plan:

- **Concerning:**

  7.3. Controls, study plan, p. 11

  Study Plan:

  [...]

  With metabolic activation

Name:	CPA, cyclophosphamide
CAS No.:	50-18-0

  [...]

  Report:

  [...]

  With metabolic activation

Name	CPA, cyclophosphamide monohydrate
CAS No.	6055-19-2

  [...]

  Reason:

  Typing error. Cyclophosphamide monohydrate was used as clastogenic positive control in main experiment I.

This deviation did not influence the quality or integrity of the present study.

12. Results and Discussion

12.1. Results

12.1.1. Pre-Experiment

According to the corresponding OECD testing guideline [4] the highest recommended concentration is 2000 µg/mL. The test item was dissolved in DMSO and rediluted with cell culture medium to achieve the final test item concentrations. No precipitate of the test item was noted. The highest dose group evaluated in the pre-experiment was 2000 µg/mL. The cytokinesis block proliferation index (CBPI) was used to calculate the cytostasis (cytostasis [%] = 100 - CBPI relative [%]). The cytostasis was used to describe cytotoxicity. The selection of concentrations used in the main experiment based on the results obtained in the pre-experiment.

Table 3: Test for Cytotoxicity, *without* and *with* metabolic activation

Dose Group	Concentration [µg/mL]	CBPI	Relative Cell Growth [%]	Cytostasis [%]	Precipitate +/-
without metabolic activation					
C	0	1.33	127	0	-
S	0	1.26	100	0	-
1	7.8	1.51	198	0	-
2	15.6	1.38	149	0	-
3	31.3	1.51	199	0	-
4	62.5	1.41	160	0	-
5	125	1.50	194	0	-
6	250	1.45	176	0	-
7	500	1.06	22	78	-
8	1000	1.02	6	94	-
9	1500	1.00	1	99	-
10	2000	1.00	2	98	-
with metabolic activation					
C	0	1.43	127	0	-
S	0	1.34	100	0	-
1	7.8	1.39	115	0	-
2	15.6	1.54	159	0	-
3	31.3	1.38	111	0	-
4	62.5	1.39	116	0	-
5	125	1.45	132	0	-
6	250	1.42	124	0	-
7	500	1.03	8	92	-
8	1000	1.00	1	99	-
9	1500	1.00	0	100	-
10	2000	1.00	0	100	-

The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent controls.

C:	Negative Control (Culture medium)
S:	Solvent Control (DMSO 1% v/v in culture medium)
CBPI:	Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative Cell Growth:	$100 \times ((CBPI_{\text{Test conc}} - 1) / (CBPI_{\text{control}} - 1))$
c_1:	mononucleate cells
c_2:	binucleate cells
c_x:	multinucleate cells
n:	total number of cells

Cytostasis [%] = 100- Relative Cell Growth [%]
the cytostasis is defined 0, when the relative cell growth exceeds 100%

12.1.2. Experiment I

Table 4: Experiment I - CBPI: 4 h treatment (*without* and *with* metabolic activation), 44 h fixation interval

Dose Group	Concentration [µg/mL]	CBPI 1/2	CBPI 2/2	Relative Cell Growth [%]	Cytostasis [%]	Precipitate +/-
without metabolic activation						
C	0	1.34	1.32	102	0	-
S	0	1.38	1.26	100	0	-
2	250	1.38	1.13	79	21	-
4	325	1.19	1.22	63	37	-
5	350	1.19	1.10	45	55	-
MMS	65	1.23	1.20	67	33	-
Colchicine	0.4	1.13	1.09	34	66	-
with metabolic activation						
C	0	1.36	1.38	80	20	-
S	0	1.48	1.45	100	0	-
1	100	1.43	1.49	99	1	-
2	250	1.37	1.15	56	44	-
3	300	1.25	1.14	41	59	-
CPA	15	1.28	1.25	57	43	-

The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent controls.

C:	Negative Control (Culture medium)
S:	Solvent Control (DMSO 1% v/v in culture medium)
MMS:	Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine:	Positive Control (without metabolic activation)
CPA:	Cyclophosphamide, Positive Control (with metabolic activation)
CBPI:	Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative Cell Growth:	$100 \times ((CBPI_{\text{Test conc}} - 1) / (CBPI_{\text{control}} - 1))$
c_1:	mononucleate cells
c_2:	binucleate cells
c_x:	multinucleate cells
n:	total number of cells
CBPI 1/2	CBPI of culture 1 of 2
CBPI 2/2	CBPI of culture 2 of 2

Cytostasis [%] = 100- Relative Cell Growth [%]
the cytostasis is defined 0, when the relative cell growth exceeds 100%

Table 5: Experiment I - Micronucleus induction in human lymphocytes, 4 h treatment, 44 h fixation interval, *without* metabolic activation

Dose Group	Concentration [µg/mL]	Culture	Scored binucleated Cells	Micronuclei	Micronucleated Cells Frequency [%]
C	0	1	1000	3	0.30
		2	1000	4	0.40
		total	**2000**	**7**	**0.35**
S	0	1	1000	4	0.40
		2	1000	3	0.30
		total	**2000**	**7**	**0.35**
2	250	1	1000	7	0.70
		2	1000	3	0.30
		total	**2000**	**10**	**0.50**
4	325	1	1000	3	0.30
		2	1000	3	0.30
		total	**2000**	**6**	**0.30**
5	350	1	1000	5	0.50
		2	1000	4	0.40
		total	**2000**	**9**	**0.45**
MMS	65	1	1000	50	5.00
		2	1000	25	2.50
		total	**2000**	**75**	**3.75**
Colchicine	0.4	1	712	8	1.12
		2	637	14	2.20
		total	**1349**	**22**	**1.66**

The micronucleated cell frequency was determined where possible in 1000 binucleated cells in each of the two separate cultures per test group, except for the positive control colchicine (712 for the 1st and 637 for the 2nd culture). In case of significant difference between both slides (generally factor > 2) additional 1000 binucleated cells of the same concentration were screened to verify this analysis (not considered necessary for dose group 2). Only the final count of all analyzed binucleated cells per culture and concentration is given in this table.

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)

Table 6: Experiment I - Micronucleus induction in human lymphocytes, 4 h treatment, 44 h fixation interval, *with* metabolic activation

Dose Group	Concentration [µg/mL]	Culture	Scored binucleated Cells	Micronuclei	Micronucleated Cells Frequency [%]
C	**0**	1	1000	4	0.40
		2	1000	5	0.50
		total	**2000**	**9**	**0.45**
S	**0**	1	1000	4	0.40
		2	1000	3	0.30
		total	**2000**	**7**	**0.35**
1	**100**	1	1000	3	0.30
		2	1000	4	0.40
		total	**2000**	**7**	**0.35**
2	**250**	1	1000	2	0.20
		2	1000	6	0.60
		total	**2000**	**8**	**0.40**
3	**300**	1	1000	2	0.20
		2	1000	4	0.40
		total	**2000**	**6**	**0.30**
CPA	**15**	1	1000	17	1.70
		2	1000	58	5.80
		total	**2000**	**75**	**3.75**

The micronucleated cell frequency was determined where possible in 1000 binucleated cells in each of the two separate cultures per test group. In case of significant difference between both slides (generally factor > 2) additional 1000 binucleated cells of the same concentration were screened to verify this analysis (not considered necessary for dose group 2). Only the final count of all analyzed binucleated cells per culture and concentration is given in this table.

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
CPA: Cyclophosphamide, Positive Control (with metabolic activation)

12.1.3. Experiment II

Table 7: Experiment II - CBPI: 44 h treatment (*without* metabolic activation), 44 h fixation interval

Dose Group	Concentration [µg/mL]	CBPI 1/2	CBPI 2/2	Relative Cell Growth [%]	Cytostasis [%]	Precipitate +/-
without metabolic activation						
C	0	1.67	1.72	120	0	-
S	0	1.52	1.64	100	0	-
1	25	1.43	1.48	79	21	-
2	50	1.57	1.42	86	14	-
3	100	1.23	1.16	34	66	-
MMS	50	1.40	1.27	58	42	-
Colchicine	0.02	1.09	1.13	19	81	-

The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.

C:	Negative Control (Culture medium)
S:	Solvent Control (DMSO 1% v/v in culture medium)
MMS:	Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine:	Positive Control (without metabolic activation)
CBPI:	Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative Cell Growth:	$100 \times ((CBPI_{Test\ conc} - 1) / (CBPI_{control} - 1))$
c_1:	mononucleate cells
c_2:	binucleate cells
c_x:	multinucleate cells
n:	total number of cells
CBPI 1/2	CBPI of culture 1 of 2
CBPI 2/2	CBPI of culture 2 of 2

Cytostasis [%] = 100- Relative Cell Growth [%]
the cytostasis is defined 0, when the relative cell growth exceeds 100%

Table 8: Experiment II - Micronucleus induction in human lymphocytes, 44 h treatment, 44 h fixation interval, *without* metabolic activation

Dose Group	Concentration [µg/mL]	Culture	Scored binucleated Cells	Micronuclei	Micronucleated Cells Frequency [%]
C	0	1	1000	5	0.50
		2	1000	3	0.30
		total	**2000**	**8**	**0.40**
S	0	1	1000	8	0.80
		2	1000	3	0.30
		total	**2000**	**11**	**0.55**
1	25	1	1000	5	0.50
		2	1000	7	0.70
		total	**2000**	**12**	**0.60**
2	50	1	1000	3	0.30
		2	1000	2	0.20
		total	**2000**	**5**	**0.25**
3	100	1	1000	5	0.50
		2	1000	8	0.80
		total	**2000**	**13**	**0.65**
MMS	50	1	1000	30	3.00
		2	864	32	3.70
		total	**1864**	**62**	**3.35**
Colchicine	0.02	1	639	20	3.13
		2	603	6	1.00
		total	**1242**	**26**	**2.06**

The micronucleated cell frequency was determined where possible in 1000 binucleated cells in each of the two separate cultures per test group, except for the positive control MMS (1000 for the 1st and 864 for the 2nd culture) and colchicine (639 for the 1st and 603 for the 2nd culture). In case of significant difference between both slides (generally factor > 2) additional 1000 binucleated cells of the same concentration were screened to verify this analysis (not considered necessary for the solvent control). Only the final count of all analyzed binucleated cells per culture and concentration is given in this table.

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)

12.1.4. Micronuclei Effects

Table 9: Summary of Micronuclei Effects: Experiment I *without* and *with* metabolic activation

Dose Group	Concentration [µg/mL]	Treatment Time	Fixation Interval	Micronucleated Cells Frequency [%]
without metabolic activation				
C	0	4 h	44 h	**0.35**
S	0	4 h	44 h	**0.35**
2	250	4 h	44 h	**0.50**
4	325	4 h	44 h	**0.30**
5	350	4 h	44 h	**0.45**
MMS	65	4 h	44 h	**3.75**
Colchicine	0.4	4 h	44 h	**1.66**
with metabolic activation				
C	0	4 h	44 h	**0.45**
S	0	4 h	44 h	**0.35**
1	100	4 h	44 h	**0.35**
2	250	4 h	44 h	**0.40**
3	300	4 h	44 h	**0.30**
CPA	15	4 h	44 h	**3.75**

The micronucleated cell frequency was determined where possible in 1000 binucleated cells in each of the two separate cultures per test group, except for the positive control colchicine (712 for the 1st and 637 for the 2nd culture). In case of significant difference between both slides (generally factor > 2) additional 1000 binucleated cells of the same concentration were screened to verify this analysis. Only the final count of all analyzed binucleated cells per culture and concentration is given in this table.

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)
CPA: Cyclophosphamide, Positive Control (with metabolic activation)

Table 10: Summary of Micronuclei Effects: Experiment II *without* metabolic activation

Dose Group	Concentration [µg/mL]	Treatment Time	Fixation Interval	Micronucleated Cells Frequency [%]
without metabolic activation				
C	0	44 h	44 h	**0.40**
S	0	44 h	44 h	**0.55**
1	25	44 h	44 h	**0.60**
2	50	44 h	44 h	**0.25**
3	100	44 h	44 h	**0.65**
MMS	50	44 h	44 h	**3.35**
Colchicine	0.02	44 h	44 h	**2.06**

The micronucleated cell frequency was determined where possible in 1000 binucleated cells in each of the two separate cultures per test group, except for the positive control MMS (1000 for the 1st and 864 for the 2nd culture) and colchicine (639 for the 1st and 603 for the 2nd culture). In case of significant difference between both slides (generally factor > 2) additional 1000 binucleated cells of the same concentration were screened to verify this analysis. Only the final count of all analyzed binucleated cells per culture and concentration is given in this table.

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)

12.1.5. Biometry

Table 11: Biometry - Experiment I *without* metabolic activation

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the non-parametric χ^2 test. The p value was used as a limit in judging for significance levels in comparison with the concurrent solvent control.

Dose Group	Concentration [µg/mL]	Treatment Time [h]	Micronuclei Frequencies [%]	Significance	P Value
S	0	4	0.35	/	/
2	250	4	0.50	-	0.4659
4	325	4	0.30	-	0.7812
5	350	4	0.45	-	0.6164
MMS	65	4	3.75	+	< 0.0001
Colchicine	0.4	4	1.66	+	< 0.0001

+: significant
-: not significant
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)

Statistical significance: statistically significant difference in micronucleated cells frequency compared to solvent control (nonparametric χ^2 test, $p < 0.05$).

Table 12: Biometry - Experiment I *with* metabolic activation

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the non-parametric χ^2 test. The p value was used as a limit in judging for significance levels in comparison with the concurrent solvent control.

Dose Group	Concentration [µg/mL]	Treatment Time [h]	Micronuclei Frequencies [%]	Significance	P Value
S	0	4	0.35	/	/
1	100	4	0.35	-	1.0000
2	250	4	0.40	-	0.7959
3	300	4	0.30	-	0.7812
CPA	15	4	3.75	+	< 0.0001

+: significant
-: not significant
S: Solvent Control (DMSO 1% v/v in culture medium)
CPA: Cyclophosphamide, Positive Control (with metabolic activation)

Statistical significance: statistically significant difference in micronucleated cells frequency compared to solvent control (nonparametric χ^2 test, $p < 0.05$).

Table 13: Biometry - Experiment II *without* metabolic activation

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the non-parametric χ^2 test. The p value was used as a limit in judging for significance levels in comparison with the concurrent solvent control.

Dose Group	Concentration [µg/mL]	Treatment Time [h]	Micronuclei Frequencies [%]	Significance	P Value
S	0	44	0.55	/	/
1	25	44	0.60	-	0.8344
2	50	44	0.25	-	0.1328
3	100	44	0.65	-	0.6822
MMS	50	44	3.35	+	< 0.0001
Colchicine	0.02	44	2.06	+	< 0.0001

+: significant
-: not significant
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)

Statistical significance: statistically significant difference in micronucleated cells frequency compared to solvent control (nonparametric χ^2 test, $p < 0.05$).

Table 14: Biometry – Trend test

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the χ^2 test for trend. The p value was used as a limit in judging for significance levels.

Experiment	Treatment Time [h]	Significance	P Value
Exp. I without metabolic activation	4	-	0.8061
Exp. I with metabolic activation	4	-	0.7889
Exp. II without metabolic activation	44	-	0.8226

+: significant
-: not significant

Statistical significance: statistically significant concentration-related increase in micronucleated cells frequency (χ^2 test for trend, $p < 0.05$).

12.2. Discussion

The test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) was investigated for a possible potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation with S9.

The selection of the concentrations used in main experiment I and II was based on data from the pre-experiment according to the guidelines.

In experiment I **without** and **with** metabolic activation 350 µg/mL and 300 µg/mL, respectively, was selected as highest concentration for the microscopic analysis of micronuclei.

In experiment II **without** metabolic activation 100 µg/mL was selected as highest concentration for the microscopic analysis of micronuclei.

The cells were prepared 44 h after start of treatment with the test item. The treatment intervals were 4 h **without** and **with** metabolic activation (experiment I) and 44 h **without** metabolic activation (experiment II). Parallel cultures were set up and 1000 binucleated cells per culture were scored for micronuclei.

The following concentrations were evaluated for micronuclei frequencies:

Experiment I with short-term exposure (4 h):

without metabolic activation: 250, 325 and 350 µg/mL

with metabolic activation: 100, 250 and 300 µg/mL

Experiment II with long-term exposure (44 h):

without metabolic activation: 25, 50 and 100 µg/mL

12.2.1. Precipitation

The test item was dissolved in DMSO and rediluted in cell culture medium (RPMI medium) at a ratio of 1:100 to obtain the final test item concentrations and a final concentration of 1% v/v DMSO in the cultures. No precipitate of the test item was noted in the cultures at the end of treatment in any concentration evaluated in experiment I and II.

12.2.2. Cytotoxicity

If cytotoxicity is observed the highest concentration evaluated should not exceed the limit of 55% ± 5% cytotoxicity according to the OECD Guideline 487 [4]. Higher levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity. The other concentrations evaluated should exhibit intermediate and little or no toxicity. However, OECD 487 does not define the limit for discriminating between cytotoxic and non-cytotoxic effects. According to laboratory experience this limit is a value of the relative cell growth of 70% compared to the solvent control which corresponds to 30% of cytostasis.

In experiment I **without** metabolic activation no increase of the cytostasis above 30% was noted up to 250 µg/mL. At 325 µg/mL a cytostasis of 37% and at 350 µg/mL a cytostasis of 55% was noted.

In experiment I **with** metabolic activation no increase of the cytostasis above 30% was noted up to 100 µg/mL. At 250 µg/mL a cytostasis of 44% and at 300 µg/mL a cytostasis of 59% was observed.

In experiment II **without** metabolic activation no increase of the cytostasis above 30% was noted up to 50 µg/mL. At 100 µg/mL a cytostasis of 66% was observed.

12.2.3. Clastogenicity / Aneugenicity

In experiment I **without** metabolic activation the micronucleated cell frequency of the negative control (0.35%) was within the historical control limits of the negative control (0.18% - 1.18%, Table 15) and the micronucleated cell frequency of the solvent control (0.35%) was within the historical control limits of the solvent control (0.02% - 1.38%, Table 15). The mean values of micronucleated cells found after treatment with the test item were 0.50% (250 µg/mL), 0.30% (325 µg/mL) and 0.45% (350 µg/mL). The numbers of micronucleated cells were within the historical control limits of the solvent control and did not show a biologically relevant increase compared to the concurrent solvent control

In experiment I **with** metabolic activation the micronucleated cell frequency of the negative control (0.45%) was within the historical control limits of the negative control (0.15% - 1.19%, Table 15) and the micronucleated cell frequency of the solvent control (0.35%) was within the historical control limits of the solvent control (0.13% - 1.22%, Table 15). The mean values of micronucleated cells found after treatment with the test item were 0.35% (100 µg/mL), 0.40% (250 µg/mL) and 0.30% (300 µg/mL). The numbers of micronucleated cells were within the historical control limits of the solvent control and did not show a biologically relevant increase compared to the concurrent solvent control

In experiment II **without** metabolic activation the micronucleated cell frequency of the negative control (0.40%) was within the historical control limits of the negative control (0.16% - 1.10%,Table 15) and the micronucleated cell frequency of the solvent control (0.55%) was within the historical control limits of the solvent control (0.06% - 1.10%,Table 15). The mean values of micronucleated cells found after treatment with the test item were 0.60% (25 µg/mL), 0.25% (50 µg/mL) and 0.65% (100 µg/mL). The numbers of micronucleated cells were within the historical control limits of the solvent control and did not show a biologically relevant increase compared to the concurrent solvent control.

The nonparametric χ^2 Test was performed to verify the results in both experiments. No statistically significant increase ($p<0.05$) of cells with micronuclei was noted in the dose groups of the test item evaluated in experiment I and II **with** and **without** metabolic activation.

The χ^2 Test for trend was performed to test whether there is a concentration-related increase in the micronucleated cells frequency in the experimental conditions. No statistically significant increase in the frequency of micronucleated cells under the experimental conditions of the study was observed in experiment I and II (Table 14).

MMS (50 and 65 µg/mL) and CPA (15 µg/mL) were used as clastogenic controls and colchicine as aneugenic control (0.02 and 0.4 µg/mL). They induced distinct and statistically significant increases of the micronucleus frequency, demonstrating the validity of the assay.

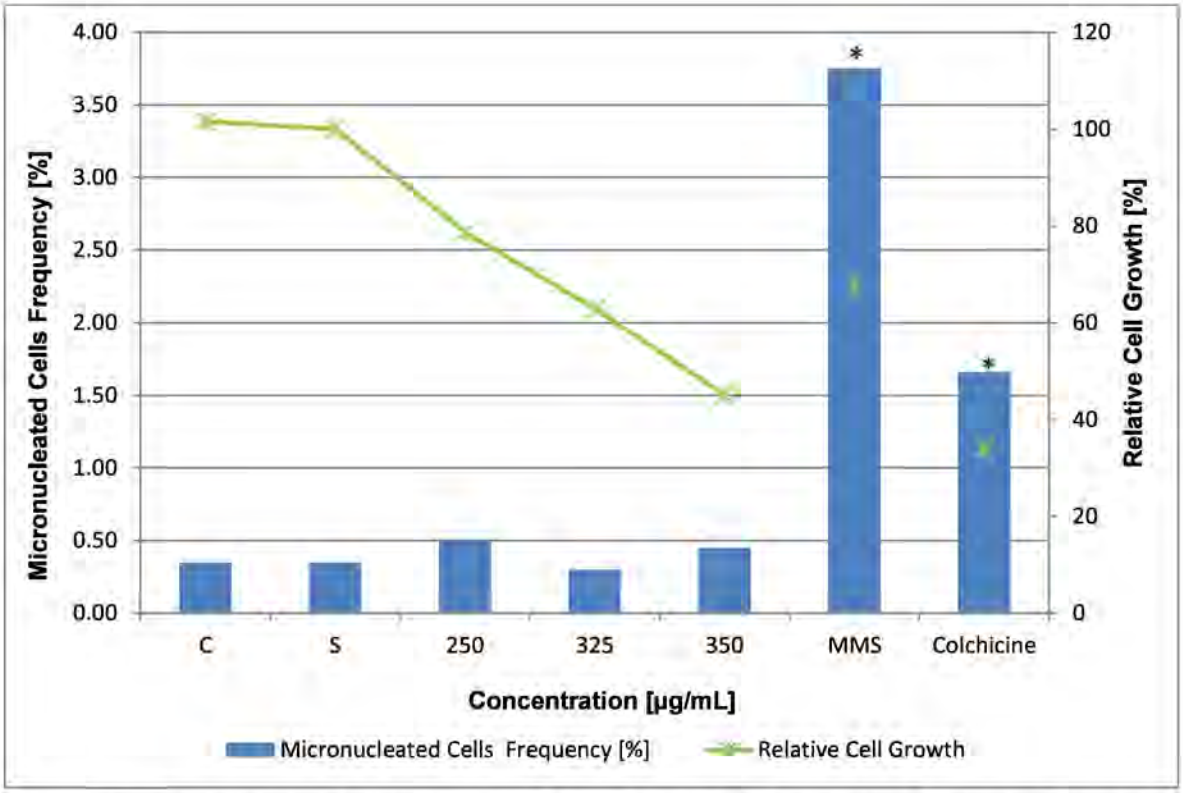

Figure 1: Micronucleus Frequency and Growth rate for Experiment I *without* metabolic activation

The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.

Micronucleated Cell Frequency was determined in 2000 cells (1000 cells per slide), except for colchicine (1349 cells).

C:	Negative Control (Culture medium)
S:	Solvent Control (DMSO 1% v/v in culture medium)
MMS:	Methylmethanesulfonate, Positive Control (without metabolic activation) [65 µg/mL]
Colchicine:	Positive Control (without metabolic activation) [0.4 µg/mL]
CBPI:	Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative Cell Growth:	$100 \times ((CBPI_{Test\ conc} - 1) / (CBPI_{control} - 1))$
c_1:	mononucleate cells
c_2:	binucleate cells
c_x:	multinucleate cells
n:	total number of cells
*:	statistically significant increase of micronucleated cells

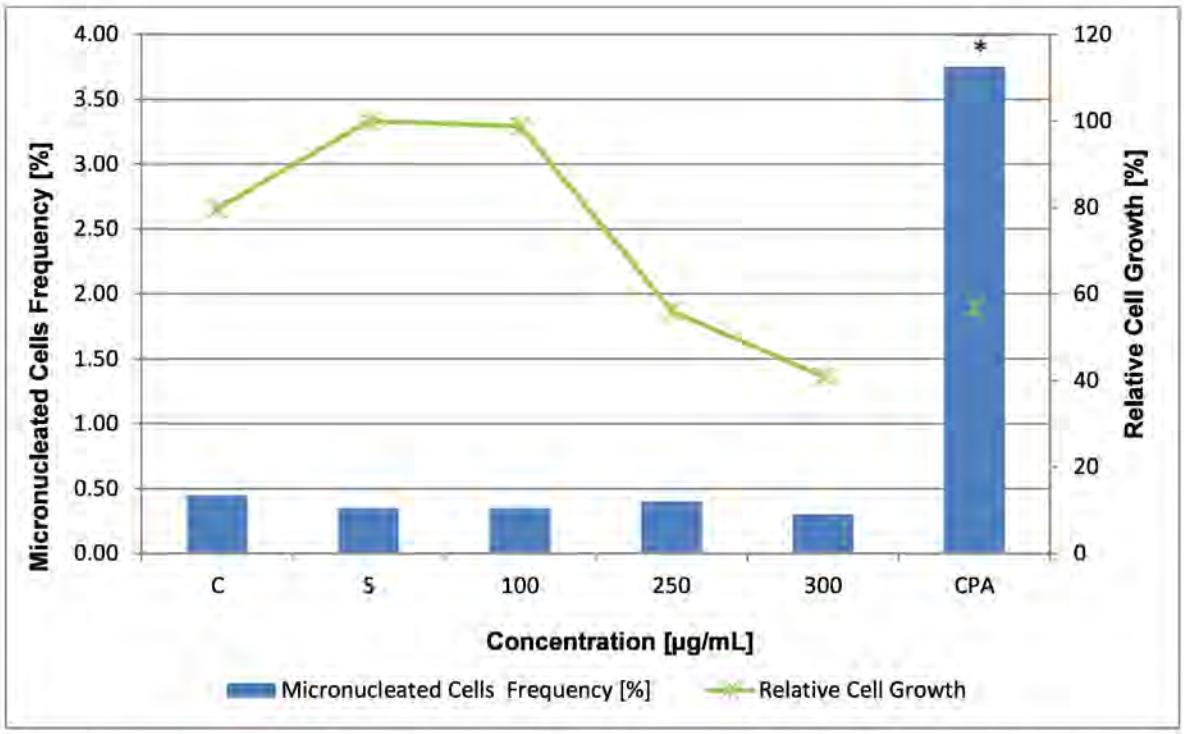

Figure 2: Micronucleus Frequency and Growth rate for Experiment I *with* metabolic activation

The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.

Micronucleated Cell Frequency was determined in 2000 cells (1000 cells per slide).

C:	Negative Control (Culture medium)
S:	Solvent Control (DMSO 1% v/v in culture medium)
CPA:	Cyclophosphamide, Positive Control (with metabolic activation) [15 µg/mL]
CBPI:	Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative Cell Growth:	$100 \times ((CBPI_{Test\ conc} - 1) / (CBPI_{control} - 1))$
c_1:	mononucleate cells
c_2:	binucleate cells
c_x:	multinucleate cells
n:	total number of cells
*:	statistically significant increase of micronucleated cells

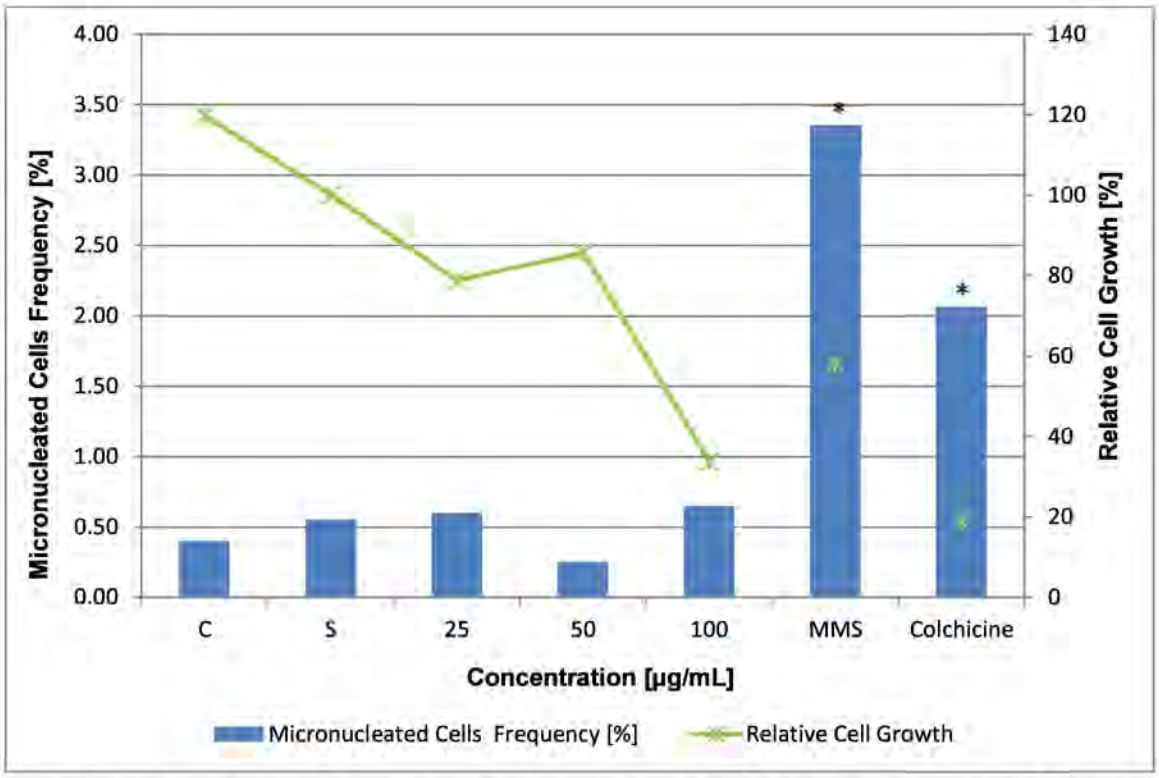

Figure 3: Micronucleus Frequency and Growth rate for Experiment II *without* metabolic activation

The CBPI was determined in 500 cells per culture of each test group.
The relative values of the CBPI are related to the solvent control.

Micronucleated Cell Frequency was determined in 2000 cells (1000 cells per slide), except for MMS (1864 cells) and Colchicine (1242 cells).

C:	Negative Control (Culture medium)
S:	Solvent Control (DMSO 1% v/v in culture medium)
MMS:	Methylmethanesulfonate, Positive Control (without metabolic activation) [50 µg/mL]
Colc:	Colchicine, Positive Control (without metabolic activation) [0.02 µg/mL]
CBPI:	Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Relative Cell Growth:	$100 \times ((CBPI_{\text{Test conc}} - 1) / (CBPI_{\text{control}} - 1))$
c_1:	mononucleate cells
c_2:	binucleate cells
c_x:	multinucleate cells
n:	total number of cells
*:	statistically significant increase of micronucleated cells

13. Conclusion

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) did not induce structural and/or numerical chromosomal damage in human lymphocytes.

Therefore, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) is considered to be non-mutagenic with respect to clastogenicity and/or aneugenicity in the *in vitro* Mammalian Cell Micronucleus Test.

14. Distribution of the Report

Original:	Eurofins Munich
Copy:	sponsor

15. References

15.1. Guidelines

[1] Act on Protection against Hazardous Substances (Chemicals Act - ChemG) "Chemicals Act in the version of the Announcement of 28 August 2013 (FLG I p. 3498, 3991), last amended by Article 115 of the Act of 10 August 2021 (FLG I p. 3436)

[2] Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998

[3] OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

[4] OECD Guideline for the Testing of Chemicals, Section 4, No. 487, "*In Vitro* Mammalian Cell Micronucleus Test", adopted 29 July, 2016

[5] Commission regulation (EU) 2017/735 B.49 "*In Vitro* Mammalian Cell Micronucleus Test", dated February 14, 2017

15.2. Literature

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[12] Matsushima, T., Sawamura, M., Hara, K. and Sugimura, T. (1976). A Safe Substitute for Polychlorinated Biphenyls as an Inducer of Metabolic Activation Systems. In: de Serres, F.J., Fouts, J.R., Bend, J.R. and Philpot R.M., (eds). In Vitro Metabolic Activation in Mutagenesis Testing. Elsevier, North-Holland, 85-88

[13] Ames, B.N., McCann; J. and Yamasaki, E. (1977). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. In: B..J. Kilbey et al (Eds.): Handbook of Mutagenicity Test Procedures, Elsevier, Amsterdam, 1-17

[14] Hagens Handbuch der Pharmazeutischen Praxis. Folgeband 1. 1995. Hrsg. H. Schumann and G. Wurm, p. 261

15.3. Internal Eurofins Munich SOP

Standard Operating Procedures (SOPs), No. 15-2-14

16. Appendix

16.1. Appendix 1: Historical Laboratory Control Data

Table 15: Historical Laboratory Control Data of the negative and solvent control in human lymphocytes (in 2016-2021)

	Negative Control				Solvent Control		
	metabolic activation				metabolic activation		
	without		with		without		with
	4 h	44 h	4 h		4 h	44 h	4 h
Mean	0.68	0.63	0.67	**Mean**	0.70	0.58	0.67
SD	0.25	0.24	0.26	**SD**	0.34	0.26	0.27
RSD	36.74	37.48	38.97	**RSD**	48.54	44.56	40.08
Min	0.25	0.23	0.20	**Min**	0.21	0.20	0.25
Max	1.50	1.30	1.30	**Max**	1.45	1.55	1.40
LCL	0.18	0.16	0.15	**LCL**	0.02	0.06	0.13
UCL	1.18	1.10	1.19	**UCL**	1.38	1.10	1.22
n	101	93	99	**n**	36	33	35

Negative Control: Cell culture medium
Solvent Control: DMSO 1% v/v or ethanol 0.5% v/v in cell culture medium
Mean: Mean number of micronucleated cells (%)
SD: Standard Deviation
RSD: Relative Standard Deviation (%)
Min: Minimum number of micronucleated cells (%)
Max: Maximum number of micronucleated cells (%)
LCL: Lower control limit (95%, mean-2SD)
UCL: Upper control limit (95%, mean+2SD)
n: Number of assays

Table 16: Historical Laboratory Control Data of the positive control in human lymphocytes (in 2016-2021)

	Positive Control				
	metabolic activation				
	without				with
	MMS		Colchicine		CPA
	4 h	44 h	4 h	44 h	4 h
Mean	3.14	3.36	2.77	3.18	3.00
SD	1.64	1.54	1.50	1.80	1.09
RSD	52.25	45.93	54.17	56.45	36.36
Min	1.00	0.85	0.85	0.80	1.00
Max	9.30	7.80	8.64	9.48	6.70
LCL	0.00	0.27	0.00	0.00	0.82
UCL	6.41	6.45	5.78	6.78	5.18
n	59	54	84	78	83

MMS:	Positive Control-clastogenicity without metabolic activation: Methylmethanesulfonate
Colchicine:	Positive Control- aneugenicity without metabolic activation
CPA:	Positive Control-clastogenicity with metabolic activation: Cyclophosphamide
Mean:	Mean number of micronucleated cells (%)
SD:	Standard Deviation
RSD:	Relative Standard Deviation (%)
Min:	Minimum number of micronucleated cells (%)
Max:	Maximum number of micronucleated cells (%)
LCL:	Lower control limit (95%, mean-2SD)
UCL:	Upper control limit (95%, mean+2SD)
n:	Number of assays

16.2. Appendix 2: Raw Data – CBPI

Table 17: Distribution of mono-, bi- and multinucleate cells - pre-experiment

Dose Group	Concentration [µg/mL]	Mono	Bi	Multi	CBPI
without metabolic activation					
C	0	353	130	17	1.33
S	0	389	93	18	1.26
1	7.8	286	173	41	1.51
2	15.6	327	154	19	1.38
3	31.3	285	173	42	1.51
4	62.5	317	160	23	1.41
5	125	298	154	48	1.50
6	250	314	151	39	1.45
7	500	471	29	0	1.06
8	1000	492	8	0	1.02
9	1500	499	1	0	1.00
10	2000	498	2	0	1.00
with metabolic activation					
C	0	329	128	43	1.43
S	0	351	129	20	1.34
1	7.8	332	141	27	1.39
2	15.6	277	177	46	1.54
3	31.3	338	136	26	1.38
4	62.5	332	140	28	1.39
5	125	321	135	44	1.45
6	250	333	125	42	1.42
7	500	486	14	0	1.03
8	1000	499	1	0	1.00
9	1500	500	0	0	1.00
10	2000	500	0	0	1.00

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
CBPI: Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Mono: number of mononucleate cells
Bi: number of binucleate cells
Multi: number of multinucleate cells

Table 18: Distribution of mono-, bi- and multinucleate cells – experiment I without metabolic activation

Dose group	Concentration [µg/mL]	Culture	Mono	Bi	Multi	CBPI
C	0	1/2	361	107	32	1.34
		2/2	373	96	31	1.32
S	0	1/2	349	110	41	1.38
		2/2	385	98	17	1.26
2	250	1/2	348	112	40	1.38
		2/2	446	45	9	1.13
4	325	1/2	418	71	11	1.19
		2/2	404	81	15	1.22
5	350	1/2	416	74	10	1.19
		2/2	451	46	3	1.10
MMS	65 µg/mL	1/2	403	78	19	1.23
		2/2	410	78	12	1.20
Colchicine	0.4 µg/mL	1/2	439	58	3	1.13
		2/2	455	43	2	1.09

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)
CBPI: Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Mono: number of mononucleate cells
Bi: number of binucleate cells
Multi: number of multinucleate cells

Table 19: Distribution of mono-, bi- and multinucleate cells - experiment I with metabolic activation

Dose group	Concentration [µg/mL]	Culture	Mono	Bi	Multi	CBPI
C	0	1/2	353	112	35	1.36
		2/2	352	106	42	1.38
S	0	1/2	314	132	54	1.48
		2/2	329	115	56	1.45
2	100	1/2	329	128	43	1.43
		2/2	308	137	55	1.49
4	250	1/2	350	113	37	1.37
		2/2	433	59	8	1.15
5	300	1/2	398	81	21	1.25
		2/2	439	54	7	1.14
CPA	15	1/2	383	94	23	1.28
		2/2	392	90	18	1.25

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
CPA: Cyclophosphamide, Positive Control (with metabolic activation)
CBPI: Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Mono: number of mononucleate cells
Bi: number of binucleate cells
Multi: number of multinucleate cells

Table 20: Distribution of mono-, bi- and multinucleate cells - experiment II without metabolic activation

Dose group	Concentration [µg/mL]	Culture	Mono	Bi	Multi	CBPI
C	0	1/2	225	217	58	1.67
		2/2	201	240	59	1.72
S	0	1/2	282	178	40	1.52
		2/2	233	215	52	1.64
1	25	1/2	308	171	21	1.43
		2/2	282	194	24	1.48
2	50	1/2	222	272	6	1.57
		2/2	297	196	7	1.42
3	100	1/2	383	117	0	1.23
		2/2	422	78	0	1.16
MMS	50	1/2	334	131	35	1.40
		2/2	385	96	19	1.27
Colchicine	0.02	1/2	457	43	0	1.09
		2/2	434	66	0	1.13

C: Negative Control (Culture medium)
S: Solvent Control (DMSO 1% v/v in culture medium)
MMS: Methylmethanesulfonate, Positive Control (without metabolic activation)
Colchicine: Positive Control (without metabolic activation)
CBPI: Cytokinesis Block Proliferation Index, CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$
Mono: number of mononucleate cells
Bi: number of binucleate cells
Multi: number of multinucleate cells

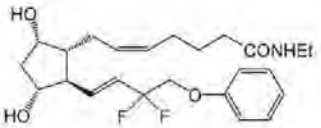
16.3. Appendix 3: Certificate of Analysis

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{33}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications.

Quality Contro [redacted] *Date:* 23NOV 2022
Approved: [redacted] *Date:* 23 Nov 2022

Quality Control Laboratory: [redacted]

16.4. Appendix 4: S9-Homogenate Certificate

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page 1 of 2

Quality Control & Production Certificate
S9-Homogenate (Eurofins Charge No. 251122)

Species, Strain, Sex, Tissue:	rat, Wistar, male, liver
Supplier:	Charles River
Weight at Delivery:	approx. 200-250 g
Inducing Agents:	Phenobarbital (Na-Salt) [Sigma, SLCD9096; 80 mg/kg bw β-Naphthoflavone [Sigma, SLCF5489]; 100 mg/kg bw
Vehicle:	Cotton Seed Oil [Sigma, MKCR3879]
Application:	per oral route on 3 consecutive days
Date of Preparation:	25 November 2022
Expiry Date:	25 November 2024
Determination of Protein Content:	BCA-Assay (BSA-calibration curve), 12 January 2023
Protein Content:	39.0 mg/mL
Sterility Test:	28 November 2022 / passed

Metabolic Activation Tests:
a) in Bacteria: Ames Test (2.5 µg 2-AA/plate):

Volume S9-Homogenate [µL]	TA 98 Revertants	TA 98 Range[a] (Min - Max)	TA 98 passed/failed	TA 100 Revertants	TA 100 Range[a] (Min - Max)	TA 100 passed/failed
0	43 47 30	15 - 140	passed passed passed	100 120 131	83 - 335	passed passed passed
20	1425 1749 1517	338 - 3065	passed passed passed	2358 2327 2824	994 - 2814	passed passed passed[b]
50	1015 1805 1627	526 - 3252	passed passed passed	2790 1773 1828	856 - 2311	passed[b] passed passed

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany
Tel. +49 (0)89 899 690-0
Fax +49 (0)89 899 690-11

GLP / GMP FDA approved

Quality Control & Production Certificate
S9-Homogenate (Eurofins Charge No. 251122)

b) in Bacteria: Ames Test (5 µg B[*a*]P/plate):

Volume S9-Homogenate [µL]	TA 98 Revertants	TA 98 Range[a] (Min - Max)	TA 98 passed/ failed	TA 100 Revertants	TA 100 Range[a] (Min - Max)	TA 100 passed/ failed
0	36 47 30	10 - 62	passed passed passed	93 98 86	40 - 160	passed passed passed
20	117 139 127	69 - 201	passed passed passed	241 254 271	153 - 479	passed passed passed
50	143 188 155	63 - 264	passed passed passed	367 349 315	211 - 711	passed passed passed

a: Range of historical control data from 2011 – June 2022 for Ames Test.
b: Considered as acceptable for inclusion in the historical control database.

C. Freitag

Dr. Christine Freitag
Deputy Head of *in vitro* Pharmacology/Toxicology

16.5. Appendix 5: Amendments to Study Plan

16.5.1. 1st Amendment to Study Plan

eurofins | BioPharma Product Testing

In vitro Mammalian Micronucleus Assay

in Human Lymphocytes

with

Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)

1st Amendment to Study Plan

Version: Final

Pages: 5

Eurofins Munich Study No.: STUGC22AA2158-3

Sponsor:

[redacted]

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany
Tel. +49 (0)89 899 650-0
Fax. +49 (0)89 899 650-11

GLP/GMP FDA approved

1. Amendments

Concerning:

4.2. General, study plan, p. 06

Before:

[…]

Study Monitors: Dr. Thomas Petry (technical matters)
ToxMinds
Thomas.Petry@toxminds.com

[redacted]

Alexis Klock
Eurofins Product Testing US

[…]

New:

[…]

Study Monitors: Dr. Thomas Petry (technical matters)
ToxMinds
Avenue de Broqueville 116
1200 Brussels - Belgium
Thomas.Petry@toxminds.com

[redacted]

Alexis Klock
Eurofins Product Testing US Inc
11822 North Creek Pkwy Suite 110
Bothell, WA 98011

[…]

Reason:

Sponsor requested inclusion of contact details of the study monitors.

Concerning:

7.1. Characterisation of the Test Item, study plan, p. 10

Before:

[…]

Name:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
CAS No.:	1185851-52-8
Batch No.:	TAF-10-1122-01

[…]

New:

[…]

Name:	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
CAS No.:	1185851-52-8
EC No.:	867-521-0
Batch No.:	TAF-10-1122-01

[…]

Reason:

Sponsor requested inclusion of the EC number of the test item.

2. Project Staff Signatures

Member of the
Quality Assurance Unit

Cornelia Kübber

Print Name: Cornelia Kübber

Date: 16 FEB 2023

Study Director

Dr. Claudia Donath

Claudia Donath

Date: 16 Feb 2023

3. Distribution of the Amendment

Original:	Eurofins Munich
Copy:	sponsor, study director, QAU

16.5.2. 2nd Amendment to Study Plan

eurofins | BioPharma Product Testing

In vitro Mammalian Micronucleus Assay

in Human Lymphocytes

with

Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)

2nd Amendment to Study Plan

Version: Final

Pages: 04

Eurofins Munich Study No.: STUGC22AA2158-3

Sponsor:

[redacted]

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany
Tel +49 (0)89 899 650-0
Fax +49 (0)89 899 650-11

GLP/GMP FDA approved

1. Amendment

Concerning:

Study Director, study plan, p. 2, 6

Before:

Dr. Claudia Donath

New:

Dr. Stephanie Lacmanski

Reason:

Project handover

2. Project Staff Signatures

Member of quality assurance unit

Cornelia Kübber

Print name: Cornelia Kübber

Date: 25 AUG 2023

Study director

J. Lacmanski

Dr. Stephanie Lacmanski

Date: 25 AUG 2023

3. Distribution of the Amendment

Original:	Eurofins Munich
Copy:	Sponsor, study director, QAU

Determination of the Ocular Safety and Irritation Potential of an Eyelash Conditioner

Final Report
Study N°09AP-1110

Study Sponsor:

[redacted]

Study Investigator:

évalulab

Represented by Andrea Sebesten
5475 Paré, Suite 206, Mont-Royal,
Québec, H4P 1P7
Tel: (514) 343-0001
Fax: (514) 343-9996
Email: asebesten@evalulab.com

This report is composed of 43 pages including appendices (32 pages).

January 8th, 2010

CONTENTS

Determination of the Ocular Safety and Irritation Potential of an Eyelash Conditioner 09AP-1110 STUDY

SUMMARY

Sponsor Code:	CW
Test:	Determination of the Ocular Safety and Irritation Potential of an eyelash conditioner tested on 20 healthy volunteers during a 28-day test period.
Product tested: Customer Lot#: Evalulab Lot#:	Enhanced Peptide Conditioner TEA .025% 100109-3 (10-7-09) 091020.CW.01
Date:	January 8th, 2010
Results:	Under the conditions described in the procedure referenced above, the test product referenced above did not produce any signs of ocular irritation or hypersensitivity of clinical magnitude, in the totality of the test panel (20 volunteers).
Conclusion:	On the basis of the results, the test product may therefore be considered as safe or as "Non-irritant to the eyes". Furthermore, given the medical supervision provided during the study, the test product may bear the claim "Tested under the control of an ophthalmologist".
Signatures:	

Doctor Jean-Pierre Chartrand, M.D.
Ophthalmologist-Surgeon

Andrea Sebesten, B.Sc.
Investigator, Evalulab Inc.

STUDY OBJECTIVE

The objective of this study was to evaluate the Ocular Safety and Irritation potential of an eyelash conditioner. The study involved 22 healthy female volunteers applying the aforementioned product once a day for a period of 28 days.
The volunteers were examined by an ophthalmologist at the beginning of the study (D0) and at the end of the study (D28).
This was followed by the completion of a detailed subjective questionnaire by the volunteers, evaluating parameters such as tolerance, sensory attributes and the effect of the test product on the appearance of the length and volume of their eyelashes.

PROTOCOL

1. Ethics Committee

This procedure and associated documents were reviewed and approved on October 14th, 2009, prior to the commencement of the study, by an Ethics Committee (an independent organization whose responsibility is to ensure the protection of the rights, security and well being of the volunteers participating in the study).

2. Duration

The ocular irritation potential test took place from November 10th to December 09th, 2009.

3. Investigation Site

Evalulab Inc. located at 5475 rue Paré, Suite 206, Mont-Royal, Québec Canada.

4. Personnel

This study was conducted by Evalulab Inc. represented by Andrea Sebesten, B.Sc. (Investigator) under the medical supervision of Doctor Jean-Pierre Chartrand, MD Ophthalmologist-Surgeon.

5. Test Product

Upon reception, the test product was registered in the "Receptions Book" and assigned a code, followed by its storage at ambient humidity and temperature in its original container (as received) in an area allocated for this purpose.
The test product was provided in 24 homogeneously labeled containers and in sufficient quantities:

24 containers x 8 ml of **Enhanced Peptide Conditioner TEA .025%,**
Lot: 100109-3 (10-7-09)
Evalulab lot: 091020.CW.01

6. Quality Assurance

Good Clinical Practices (GCP) are defined by the totality of the pronouncements put in place for ensuring the quality and authenticity of the trials and the obtained data on one hand and the respect for the ethics on the other.
The data obtained for each volunteer is recorded in individual Case Report Forms. The data entry is made in black ink. In case of errors or omissions, the initial entry is crossed out and initialled by the investigator.
All recorded data is validated by the investigator, who assumes responsibility for the quality of the work presented and verifies that all gathered data is in agreement with the protocol.
The records obtained during the study will be kept by Evalulab Inc. for a period of 2 years.

7. Adverse Events or Serious Adverse Events

Volunteers were asked to immediately communicate any reactions to Evalulab. Tolerance to the test product was evaluated based on the observed reactions and their degree of severity, as well as on the reproducibility from one volunteer to another.

An "Adverse Event" is defined as any noxious and unintended response observed in a volunteer testing a product that does not necessarily have a causal relation with the test product or the treatment in question.

The risks for adverse events associated with this test, both cutaneous and ocular may vary amongst the volunteers. Volunteers may be subject to ocular discomfort, rash (intense redness), cracking, exfoliation effect, dryness, or even pain if the product to be tested is strongly irritant or if the volunteer is particularly sensitive to the product. Volunteers may also develop an allergic sensitization to the test product or to its components.

The term "Severe Adverse Event" refers to any untoward medical occurrence, related or not to the test product that may lead to death, persistent or significant disability, that requires hospitalization or prolongation of a hospitalization period or that provokes invalidity, significant or permanent incapacity, or that translates to congenital anomaly or malformation.

8. Amendments to Protocol

There were no amendments to the protocol.

9. Ocular Safety Evaluation

• Type of Study

Monocentric and open-ended, meaning the evaluator, volunteers, and sponsors alike, were aware of the nature of the test material.

• Volunteers

Volunteer Recruitment:
A total of 22 female volunteers were recruited for the purpose of this study. The profile of each volunteer is presented in Table I in the Appendices.

Informed Consent Forms:
All volunteers had to read, sign and date the Informed Consent Forms explaining the conditions of the test, the risks involved and briefly describing the product to be tested. Each volunteer was informed verbally and in writing about the nature of the test and of the potential risks involved.

Confidentiality:
Participation of the volunteers in this study is confidential. The information gathered in the course of the study was recorded in individual Case Report Forms, which are numerically coded and do not contain the names of the volunteers.
Only the employees of Evalulab, auditors of the sponsor, and regulatory bodies (FDA, Health Canada and the ethics committee) may have access to the confidential information.

Inclusion Criteria:

1. Volunteers of the feminine sex, between18 and 60 years of age,
2. With self-declared small and/or sparse appearing eyelashes,
3. Without any dermal anomalies in the areas to be tested that may interfere with the results of the study,
4. Cooperating in the study, able to be monitored at each visit, aware of the demands and duration of the controls, thus allowing perfect adherence to the established protocol,
5. Who agree to sign the two Informed Consent Forms for the study with full knowledge of the details of the study and the risks involved,
6. Who use an adequate method of contraception (contraceptive pill, condoms, spermicidal creams, an intra-uterine device (IUD), abstinence ...).

Exclusion Criteria:

1. Women with a history of skin irritation or allergies to the type of product to be tested or in general, with allergies to certain food, to certain chemical products, to jewellery...,
2. With a history of eye diseases (glaucoma, seasonal conjunctivitis...) or currently suffering from ocular anomalies as judged by the certified ophthalmologist during the initial eye examination. Women wearing eye glasses or lenses are admissible,
3. Who suffer from a serious illness or health problem, or a critical or progressive disease (asthma, diabetes, cancer, immunological deficiency, removed organ ...),
4. Who have taken prescription or over the counter medication (at a frequency equal to or more than 3 doses per week) that could affect skin characteristics or could bias the study (i.e. antibiotics, steroids, antihistamines, anti-inflammatories,...) within 7 days of study start,
5. Who frequent tanning salons or foresee exposure to the sun during the study,
6. Who abuse alcohol, drugs or/and tobacco,
7. Women who are pregnant, lactating or expecting to become pregnant during the study.

- **Study Design**

At Day D=0, the volunteers visited the laboratory for verification of the inclusion/exclusion criteria, which was followed by the signing of the Informed Consent Forms explaining the conditions of the test, the risks involved and a description of the treatment.

Next, each volunteer was given the test product, a follow-up sheet to be completed after every application and a self-evaluation questionnaire to be completed after four weeks of treatment. The volunteers were instructed to apply the test product, once a day in the evening after having washed their face, along the upper eyelashes, in the same way that they would apply eyeliner.
The use of all other skin care products (regular cleansing products, and makeup) was permitted during the study. Changes regarding the brand of their regular facial cleanser or makeup products were not permitted during the study.

Furthermore, during the first visit (D=0), the volunteers' eyes were examined by a certified ophthalmologist to discern any ocular anomalies. They were again examined during the last visit (D=28) for signs of irritation or intolerance due to the test product.

In addition, the volunteers had to bring with them their completed follow-up sheet and self-evaluation questionnaire, as well as the test product container to their last visit.

- **Evaluation**

Volunteer Participation
Volunteer participation is defined by the overall participation of the volunteers in the study and the level of tolerance as reported by the volunteers to the treatment in question. At the end of the treatment period, each volunteer completed a self-evaluation questionnaire that included a section on tolerance.

Self Evaluation Questionnaire
Each volunteer completed a Self Evaluation Questionnaire at the end of the study detailing the tolerance to and evaluation of the test product on the appearance of their eyelashes. The Self Evaluation Questionnaire concerns the cosmetic qualities (texture, odour, application, absorption...) of the test product as well as a subjective assessment of the effect on the appearance of the eyelashes. Furthermore, the volunteers were encouraged to write down the comments that they may have concerning the product.

Evaluation of Ocular Safety and Irritation Potential by Ophthalmologist Assessment
Prior to acceptance into the study, each volunteer received a complete ophthalmic examination by a certified ophthalmologist to ensure ocular health and if appropriate, the correct fit of their contact lenses.
Evaluation for the potential of ocular irritation was based on questions asked to the volunteers and on an eye examination by the ophthalmologist:

Questioning: by the ophthalmologist to the volunteers
Questions regarding the following discomforts were asked: cloudy vision, pain, redness and other types of discomfort (itching, watery eyes, sensations of heat and burning).

Ophthalmic Examination
The examination was performed with a slit lamp and included the subject's eyelids, cornea, conjunctiva, anterior chambers and papillary reactions, and vision acuity. The procedure was repeated for each volunteer after 28 days of product use.
Apparition of any reaction in the eyes and or in the eye contour area was evaluated by the ophthalmologist and observations (if any) were recorded in each volunteer's Case Report Form.

RESULTS

All individual data collected from the self-evaluation questionnaires are presented in Tables II.A, II.B and II.C in the Appendices.
A summary of each section for which results were obtained is provided below, with a discussion.

1. Volunteer Participation

◦ Participation

Twenty-two (22) female volunteers between 18 and 60 years of age (Average Age = 34.23) were recruited.
Three (3) volunteers did not complete the study for various reasons:
- Volunteer #01-1110-003 did not return to the laboratory for the second assigned visit because of schedule incompatibility.
- Volunteer #01-1110-016 did not return to the laboratory for the second assigned visit without expressing her reasons.
- Volunteer #01-1110-021 did not return to the laboratory for the second assigned visit because of a medical problem unrelated to the test product.
The remaining 19 volunteers completed the study.

◦ Tolerance as reported by the volunteers

Four volunteers reported the presence of different signs of intolerance, as follows:
- Volunteer (#01-1110-005) experienced a slight redness, itching, stinging and burning sensation 3 days during the study. These intolerance signs were not observed by the ophthalmologist at D28, but they were noted in the volunteer's case report form as slight in intensity, as having a possible connection with the test product and as having disappeared spontaneously. The intolerance signs were experienced only when a larger amount of product was applied along the upper eyelashes.
-Volunteer (#01-1110-006) experienced a slight burning sensation once during the treatment. This incident was not observed by the ophthalmologist at D28, but it was noted in the volunteer's case report form as slight in intensity, as having a probable connection with the test product and as having disappeared spontaneously. The burning sensation was experienced only when the volunteer applied a greater amount of product along the upper eyelashes.
-Volunteer (#01-1110-007) experienced a slight itching sensation 2 days during the study. This was noted in the volunteer's case report form by the ophthalmologist at the second assigned visit as slight in intensity and as not having any connection with the test product.
-Volunteer (#01-1110-014) experienced a slight stinging sensation 2 days during the treatment. This incident was not observed by the ophthalmologist at D28, but it was noted in the volunteer's case report form as very slight in intensity and as having a probable connection with the test product.

The overall tolerance scores to the test product after 28 days of treatment are summarized below in Figure 1.

Figure 1: Overall scores for tolerance at D28

Intolerance Criteria	"None"	"Slight"	"Moderate" + "High"
Puffiness	100%	0%	0%
Other: dryness around the eyes	95%	5%	0%
Redness	89%	11%	0%
Eye watering	89%	11%	0%
Stinging	84%	16%	0%
Itching	79%	21%	0%
Burning sensation	74%	21%	5%

The test product was moderately tolerated by the majority of the volunteers.
According to the results presented in Figure 1, 5% of the participants experienced a "Moderate" + "High" burning sensation, 21% of the volunteers experienced a "Slight" itching sensation, 16% a "Slight" stinging sensation and 11% "Slight" eye watering and redness. Additionally, 5% of the participants experienced a "Slight" dryness sensation around the eyes.

2. Evaluation of consumer satisfaction by subjective self-evaluation questionnaire

- **Sensory Attributes**

The scores obtained for the sensory attributes at day 28 are summarized below in Figure 2. The results are expressed as the combined percentage of volunteers who indicated either "Appreciated" or "Highly Appreciated" (Total appreciated) or "Indifferent" for each sensory attribute.

Figure 2: Overall scores for sensory attributes at D28

Criteria	Total Appreciated	Indifferent
Ease of application	94%	6%
Texture	79%	16%
Fragrance	63%	32%
Feeling after application	53%	26%

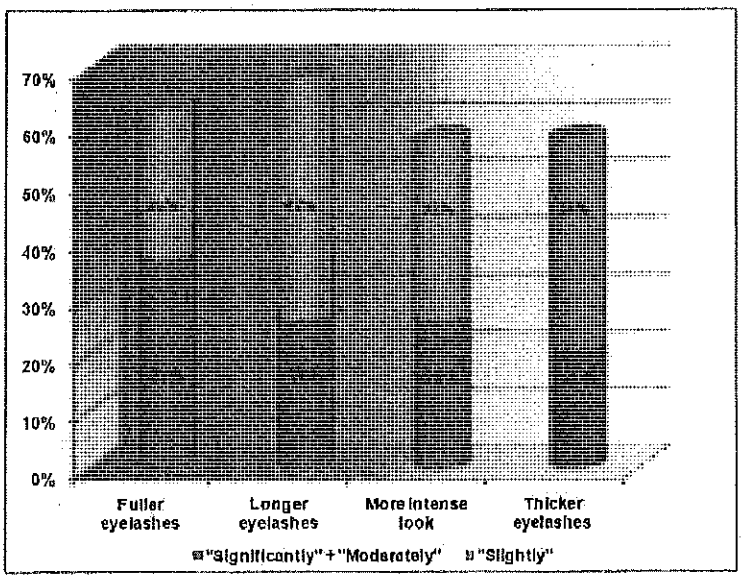
The overall level of appreciation for the sensory attributes was good for the test product. Ninety-four percent (94%) the volunteers appreciated the ease of application of the test product. Seventy-nine percent (79%) of the participants appreciated the texture of the test product, while 16% were "Indifferent" to the previously mentioned parameter. The fragrance was appreciated by 63% of the volunteers. Additionally, 32% of the participants were "Indifferent" to the fragrance of the test product. The feeling after application was the least appreciated attribute, receiving a score of only 53%. However, it is important to note that 26% of the volunteers were "Indifferent" to the feeling after application.

- **Subjective assessment of the effect on the appearance of the eyelashes**

To assess the effect of the product on the appearance of the eyelashes at D28, the volunteers were to complete questions pertaining to the appearance of longer, thicker and fuller eyelashes and to a more intense look at day 28. The responses for each parameter expressed as "Significantly", "Moderately", and "Slightly" have been combined and summarized as the total percentage of positive responses in Figure 3.

All scores for performance collected from the self-evaluation questionnaires completed by the volunteers are presented in Table II.C in the Appendices.

Figure 3: Overall scores for the effect on the appearance of the eyelashes at D28



From the results presented in Figure3, it is shown that 37% of the volunteers considered their eyelashes as appearing "Significantly" + "Moderately" fuller, while 26% of the participants noticed a "Slightly" improvement in the same criteria at day 28, resulting in a 63% positive response rate.

Twenty-six percent (26%) of the volunteers noticed that the appearance of the length of their eyelashes was "Significantly" + "Moderately" improved, while 42% of the volunteers observed a "Slightly" improvement for the same parameter at day 28, resulting in a 68% positive response rate.

Scores for the parameter "More Intense Look" showed that a total of 58% of the volunteers observed an improvement for this parameter (26% "Significantly" + "Moderately" and another 32% "Slightly").

Additionally, 21% of the volunteers considered their eyelashes as appearing "Significantly" + "Moderately" thicker, while 37% of the participants noticed a "Slightly" improvement in the same criteria at day 28, resulting in a 58% positive response rate.

Other data not shown in the figures above:
Overall scores for the question **"After how many days of use of the test product did you first notice an improvement in the appearance of your lashes?"** indicated that:
- None of the volunteers noticed an improvement after the first 7 days of use,
- Thirty-two percent (32%) observed an improvement after 14 days of use,
- Twenty-six percent (26%) saw an improvement after 21 days of use,
- Ten percent (10%) perceived an improvement after 28 days of use, and the remaining 32% of the participants did not see any improvements at the end of the study.

Responses to additional questions at the completion of the study pertained to global performance, recommendation to others, and readiness to purchase the test product.
The global performance of the test product was rated as follows: 0% "Excellent", 16% "Very Good" and 47% "Good" resulting in a total of sixty-three percent (63%) of the participants who considered the test product as a performing eyelash conditioner. However, it is important to note that an additional 11% of the volunteers rated the test product as an "Acceptable" eyelash conditioner.

Seventy-three percent 73% (5% "Strongly agree" and 68% "Somewhat agree") would recommend the test product to others, while 68% of the participants would buy the test product if it was offered at a competitive price (10% "Certainly" and 58% "Maybe").

- **Comments**

The complete list of all the comments expressed by the volunteers about the treatment is presented in Table III in the Appendices.
None of these comments need to be underscored or discussed at any length.

In addition, the description of the habitual use of an eye makeup remover and eye makeup is presented in Table IV in the Appendices.

3. Evaluation of Ocular Safety and Irritation Potential by Ophthalmologist Assessment

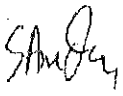
No "Adverse Events" or "Serious Adverse Events" were reported during the entire length of the study by the ophthalmologist. All completed ophthalmic examination records are provided in the Appendices.

CONCLUSION

Under the conditions of the study, the test product did not produce any ocular irritation or hypersensitivity of clinical magnitude, in the totality of the test panel (19 volunteers). Therefore the test product may be considered as safe for use as an eyelash conditioner.

Given the supervision provided by an ophthalmologist, the test product may bear the claim "Tested under the control of an ophthalmologist".

I, the undersigned, Andrea Sebesten, declare that this study was conducted under my supervision, in accordance with the principles of *"Good Clinical Practices"*. The recorded results show exactly and completely the raw data of the study.



Signature
Andrea Sebesten, B. Sc.
Investigator, Laboratory Director

Date: Mont-Royal, January 8th, 2010.

I the undersigned, Elisabeth Fiquet, declare that the information provided in this report reflects in a complete and exact manner the results obtained during the study.

Signature
Elisabeth Fiquet, B. Sc.
Quality Assurance Director, President

Date: Mont-Royal, January 8th, 2010.

APPENDICES

Table I: Volunteer Profile

Volunteer #			Initials	Age	Sex	Contact Lenses
01	-1110-	001	MC	44	F	No
01	-1110-	003	PB	25	F	No
01	-1110-	004	MD	36	F	No
01	-1110-	005	VP	18	F	Yes
01	-1110-	006	LM	20	F	No
01	-1110-	007	EM	33	F	No
01	-1110-	008	AK	26	F	Yes
01	-1110-	009	GS	36	F	No
01	-1110-	010	LC	60	F	No
01	-1110-	011	SB	33	F	No
01	-1110-	012	PT	33	F	No
01	-1110-	013	CO	54	F	No
01	-1110-	014	KP	32	F	Yes
01	-1110-	015	YG	35	F	No
01	-1110-	016	MB	36	F	No
01	-1110-	017	DM	33	F	No
01	-1110-	018	MB	34	F	No
01	-1110-	019	SR	21	F	No
01	-1110-	020	SR	20	F	No
01	-1110-	021	MG	55	F	No
01	-1110-	022	MP	22	F	No
01	-1110-	023	ES	47	F	No

Table II.A: Scores for tolerance at D28

0=None, 1=Slight, 2=Moderate and 3= High

Intolerance Criteria	001	004	005	006	007	008	009	010	011	012
Redness	0	0	1	0	0	0	0	0	0	0
Itching	0	0	1	0	1	0	0	0	0	0
Stinging	0	0	0	0	0	0	0	0	0	0
Burning sensation	0	0	1	2	1	0	0	0	0	0
Eye watering	0	0	0	0	0	0	0	0	0	0
Puffiness	0	0	0	0	0	0	0	0	0	0
Other	0	0	0	0	0	0	0	0	0	0

0=None, 1=Slight, 2=Moderate and 3= High

Intolerance Criteria	013	014	015	017	018	019	020	022	023
Redness	0	0	0	0	0	0	0	0	1
Itching	0	1	1	0	0	0	0	0	0
Stinging	0	1	0	0	0	0	0	1	1
Burning sensation	0	0	1	0	0	1	0	0	0
Eye watering	0	0	1	0	0	0	0	0	1
Puffiness	0	0	0	0	0	0	0	0	0
Other	0	0	0	0	0	0	0	0	1*

**dryness around the eyes*

Table II.B: Scores for sensory attributes at D28

3=Highly appreciated, 2=Appreciated, 1=Indifferent and 0=Did not appreciate

Criteria	001	004	005	006	007	008	009	010	011	012
Fragrance	2	2	0	3	2	1	1	2	2	3
Texture	2	3	2	3	2	2	1	2	2	3
Ease of application	2	3	2	3	2	3	3	2	2	3
Feeling after application	2	2	0	0	1	1	1	1	2	3

3=Highly appreciated, 2=Appreciated, 1=Indifferent and 0=Did not appreciate

Criteria	013	014	015	017	018	019	020	022	023
Fragrance	1	1	2	3	3	1	1	2	2
Texture	2	0	2	3	3	1	1	2	2
Ease of application	3	2	2	3	3	3	1	2	2
Feeling after application	2	0	2	3	2	0	1	2	2

Table II.C: Scores for performance at D28

7 = After 7 days, 14 = After 14 days, 21 = After 21 days, 28 = After 28 days and None

Criteria	001	004	005	006	007	008	009	010	011	012
First improvement in appearance of eyelashes observed after:	28	21	14	14	21	14	14	None	28	None

7 = After 7 days, 14 = After 14 days, 21 = After 21 days, 28 = After 28 days and None

Criteria	013	014	015	017	018	019	020	022	023
First improvement in appearance of eyelashes observed after:	None	14	14	21	21	None	None	21	None

3= Significantly, 2= Moderately, 1= Slightly and 0= Not at all

Criteria	001	004	005	006	007	008	009	010	011	012
Longer looking eyelashes	2	1	2	1	1	2	1	0	1	0
Thicker looking eyelashes	1	2	1	0	1	2	1	0	1	0
Fuller looking eyelashes	1	2	2	1	1	2	2	0	2	0
More intense look	1	0	2	0	1	2	2	0	1	0

3= Significantly, 2= Moderately, 1= Slightly and 0= Not at all

Criteria	013	014	015	017	018	019	020	022	023
Longer looking eyelashes	0	1	1	1	2	0	0	2	0
Thicker looking eyelashes	0	0	2	1	1	0	0	2	0
Fuller looking eyelashes	0	0	2	1	2	0	0	1	0
More intense look	0	1	1	1	2	0	0	2	0

EVALUATION 4=Excellent, 3=Very good, 2=Good, 1=Acceptable and 0=Bad

Criteria	001	004	005	006	007	008	009	010	011	012
Global performance	1	2	3	2	2	2	2	0	2	2

4=Excellent, 3=Very good, 2=Good, 1=Acceptable and 0=Bad

Criteria	013	014	015	017	018	019	020	022	023
Global performance	0	2	2	1	3	0	0	3	0

RECOMMENDATION 3= Strongly agree, 2= Somewhat agree, 1= Somewhat disagree, 0= Fully disagree

Criteria	001	004	005	006	007	008	009	010	011	012
Recommendation	2	2	3	2	2	2	2	1	2	2

3= Strongly agree, 2= Somewhat agree, 1= Somewhat disagree, 0= Fully disagree

Criteria	013	014	015	017	018	019	020	022	023
Recommendation	0	2	2	2	2	0	0	2	0

PURCHASE 2= Certainly, 1= Maybe, 0= Certainly not

Criteria	001	004	005	006	007	008	009	010	011	012
Purchase at competitive price	0	1	1	1	1	2	1	0	1	1

2= Certainly, 1= Maybe, 0= Certainly not

Criteria	013	014	015	017	018	019	020	022	023
Purchase at competitive price	0	1	1	1	1	0	0	2	0

Table III: Comments perceived by the volunteers for the product at D28

Volunteer #	Negative comments	Positive comments
01-1110-001	Not really effective.	-
01-1110-005	There is a slight stinging sensation during the application. I also experienced a slight burning sensation when I applied a greater amount of product.	When I applied only a small amount of product, I did not experience an adverse effect.
01-1110-006	Sometimes it burns the eyes.	-
01-1110-010	I experienced a tightening sensation on the upper eyelid.	-
01-1110-013	I did not notice any changes. However, ...	... this product is easy to use. I would like to continue to use it. In my opinion one month of use is not enough to obtain an improvement.
01-1110-014	I expected more from this product. I experienced a slight stinging sensation. The texture of the product was too liquid and sticky at the same time.	-
01-1110-023	The product was not effective. I did not noticed a difference.	-

Table IV: Habitual use of an eye makeup remover and eye makeup for each volunteer

Y= Yes and N=No; -1 = less than 1 month, 2 = 2 months and +2 = more than 2 months

Criteria	001	004	005	006	007	008	009	010	011	012
Regular use of an eye makeup remover	Y	N	Y	Y	Y	N	Y	N	Y	Y
If yes, since when	+2	-	+2	+2	+2	-	+2	-	+2	+2

Y= Yes and N=No; -1 = less than 1 month, 2 = 2 months and +2 = more than 2 months

Criteria	013	014	015	017	018	019	020	022	023
Regular use of an eye makeup remover	N	Y	N	N	Y	Y	Y	Y	N
If yes, since when	-	+2	-	-	+2	+2	+2	2	-

Y= Yes and N=No

Criteria	001	004	005	006	007	008	009	010	011	012
Regular use of eye makeup	Y	Y	Y	Y	Y	Y	Y	Y	Y	N

Y= Yes and N=No

Criteria	001	004	005	006	007	008	009	010	011	012
If yes, with what kind of products										
Eye shadow	N	N	Y	Y	N	N	N	N	Y	N
If yes: 1. Brand of the eye shadow	-	-	Lise Watier	L'Oréal	-	-	-	Rimmel	Maybelline	-
2. Name of the eye shadow	-	-	Quatuor Eye shadow quartet	Wear Infinité	-	-	-	Colour Rush Eye shadow	Expert Wear Eye Shadow	-
Mascara	Y	Y	Y	Y	Y	Y	N	Y	Y	N
If yes, 1. Brand of the mascara	L'Oréal	L'Oréal	Maybelline	L'Oréal	L'Oréal	Maybelline	-	Maybelline	Maybelline	-
2. Name of the mascara	Voluminous Original	Voluminous Original	Intense XXL	Voluminous Original	Volume Shocking	Colossal VolumExpress	-	Great Lash	Great Lash Waterproof Mascara	-
Liquid eye liner	Y	Y	Y	N	N	N	Y	N	N	N
If yes, 1. Brand of the liquid eye liner	L'Oréal	L'Oréal	Inglot	-	-	-	Maybelline	-	-	-
2. Name of the liquid eye liner	Lineur Intense Brush Tip	Lineur Intense Brush Tip	Black Liquid Eyeliner	-	-	-	Ultra Liner Waterproof	-	-	-
Other product	N	N	Y	Y	N	N	N	N	N	N
If yes, 1. Brand of the product	-	-	Smashbox	L'Oréal	-	-	-	-	-	-
2. Name of the product	-	-	Blush - Baked Fusion Soft Lights	Pencil Perfect	-	-	-	-	-	-

Table IV: Habitual use of an eye makeup remover and eye makeup for each volunteer (cont'd & end)

Y= Yes and N=No

Criteria	013	014	015	017	018	019	020	022	023
Regular use of eye makeup	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y= Yes and N=No

Criteria	013	014	015	017	018	019	020	022	023
If yes, with what kind of products									
Eye shadow	N	Y	N	Y	Y	Y	Y	Y	N
If yes: 1. Brand of the eye shadow	-	Annabelle	-	Rimmel	MAC	Lancôme	Quo	TCW	-
2. Name of the eye shadow	-	Pigment dust	-	Colour Rush Eye shadow	Eye Shadow	Color Design	Eye Shadow	Eye Shadow	-
Mascara	Y	Y	Y	Y	Y	Y	Y	Y	N
If yes, 1. Brand of the mascara	Maybelline	Bonne Bell	Cover Girl	L'Oréal	L'Oréal	Bourjois	Maybelline	N.Y.C.	-
2. Name of the mascara	Define-A-Lash	Eye Style Mascara	Lash Exact	Lash Out	Voluminous Original	Coup de Théâtre Mascara	Great Lash	Lash Precise Defining Mascara	-
Liquid eye liner	N	N	N	Y	N	Y	Y	N	N
If yes, 1. Brand of the liquid eye liner	-	-	-	Maybelline	-	Smash box	N.Y.C.	-	-
2. Name of the liquid eye liner	-	-	-	Waterproof Liquid Eyeliner	-	Cream Eye Liner	Liquid Eyeliner	-	-
Other product	Y	N	Y	Y	Y	N	Y	N	Y
If yes, 1. Brand of the product	Use Watier	-	Marcelle	Cover Girl	Chanel	-	Physician's formula	-	Profound Cosmetics
2. Name of the product	Eye Shine Metallic Liner	-	Face powder	Brow & Eye Makers Pencil	Intense Eye pencil	-	Powder Palette Multi-Colored Blush	-	Lip + Eyeliner Pencil

évalu

Ocular Pressure – Study 09AP-1110				Date: January 8th, 2010
Volunteer Code	Ocular Pressure (Right Eye) – D0	Ocular Pressure (Left Eye) – D0	Ocular Pressure (Right Eye) – D28	Ocular Pressure (Left Eye) – D28
01-1110-01	11	11	11	11
01-1110-03	13	13	-	-
01-1110-04	17	17	17	17
01-1110-05	12	12	12	12
01-1110-06	16	16	16	16
01-1110-07	12	12	12	12
01-1110-08	17	17	16	16
01-1110-09	15	15	15	15
01-1110-10	15	15	15	14
01-1110-11	15	14	13	14
01-1110-12	14	14	15	15
01-1110-13	13	13	13	13
01-1110-14	15	15	15	15
01-1110-15	15	15	15	15
01-1110-16	15	15	-	-
01-1110-17	14	14	14	14
01-1110-18	16	16	12	12
01-1110-19	15	15	15	15
01-1110-20	18	18	19	19
01-1110-21	17	17	-	-
01-1110-22	17	17	17	17
01-1110-23	20	20	20	20

Final Report

Original 1 of 1

Determination of Skin Irritation Potential of
Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
(Neat Oil)
in the Reconstructed human Epidermis (RhE)
Test Method
following OECD Guideline 439 and EU-Method B.46

Study No.: 22120103G840

Sponsor:

[redacted]

Monitor:

ToxMinds BVBA
Dr. Thomas Petry
Avenue de Broqueville, 116
1200 Brussels
Belgium

Test Facility:

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler
Germany

Study Director:

Diana Brandt

1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following documents:

- OECD Principles of Good Laboratory Practice and Compliance Monitoring, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998 and subsequent advisory/consensus OECD GLP documents (where appropriate).
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemicals Act of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 from 28. Aug. 2013, published in Federal Law Gazette, Germany (BGBl) No. 55/2013 as of 06. Sep. 2013, and further revisions.

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.
There were no circumstances that may have affected the quality or integrity of the study.

This report contains the following parameter which was not performed under GLP conditions: Determination of the proficiency chemicals and the pre-tests.

Diana Brandt Study Director	19 JUN 2023 Date

Information on Study Organisation:

Study Director	Diana Brandt
Deputy Study Director	Caroline Przewalla
Study Plan dated	28. Feb. 2023
Experimental Starting Date	08. Mar. 2023
Experimental Completion Date	10. Mar. 2023

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice.

All phases of the study (study plan, performance of the study and final report) were checked by the quality assurance. Dates of inspections are given below. Findings are reported to the study director and test facility management.

The inspection of the performance of short-term studies (duration less than four weeks) may be carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the final report was written in accordance with the study plan and the Standard Operating Procedures of the test facility.

Deviations from the study plan (if any) were acknowledged and assessed by the study director and included in the final report.

The reported results reflect the raw data of the study.

Phases of Study	Inspected on	Findings reported on	Audit report no.
Study plan	22. Feb. 2023	22. Feb. 2023	230222-06
Study plan Amendment No. 1	13. Apr. 2023	13. Apr. 2023	230413-02
Study plan Amendment No. 2	14. Jun. 2023	14. Jun. 2023	230614-06
Performance of study	10. Mar. 2023	10. Mar. 2023	230310-07
Final report	06. Jun. 2023	06. Jun. 2023	220606-05

Schedler

Dr. Anette Schedler
Quality Assurance

19 JUN 2023

Date

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3 SUMMARY

Title of Study: Determination of Skin Irritation Potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in the Reconstructed human Epidermis (RhE) Test Method following OECD Guideline 439 and EU-Method B.46

Findings and Results:

This study was conducted to determine the skin irritation potential of the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil), using the *in vitro* EpiDerm™ reconstructed human epidermis (RhE) assay according to OECD Guideline 439, in compliance with GLP.One valid experiment was performed.

The test item was applied to a three-dimensional human epidermis tissue model in triplicate for an exposure time of 60 minutes. DPBS buffer was used as negative control and 5 % SDS solution was used as positive control.

After treatment, the respective substance was rinsed from the tissue. Cell viability of the tissues was then evaluated by addition of MTT, which can be reduced to formazan. The formazan production was evaluated by measuring the optical density (OD) of the resulting solution.

After treatment with the negative control, the mean absorbance value was within the required acceptability criterion of 0.8 ≤ mean OD ≤ 2.8, OD was 1.717. The positive control showed clear irritating effects and the mean value of relative tissue viability was 2.6 % (required: ≤ 20 %).

The variation within the tissue replicates of negative control, positive control and test item was acceptable (required: ≤ 18 %).

After the treatment with the test item, the mean value of relative tissue viability was 16.0 %. This value is below the threshold for skin irritation potential (50 %). Test items which induce values below the threshold of 50 % are considered at least irritant to skin.

Under the conditions of the test, the test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) is considered at least irritant to skin in the Reconstructed human Epidermis (RhE) test method.

The % viability of the test item tissues are 3.0 %, 17.2 % and 27.7 % at the end of the test.

As the test item is highly viscous and colourless, it is possible that test item could not be completely washed off from all tissues equal. Therefore, it remained on one tissue more than on the others and this could not be seen.

Another explanation for this fluctuation could be that the tissues are biological systems and therefore their behaviour is not predictable. Some tissues are more sensitive than others (biological fluctuation).

It is also possible that both cases (test item remained on the tissue and this tissue was more sensitive than the others) worked together.

As all three values of the viability of the test item lay below the threshold of 50 %, this fluctuation can be stated as uncritical and the evaluation is clear.

4 PURPOSE AND PRINCIPLE OF THE STUDY

This *in vitro* study will be performed in order to evaluate the potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) to evoke skin irritation in a Reconstructed human Epidermis (RhE) test model (e. g. EpiDerm™ Skin Irritation Test).

Skin irritation refers to the production of reversible damage to the skin following the application of a test chemical.

The EpiDerm™ Skin Irritation Test (EpiDerm™ SIT) can be used for hazard identification of irritant chemicals in accordance with UN GHS Category 2 and also to identify non-classified chemicals. The EpiDerm™ SIT can be used as stand-alone replacement test for *in vitro* skin irritation testing or within a testing strategy as a partial replacement test.

The liquid test item was applied topically to a three-dimensional RhE tissue construct in triplicate for an exposure time of 60 minutes.

Skin irritant materials are identified by their ability to produce a decrease in cell viability, measured by dehydrogenase conversion of MTT (3-(4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazolium bromide), present in cell mitochondria, into a blue formazan salt. The formazan is quantitatively measured after extraction from tissues. The percentage reduction of cell viability in comparison with untreated negative controls is used to predict the skin irritant potential.

5 LITERATURE

The study was conducted in compliance with the following guideline(s):

- OECD Guideline for the Testing of Chemicals, Version 439, adopted 14. Jun. 2021, "*In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method"
- Commission Regulation (EU) No. 640/2012 amending Regulation (EC) No. 761/2009, Annex III, EU method B.46 "*IN VITRO* SKIN IRRITATION: RECONSTRUCTED HUMAN EPIDERMIS MODEL TEST", adopted 06. Jul. 2012

Corresponding SOP of LAUS GmbH:

- SOP 118 008 40, edition 11, valid from 04. Jul. 2022, "Prüfung auf Hautreizung mit dem Human Skin Model EpiDerm™"

Additional literature:

- ECVAM international validation study on *in vitro* tests for acute skin irritation: "Report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test" (Altern Lab Anim. 2007 Dec; 35 (6): 559-601).
- MatTek Protocol: for In Vitro EpiDerm™ Skin Irritation Test (EPI-200-SIT) for use with MatTek Corporation´s reconstructed human epidermal model EpiDerm ™ (EPI-200-SIT), 15. Aug. 2022

6 MATERIALS AND METHODS

6.1 Test Item

Designation in Test Facility:	22120103G
Date of Receipt:	01. Dec. 2022
Condition at Receipt:	cooled, in proper conditions

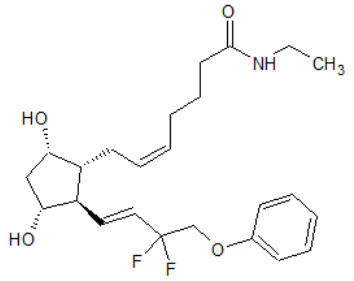
6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Batch no.	TAF-10-1122-01
CAS no.	1185851-52-8
EC no.	867-521-0
Composition	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
Storage	fridge (2 - 8 °C); keep under inert gas
Expiry date	23. Nov. 2026
Stability	stable under storage conditions
Appearance	clear, colorless to light yellow liquid
Purity	99.78 %
Homogeneity	homogeneous
Production date	18. Nov. 2022
Molecular formula	$C_{24}H_{33}F_2NO_4$
Molecular weight	437.52 g/mol
Vapour pressure	unknown
Solubility in solvents	water: not stated; ethanol: >1g/L; acetone: not stated; acetonitrile: not stated; DMSO: >1g/L; methanol: >1g/L; dimethyl fumarate: 0.1-1g/L
Stability in solvents	water: not stated; ethanol: not stated; acetone: not stated; acetonitrile: not stated; DMSO: not stated; methanol: not stated; dimethyl fumarate: not stated

A certificate of analysis was provided by the sponsor and is attached (in copy) in annex 5.

6.1.2 Structural Formula



O[C@@H]2C[C@H](O)[C@H](C/C=C\CCCC(=O)NCC)[C@H]2/C=C/C(F)(F)COc1ccccc1

6.1.3 Storage in Test Facility

The test item was stored in a closed vessel in a fridge (2 – 8 °C), kept under inert gas.

6.2 Test System

6.2.1 Specification

The test system is a commercially available EpiDerm™ tissue kit (e. g. EPI-200-SIT / EPI-212-SIT), procured by MatTek In Vitro Life Science Laboratories.

The EpiDerm™ tissue consists of human-derived epidermal keratinocytes which have been cultured to form a multi-layered, highly differentiated model of the human *epidermis*. It consists of organized basal, spinous and granular layers, and a multi-layered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*. The EpiDerm™ tissues are cultured on specially prepared cell culture inserts with a porous membrane through which nutrients can pass to the cells. The tissue surface is 0.6 cm^2.

6.2.2 Origin

EpiOcular™ tissues were procured from MatTek In Vitro Life Science Laboratories, Mlynské Nivy 73, 82105 Bratislava, Slovakia and used for this study.

Designation of the kit:	EPI-218-SIT
Day of delivery:	07. Mar. 2023
Batch no.:	38712

6.3 Controls

6.3.1 Negative Control

"Dulbecco's Phosphate Buffered Saline" (DPBS, without Ca^{2+} and Mg^{2+}) buffer was portioned and delivered from MatTek In Vitro Life Science Laboratories. Composition and batch number see chapter 6.4.1.

6.3.2 Positive Control

Sodium dodecyl sulphate (SDS), CAS-No.: 151-21-3, solution in demineralised water, concentration 50 g/L (5 % SDS), delivered from MatTek In Vitro Life Science Laboratories, batch no.: 120622NMB.

6.4 Chemicals and Media

6.4.1 MTT-Solution

Contained 1 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (=MTT), which can be reduced to a blue formazan salt/dye and was prepared by LAUS GmbH.

A MTT stock solution of 5 mg/mL in DPBS was prepared and stored in aliquots of 2 mL in the freezer (– 20 ± 5 °C). One aliquot of 2 mL of the stock solution was thawed and diluted with 8 mL assay medium (resulting in 1 mg/mL). This MTT-solution with the concentration of 1 mg/mL was used in the test.

6.4.1 DPBS-buffer

"Dulbecco`s Phosphate Buffered Saline" (DPBS, without Ca^{2+} and Mg^{2+}) was used for the rinsing the test item of the tissues and as solvent for the MTT concentrate. A subset was portioned and delivered from MatTek In Vitro Life Science Laboratories; the other subset was prepared by LAUS GmbH.

Composition of the subset prepared at LAUS GmbH (batch no.: T20230130):

KCl	0.4 g
KH_2PO_4	0.4 g
NaCl	16.01 g
$Na_2HPO_4 * 2H_2O$	2.90 g
H_2O	ad 2 L

pH was adjusted to 6.997 using 1M HCl.

Molar composition of the subset from MatTek In Vitro Life Science Laboratories (batch no.: 022123MSA) is the same, but different salts (crystal water) may have been used.

The buffer which was delivered from MatTek In Vitro Life Science Laboratories was used as negative control and for rinsing the test item from the tissues. The buffer which was prepared by LAUS GmbH was only used for preparing the MTT concentrate.

6.4.2 Assay Medium

Serum-free DMEM (Dulbecco's Modified Eagle's Medium) was used as assay medium and was procured from MatTek In Vitro Life Science Laboratories, batch no.: 030223LHC.

6.4.3 Isopropanol

CH_3-CH(OH)-CH_3, for synthesis., ≥99.5 %, batch no.: 190296551, used as extracting solvent for formazan

6.5 Test Vessels

All vessels used are made of glass or plastic (steralised). The glassware was sterilised before use by autoclaving.

The following vessels were used:

- 6-well-plates
- 24-well plates
- 96-well-plate

6.6 Instruments and Devices

The instruments and devices that were used in the test are listed in the following table.

Table 6.6-a Instruments and Devices

Device	Device name	Manufacturer
Autoclave	Autoklav 3870 ELV-B	Tuttnauer
Microtiter plate photometer	Photometer Anthos Reader 2010 Flexi	Anthos Microsysteme GmbH
Clean bench	Mars 1200	Scanlaf
Suction Pump	VacuSip	Integra
Pipetting device	AccuJet pro	Brand
Pipetting device	AutoRep E	Rainin
Precision scales	ME5002T/M00	Mettler Toledo
Analytical scales	XS205 Dual Range	Mettler Toledo
Incubation chamber	CO_2 Inkubator CB-150 (E3)	Binder
Glass thermometer	Glass thermometer 20210422-1	--
Glass thermometer	Glass thermometer 20020912-15	--
Thermohygrometer	Thermohygrometer Dewpoint Pro	DOSTMANN electronic GmbH
Table water bath	WBS-11	neolab
Pipette 20 – 200 µL	Pipet-lite XLS	Rainin
Pipette 200 – 2000 µL	Pipet-lite XLS	Rainin
Orbital shaker	Schüttelapparat 3005	GFL GmbH
Freezer	LGex 3410-23A-001	Liebherr
Stop watch	Stop watch	Roth
pH meter	3310	wtw

-- = various suppliers

Usage and, if applicable, calibration followed the corresponding SOP in the current edition. Standard laboratory material and equipment was also used.

6.7 Demonstration of Proficiency

The validity of the EpiDerm™ Skin Irritation Test at LAUS GmbH was demonstrated in a non-GLP proficiency study. For this purpose, 10 proficiency chemicals (indicated by the OECD guideline 439) were tested.

All of the 10 proficiency chemicals were correctly categorized. Therefore, the proficiency of the EpiDerm™ Skin Irritation Test was demonstrated (see chapter 15).

7 PERFORMANCE OF THE STUDY

The test item was heated up to 37 ± 1 °C for 15 minutes before usage. Afterwards, the test item was handled as liquid substance.

7.1 Non-GLP Pre-Tests

7.1.1 Non-GLP pre-test: Nylon Mesh Compatibility

It was tested whether the test item reacts with the nylon mesh (EPI-MESH). The mesh was brought onto a slide, then, 30 µL test item were applied. After 1 hour, the mesh was evaluated microscopically.

No reaction with the mesh has occurred, the nylon mesh was used in the main test to ensure spreading.

7.1.2 Non-GLP Pre-Test: Assessment of Colored or Staining Test Items

The test item is colorless (which was visually determined). To assess, whether the test item will become colored after contact with demineralised water, 30 µL test item and 0.3 mL demineralized water were given in a test tube and incubated in the dark for 1 hour at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

The color of the solution was not significantly changed; therefore, no additional test was performed.

In a parallel running OECD 492 non-GLP pre-test, the color of the solution of the test item in isopropanol was also not significantly changed.

7.1.3 Non-GLP Pre-Test: Assessment of Direct Reduction of MTT by the Test Item

To assess, whether the test item has the ability of direct MTT reduction, 30 µL test item were added to 1 mL of MTT solution in a 6-well plate and the mixture was incubated in the dark for 1 hour at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity. Untreated MTT solution was used as control. After incubation a potential color formation was assessed.

The color of the MTT solution was not changed to blue/purple, therefore, the test item was not presumed to have reduced the MTT and no additional test on freeze killed tissues was performed.

7.2 Main Test

7.2.1 Pre-Incubation of Tissues

All working steps were performed under sterile conditions.

The assay medium was pre-warmed to room temperature.

6-well-plates were labelled, one with “negative control”, one with “test item number” and one with “positive control”. For each treatment group, one 6-well plate was used. The wells of the upper row of each plate were filled with 0.9 mL assay medium.

After arrival, all tissues were inspected for viability and for presence of air bubbles between agarose gel and insert. Cultures with air bubbles under the insert covering more than 50 % of the insert area were discarded. Viable tissues were transferred (3 tissues per plate) in the upper row of the 6-well-plates and incubated for 1 hour at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

After the pre-incubation, every tissue was transferred to the lower well of the 6-well plate which was previously filled with 0.9 mL fresh assay medium. All 6-well-plates were incubated for 20 hours at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity. The rest of the assay medium was stored in the refrigerator.

7.2.2 Exposure

The assay medium was pre-warmed to 37 ± 1 °C.

After overnight pre-incubation, one 6-well-plate (three tissues) was used as negative control (30 µL DPBS buffer each), one as positive control (30 µL SDS-solution each) and one for the test item. 0.9 mL assay medium were filled in the upper row of the used 6-well-plates. For both controls, a nylon mesh was added in order to ensure sufficient contact with the tissue surface.

At the beginning of each experiment (application of negative control), a stop watch was started. Afterwards, 30 µL of the controls and the test item were applied in triplicate in 1- minute-intervals. This was done in such a fashion that the upper surface of the tissue was covered. As the liquid test item did not react with the nylon mesh in the non-GLP pre-test, a nylon mesh was added to the tissues treated with the test item in order to ensure sufficient contact with the tissue surface.

After dosing all tissues, all plates were transferred into the incubator for 35 minutes at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity. Afterwards they were placed into the sterile hood and it was waited until a 60 minutes period was completed for the first tissue.

1 hour after the first application, each insert was removed from the plates in 1-minute-intervals using sterile forceps and rinsed immediately. The inserts were thoroughly rinsed with DPBS buffer, blotted on absorbent material and transferred into the pre-filled wells of the pre-labelled 6-well plates (containing 0.9 mL fresh assay medium). Afterwards, the tissues were incubated for 25 hours at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

7.2.3 Medium Renewal

After overnight incubation, the tissues were removed from the incubator and shaken for 5 minutes (120 rpm). Then, the lower row of each incubated 6-well-plate was filled with 0.9 mL fresh assay medium. Afterwards, the inserts were transferred in the fresh assay medium from the upper to the lower row and the 6-well-plates were post-incubated for 18 hours at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

7.2.4 MTT Assay

On the day of the MTT assay, the MTT stock solution was thawed. The thawed MTT stock solution was diluted with the MTT solvent directly before use.

A 24-well-plate was prelabeled and filled with 300 µL freshly prepared MTT-solution in the respective wells. After overnight post-incubation, the tissues were blotted on absorbent material and then transferred into the MTT-solution. Afterwards, the 24-well-plate was incubated for 3 hours at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

Then, the MTT-solution was aspirated and replaced by DPBS buffer, which was aspirated, too, and replaced several times. At last, each tissue was thoroughly blotted on absorbent material and set into an empty 24-well-plate. Into each well, 2 mL isopropanol were pipetted, taking care to reach the upper rim of the insert.

Afterwards, the plate was sealed, placed in an airtight box and then shaken directly for 2 hours at room temperature.

7.2.5 Measurement

After the extraction, the tissues extracted from top and bottom were pierced with an injection needle, taking care that all color was extracted. The inserts were then discarded and the content of each well was thoroughly mixed in order to achieve homogenization.

From each well, two replicates with 200 µL solution were pipetted into a 96-well-plate which was read in a plate spectrophotometer at 570 nm. In addition, eight wells were filled with 200 µL isopropanol, serving as blank.

8 EVALUATION

The values of the 96-well-plate-reader were transferred into a validated spreadsheet (Microsoft Excel®).

Note: All calculations are performed with unrounded values. Therefore, re-calculation with rounded values may lead to slightly different results.

8.1 Calculations

Calculation was performed as follows:

- Calculation of mean OD of the blank control wells (Isopropanol) (= OD_{Blk}) and of the negative and positive control and the test item wells
- Subtraction of OD_{Blk} from the mean OD values (= blank corrected values)
- Calculation of mean value of the two replicates for each tissue (= e. g. OD corrected test item)
- Calculation of mean value of the three relating tissues for each control and the test item (= e. g. OD corrected mean negative control)

Note: Corrected mean OD value of the negative control corresponds to 100 % viability. For the mean of the 3 replicates of test item and positive control, tissue viability was calculated as % photometric absorbance compared to the negative control.

To calculate the relative tissue viability of each test item and positive control replicate, the following equation was used:

$$\%\ \text{Viability} = \left[\frac{\text{OD corrected test item or positive control}}{\text{OD corrected mean negative control}}\right] \cdot 100\ \%$$

9 FINDINGS AND RESULTS

9.1 Measured Values

As blank, the optical density of isopropanol was measured in 8 wells of the 96-well-plate. The measured values and their mean are given in the following table:

Table 9.1-a Absorbance values blank isopropanol (OD 570 nm)

Replicate	1	2	3	4	5	6	7	8	Mean
Absorbance	0.045	0.044	0.045	0.044	0.044	0.043	0.044	0.044	**0.044**

The absorbance values of negative control, test item and positive control are given in the following table:

Table 9.1-b Absorbance Values negative control, test item and positive control (OD 570 nm)

Designation	Measure-ment	Negative Control	Test Item	Positive Control
Tissue 1	1	1.702	0.340	0.088
	2	1.692	0.339	0.088
Tissue 2	1	1.706	0.519	0.087
	2	1.700	0.519	0.087
Tissue 3	1	1.885	0.098	0.091
	2	1.881	0.094	0.090

From the measured absorbances, the mean of each tissue was calculated, subtracting the mean absorbance of isopropanol as given in table 9.1-a. The mean of the three tissues was also calculated.

Table 9.1-c Mean Absorbance Values

Designation	Negative Control	Test Item	Positive Control
Mean – blank (tissue 1)	1.653	0.296	0.044
Mean – blank (tissue 2)	1.659	0.475	0.043
Mean – blank (tissue 3)	1.839	0.052	0.047
Mean of the three tissues	**1.717**	**0.274**	**0.045**

9.2 Comparison of Tissue Viability

For the test item and the positive control, the following percentage values of tissue viability were calculated in comparison to the negative control:

Table 9.2 % Tissue Viability

Designation	Test Item	Positive Control
% Tissue viability (tissue 1)	17.2 %	2.6 %
% Tissue viability (tissue 2)	27.7 %	2.5 %
% Tissue viability (tissue 3)	3.0 %	2.7 %
% Tissue viability (mean)	**16.0 %**	**2.6 %**
± SD of mean tissue viability (%)	**12.4 %**	**0.1 %**

9.3 Assessment

Skin irritation potential of the test item is assessed as given in the following table:

Table 9.5-a Assessment of Skin Irritation Potential

% Tissue viability	Assessment	UN GHS classification
≤ 50 % of negative control	Corrosive/ Irritant to skin	UN GHS Category 1 or 2
> 50 % of negative control	Non-irritant to skin	No Category for Skin Irritation

The mean value of relative tissue viability of the test item was reduced to 16.0% after the treatment. This value is below the threshold for skin irritation (50 %).

Therefore, the test item is considered as at least irritant to skin.

9.4 Validity

Validity criteria and results are stated in the following table:

Table 9.6-a Validity

Criterion	Demanded	Found
OD of the Negative Control	≥ 0.8 and ≤ 2.8 not below the 95.5 % confidence interval of the historical data (1.143 – 2.327)	1.717
% Tissue Viability of the Positive Control	≤ 20 % of negative control within 95.5 % confidence interval of the historical data (0.0 %* – 9.6 %)	2.6 %
SD of the Mean Viability of the Tissue Replicates (%)	≤ 18 %	6.2 % (negative control) 0.1 % (positive control) 12.4 % (test item)

*** Calculated value is < 0. Since these values have no biological relevance, they are set equal to 0.**

All validity criteria were met.

The values for negative control and for positive control were within the range of historical data of the test facility (see annex 2).

Therefore, the experiment is considered valid.

10 DISCUSSION

In this study, one valid experiment was performed.

Three tissues of the human skin model EpiDerm™ were treated with the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (neat oil), for 60 minutes.

The % viability of the test item tissues are 3.0 %, 17.2 % and 27.7 % at the end of the test.

As the test item is highly viscous and colourless, it is possible that test item could not be completely washed off from all tissues equal. Therefore, it remained on one tissue more than on the others and this could not be seen.

Another explanation for this fluctuation could be that the tissues are biological systems and therefore, their behaviour is not predictable. Some tissues are more sensitive than others (biological fluctuation).

It is also possible that both cases (test item remained on the tissue and this tissue was more sensitive than the others) worked together.

As all three values of the viability of the test item lay below the threshold of 50 %, this fluctuation can be stated as uncritical and the evaluation is clear.

After the treatment with the test item, the mean value of relative tissue viability was reduced to 16.0 %. This value is below the threshold for skin irritation potential (50 %). Test items that induce values below the threshold of 50 % are considered at least irritant to skin.

The optical density of the negative control was well within the required acceptability criterion of $0.8 \leq$ mean OD ≤ 2.8.

The positive control has met the acceptance criterion too, for thus ensuring the validity of the test system.

For these reasons, the result of the test is considered valid.

11 DEVIATIONS

11.1 Deviations from the Study Plan

No deviations were ascertained.

11.2 Deviations from the Guidelines

No deviations were ascertained.

12 RECORDING AND ARCHIVING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP-Document-Archive of the test facility for 15 years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP-Substance Archive for 15 years and then discarded.

Number of originals of the final report to be sent to the sponsor: 0, PDF-file only

13 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6, 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien / toxicological in vitro studies on mammalian cells and bacteria)

Datum der Inspektion / Date of Inspection
(Tag.Monat.Jahr / day.month.year)
28. und 29.04.2021

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist spätestens drei Jahre nach der letzten Inspektion zu beantragen. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for not later than three years after the last inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Landesamt für Umwelt Rheinland-Pfalz

Unterschrift, Datum / Signature, Date Mainz, 21.06.21

Sabine Riewenherm - Präsidentin -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

14 ANNEX 2: HISTORICAL DATA

In the following table, the means of the negative controls and positive controls of all performed experiments up to 01. Mar. 2023 are stated and compared with the values which were found in this study.

Table 14 Historical Data

Parameter	Negative Control (OD)	Positive Control (% OD compared to Negative Control)
Substance	DPBS buffer	Sodium Dodecyl Sulphate Solution 5 %
Mean	1.735	4.1 %
SD	0.296	2.8 %
Range min-max	0.476 - 2.471	1.7 - 17.1 %
Range mean ± 2 SD	1.143 - 2.327	0.0 - 9.6 %
Study 22120103G840	**1.717**	**2.6 %**

SD = Standard Deviation
*** Calculated value is < 0. Since these values have no biological relevance, they are set equal to 0.**

15 ANNEX 3: LIST OF PROFICIENCY CHEMICALS

In the following table the outcome of the proficiency chemical testing is stated.

All 10 proficiency chemicals were correctly classified.

The demonstration of proficiency was performed under non-GLP conditions but within the GLP-environment at LAUS GmbH.

Table 15 Results of Proficiency Chemicals

Chemical Name	CAS No.	Physical State	Prediction OECD 439 UN GHS Category	Findings LAUS GmbH
Naphthalene acetic acid	86-87-3	solid	No category	No category
Isopropanol	67-63-0	liquid	No category	No category
Methyl stearate	112-61-8	solid	No category	No category
Heptyl butyrate	5870-93-9	liquid	No category	No category
Hexyl salicylate	6259-76-3	liquid	No category	No category
Cyclamen aldehyde	103-95-7	liquid	Category 2	Category 2
1-bromohexane	111-25-1	liquid	Category 2	Category 2
Potassium hydroxide (5% aq.)	1310-58-3	liquid	Category 2	Category 2
1-methyl-3-phenyl-1-piperazine	5271-27-2	solid	Category 2	Category 2
Heptanal	111-71-7	liquid	Category 2	Category 2

16 ANNEX 4: QUALITY CONTROL DATA OF TEST SYSTEM

Provided by MatTek Corporation (supplier)

16.1 Certificate of Analysis

MATTEK
A BICO COMPANY

Certificate of Analysis

Product: EpiDerm™ Reconstructed Human Epidermis

Lot Number: 38712

Part#: EPI-200, EPI-212, EPI-218

Description: Reconstructed human epidermis tissue containing normal human keratinocytes. This product is for research use only. Not for use in animals, humans or diagnostic purposes.

I. Cell source

All cells used to produce EpiDerm™ are purchased or derived from tissue obtained by MatTek Corporation from accredited institutions. In all cases, consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the tissues or derivatives of the tissue for research purposes.

Keratinocyte Strain: 00267

II. Analysis for potential biological contaminants

The cells used to produce EpiDerm™ tissue are screened for potential biological contaminants. Tests for each potential biological contaminant listed below were performed according to the test method given. Results of "Not detected" indicate that testing for the potential biological contaminant was not observed as determined by the stated test method.

Keratinocytes:

HIV-1 virus – Oligonucleotide-directed amplification	Not detected
Hepatitis B virus – Oligonucleotide- directed amplification	Not detected
Hepatitis C virus – Oligonucleotide- directed amplification	Not detected
Bacteria, yeast, and other fungi – long term antibiotic, antimycotic free culture	Not detected

III. Analysis for tissue functionality and quality

Test	Specification	Acceptance criteria	Result and QA Statement	
Tissue viability	MTT QC assay, 4 hours, n=3	OD (540-570 nm) <1.0-3.0>	1.586 ± 0.091	**Pass**
Barrier function	ET-50 assay, 100 µl 1% Triton X-100, 4 time-points, n=3, MTT assay	ET-50 <4.77-8.72 hrs>	5.95 hrs	**Pass**
Sterility	Long term antibiotic and antimycotic free culture	No contamination	Sterile	**Pass**

Tissue viability and the barrier function test are within the acceptable ranges and indicate appropriate formation of the epidermal barrier, the presence of a functional stratum corneum, a viable basal cell layer, and intermediate spinous and granular layers. Results obtained with this lot conform to the requirements of the **OECD TG 431, 439 and 498.**

Initials: SL
Date: 3/8/23

Nelson Rivas
Quality Assurance Department
Document Control Manager

March 8, 2023
Date

CAUTION: Whereas all information herein is believed to be correct, no absolute guarantee that human derived material is non-infectious can be made or is implied by this certificate of analysis. All tissues should be treated as potential pathogens. The use of protective clothing and eyeware and appropriate disposal procedures are strongly recommended.

MatTek Corporation	**www.mattek.com**	**support@mattek.com**
MatTek Headquarters	200 Homer Avenue, Ashland, MA - USA	+1-508-881-6771
MatTek Europe	Mlynské Nivy 73, Bratislava - Slovakia	+421-2-3260-7401

QC-10-012-0005 Rev. B

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16.2 Functionality Test

MatTek Corporation

EpiDerm QC (EPI-200)

LOT 38712 TESTING DATE 8-Mar-2023
TESTED Post Refrigerated Storage
COMMENTS NO

Dosed with: 1.0% Triton X-100 (100uL)

Exposure Time (hr)	Well	OD	MTT (OD)	Std Dev (OD)	Viability %	Std Dev (%)
4	1	1.4139	1.197	0.204	75.5	12.8
	1	1.0099				
	1	1.1677				
6	1	0.7744	0.783	0.043	49.4	2.7
	1	0.7455				
	1	0.8305				
8	1	0.151	0.191	0.080	12.0	5.1
	1	0.2829				
	1	0.1378				
10	1	0.0526	0.048	0.005	3.0	0.3
	1	0.0487				
	1	0.0418				
H20 (4 hr)	1	1.6671	1.586	0.091	100.0	5.7
	1	1.6018				
	1	1.4879				
	Avg. cv (%):		16.4	11.1	Exp. Cv (%):	13.7

ET-50 (hr): 5.95

EPIDERM (EPI-200) Acceptance Criteria
1996 EpiDerm Database (n=184)

	TRI (hr)	SDS (hr)	H2O (OD)	H2O CV	Exp CV
avg	6.74	0.92	1.47	4.6	9.6
s.d.	0.99	0.23	0.13		
c.v.	14.60	24.60	8.80		
avg +2*sd	8.72	1.38	1.73		
avg - 2*sd	4.77	0.47	1.21		

QC Evaluation: PASS
Initials: GF
Date: 3/8/2023

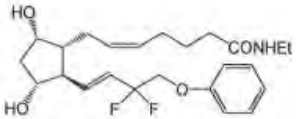
17 ANNEX 5: COPY OF THE CERTIFICATE OF ANALYSIS OF THE TEST ITEM

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{33}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications.

Quality Control: [redacted] *Date:* 23NOV2022

Approved [redacted] *Date:* 23Nov2022

Quality Control Laboratory
[redacted]

TC

TOXI-COOP ZRT.

TOXI-COOP ZRT.

Address: 8230 Balatonfüred, Arácsi út 97-99. Hungary
Phone: +36-30-678-2994

Final Report

In Vitro Skin Irritation Test with Tafluprost ethyl amide in the EPISKIN Model

Study No.: **147-439-7155**

Date of Final Report: **November 11, 2022**

(Report including Appendices total pages 30)

Sponsor:

[redacted]

Author:

István Buda
TOXI-COOP ZRT.
Arácsi út 97-99.
8230 Balatonfüred
Hungary

Table of Contents Page

The following prints of this report are issued:

Paper prints:

Original 1 of 2	Archived at TOXI-COOP ZRT.
Original 2 of 2	Released to the Sponsor

Electronic copy:

Electronic copy 1 of 1	An electronic copy in PDF format is released to the Sponsor.

The electronic file is an unaudited copy, generated after finalization of the report. The Sponsor is reminded that PDF files are not sufficiently protected against modification. Therefore, TOXI-COOP ZRT. cannot take any responsibility for the content of the electronic copy. The Sponsor is using the electronic copy on his own responsibility.

List of Abbreviations

OECD	–	Organisation for Economic Cooperation and Development
UN GHS	–	United Nations Globally Harmonized System of Classification and Labelling of Chemicals
EC	–	European Commission
GLP	–	Good Laboratory Practice
i.e.	–	id est (that is)
e.g.	–	exempli gratia (for example)
SOP	–	Standard Operating Procedure
EU	–	European Union
MTT	–	Thiazolyl Blue Tetrazolium Bromide
PBS	–	Phosphate Buffered Saline
HCl	–	Hydrochloric acid

Statement of the Study Director

This study has been performed in accordance with the agreed study plan, the OECD Guideline for the Testing of Chemicals (No. 439, 14 June 2021); Commission Regulation (EU) No 2019/1390 of 31 July 2019 amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), B.46. *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method; EURL ECVAM DB-ALM Protocol n° 131 (09 June 2012) and SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21 and the Good Laboratory Practice Regulations as specified by national Hungarian Good Laboratory Practice Regulation: 42/2014 (VIII. 19.) EMMI decree of the Minister of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM(98)17.
I declare that this report constitutes a true record of the actions undertaken and the results obtained in this study.

Signature: _____ Date: 11 NOVEMBER 2022

István Buda

Statement of the Management

I, the undersigned managing director hereby declare that the "*In Vitro* Skin Irritation Test with Tafluprost ethyl amide in the EPISKIN Model" was performed in accordance with the agreed study plan in TOXI-COOP ZRT. as a GLP study.

Signature: _____ Date: Nov 11, 2022

Dr. Gábor Hirka

Quality Assurance Statement

Study Number: **147-439-7155**

Study Title: ***In Vitro* Skin Irritation Test with Tafluprost ethyl amide in the EPISKIN Model**

Test Item: **Tafluprost ethyl amide**

This study has been inspected and this report audited by the Quality Assurance in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in writing to the study director and to management. The dates of such inspections and of the report audit are given below:

Date	Inspection/Audit	Date of report to Management	Date of report to Study Director
13 September 2022	Study Plan	13 September 2022	13 September 2022
14 – 16 September 2022	Handling of Test Item, Adequacy of Test System, Identification, Treatment, MTT Test, Formazan Extraction, Measurement of Samples, Tools & Equipment (Process-Based Inspection)	16 September 2022	16 September 2022
20 October 2022	Draft Report	20 October 2022	20 October 2022
11 November 2022	Final Report	11 November 2022	11 November 2022

Signature: _____ Date: 11 November 2022

Pálma Nagy
Quality Assurance

General Statements and Responsibilities

Study title:	***In Vitro* Skin Irritation Test with Tafluprost ethyl amide in the EPISKIN Model**
Study number:	**147-439-7155**
Sponsor:	[redacted]
Sponsor representative:	Dr. Karsten Schilling **IRSC - International Regulatory & Scientific Consulting** Paul-Lincke-Str. 36 D-67304 Eisenberg Germany
Test facility:	**TOXI-COOP ZRT.** Arácsi út 97-99., 8230 Balatonfüred Hungary Phone: +36-30-678-2994
Test facility management:	**Dr. Gábor Hirka**
Study director:	**István Buda**
Quality Assurance:	**Ildikó Hermann** **Pálma Nagy** **Zsuzsanna Szabó** **Katalin Böröczki**
Technical Assistance:	**Krisztina Fejes**

Study Schedule

Start of experimental phase:	**14 September 2022**
End of experimental phase:	**16 September 2022**
Date of Draft Report:	**25 October 2022**
Date of Final Report:	**11 November 2022**

1.0 Summary

EpiSkin™ SM test of Tafluprost ethyl amide has been performed to predict its irritation potential by measurement of its cytotoxic effect, as reflected in the MTT assay, according to the OECD Test Guideline No. 439, 14 June 2021.

Disks of EPISKIN (three units) were treated with test item and incubated for 15 minutes (± 0.5 min) at room temperature. Exposure of test material was terminated by rinsing with 1xPBS solution. Epidermis units were then incubated at 37±1 °C for 42 hours (± 1 h) in an incubator with 5±1 % CO_2, ≥95 % humidified atmosphere. The viability of each disk was assessed by incubating the tissues for 3 hours (± 5 min) with MTT solution at 37±1 °C in 5±1 % CO_2, ≥95 % humidified atmosphere, protected from light. The precipitated formazan was then extracted using acidified isopropanol and quantified spectrophotometrically.

SDS (5 % aq.) and 1×PBS treated (three units / positive and negative control) epidermis were used as positive and negative controls, respectively. For each treated tissue viability was expressed as a percentage relative to negative control.

The test item is identified as requiring classification and labelling according to UN GHS (Category 2 or Category 1), if the mean relative viability after 15 minutes exposure and 42 hours post incubation is less or equal (≤) to 50 % of the negative control.

In this *in vitro* skin irritation test using the EPISKIN model, the test item Tafluprost ethyl amide did not show significantly reduced cell viability in comparison to the negative control (mean viability: 96 %). All obtained test item viability results were above 50 % when compared to the viability values obtained from the negative control. Therefore, the test item was considered to be non-irritant to skin.

Positive and negative controls showed the expected OD and cell viability values within acceptable limits and indicated the suitability and sensitivity of the test system. Standard deviation of all calculated viability values (test item and controls) was below 18. The mean OD value of the blank sample was below 0.1. The experiment was considered to be valid.

The results obtained from this *in vitro* skin irritation test, using the EPISKIN model, indicated that the test item reveals no skin irritation potential under the utilised testing conditions. According to the current OECD Guideline No. 439, the test item Tafluprost ethyl amide is considered as non-irritant to skin. Moreover, there is no need for classification (UN GHS No Category).

2.0 Study purpose and introduction

The irritation potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the EPISKIN reconstituted human epidermis. This method is approved by international regulatory agencies as a replacement for the identification of irritants / corrosives in the *in vivo* Rabbit skin assay (OECD 404).
The test is designed to predict and classify the skin irritant potential of chemicals according to chemical safety regulations, using the reconstructed human epidermis model EpiSkin™ Small Model and parameters related to skin irritation.

EpiSkin™ Small Model (EpiSkin™SM) is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis, and the subsequent assessment of their effects on cell viability. Cell viability determination is based on cellular mitochondrial dehydrogenase activity, measured by MTT reduction and conversion into a blue formazan salt that is quantitatively measured after extraction from tissues (Faller C. *et al*., 2002, Mosmann T., 1983). The reduction of cell viability in treated tissues is compared to negative controls and expressed as a percent. The percent reduction in viability is used to predict the irritation potential.

3.0 Regulatory guidelines and test methods

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD Guideline for the Testing of Chemicals, Section 4, No. 439, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Methods" 14 June 2021.
- Commission Regulation (EU) No 2019/1390 of 31 July 2019 amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), B.46. *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method.
- EURL ECVAM DB-ALM Protocol n° 131 (09 June 2012): EpiSkin™ Skin Irritation Test 15 min – 42 hours
- SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21

4.0 Archiving

The study documents and samples as listed below will be archived according to the OECD GLP and to the TOXI-COOP ZRT.'s SOPs in the archives of TOXI-COOP ZRT. (Galamb u. 12/A., 8230 Balatonfüred, Hungary):

- Study Plan and Amendment 1 to the Study Plan (15 years)
- All raw data (15 years)
- Retained sample of the test item and reference item (5 years)
- Correspondence (15 years)
- Study report and any amendments (15 years)

For the first 5 years archiving is included, thereafter archiving occurs at additional costs of the Sponsor. After this period, the Sponsor will be notified to decide on further archiving to comply with current legal requirements.
After the retention time all the archived materials listed above will be returned to the Sponsor or retained for a further period if agreed by a contract or destroyed on their behalf.
None of the above cited documents or material will be discarded without the explicit written consent of the Sponsor.
At the end of the study, any remaining test item will be returned to the Sponsor or will be discarded, unless otherwise instructed by the Sponsor.

5.0 Materials and methods

5.1 Test Item

5.1.1 Name and Data of Test Item

Name:	Tafluprost ethyl amide
Batch No.:	0652603-2
Appearance:	colourless to pale yellow, oily consistency
Expiry date:	24 June 2023
CAS number:	1185851-52-8
Molecular weight:	437.5 g/mol
Purity (HPLC):	98.5 %
Storage:	Refrigerator (5±3 °C)
Safety precautions:	According to SDS

Test item information is based on written information given by the Sponsor.

5.1.2 Identification, Receipt

The test item of a suitable chemical purity was supplied by the Sponsor. All precautions required in the handling and disposal of the test item were outlined by the Sponsor. These documents are part of the raw data. Identification of the test item was performed in the laboratory of TOXI-COOP ZRT. on the basis of the information provided by the Sponsor.

5.1.3 Formulation

The test item was applied in its original form, no formulation was required.

5.2 Controls

Negative and positive controls were used in the experiment parallel, such that multiple test items can be tested with the reference values from the common parallel controls. These negative and positive controls were performed in the same experimental period using the same batch of chemicals and same batch of skin units.

5.2.1 Negative Control

Phosphate Buffered Saline (1x PBS):

Name: Phosphate Buffered Saline (10x PBS)
Supplier: SIGMA-ALDRICH
Batch No.: SLCL4813
Retest date: October 2022
Storage: Room temperature

Diluted with ultra-pure water (prepared by Synergy Smart UV HF ASTM Type 1: F8JA80461C water purification system) in TOXI-COOP ZRT.

5.2.2 Positive Control

Sodium Dodecyl Sulfate (SDS) 5 % aq. solution:

Name: Sodium dodecyl sulfate - ReagentPlus®, ≥98.5% (GC)
Supplier: SIGMA-ALDRICH
Batch No.: SLCJ3654
Appearance: White powder
Retest date: August 2023
Storage: Room temperature, protected from humidity

Diluted with ultra-pure water (prepared by Synergy Smart UV HF ASTM Type 1: F8JA80461C water purification system) in TOXI-COOP ZRT.

5.2.3 Reference Item Identification, Receipt

The reference item (positive control) of a suitable chemical purity was supplied by the Supplier. All precautions required in the handling and disposal of the reference item were outlined by the Supplier. These documents are part of the raw data.
Identification of the reference item was performed in the laboratory of TOXI-COOP ZRT., on the basis of the information included in the cover documentation that was provided by the Supplier.

5.3 Additional materials

5.3.1 MTT stock solution

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1] was dissolved to a final concentration of 3 mg/mL in saline buffer (1x PBS). The obtained stock solution can be stored in refrigerator (2-8 °C), protected from light up to 15 days.

5.3.2 MTT ready to use solution

The MTT stock solution was diluted with pre-warmed (37 °C) “assay medium” to a final concentration of 0.3 mg/mL. The obtained solution was used within two hours; it was protected from light before used.

5.3.3 Acidified isopropanol

Isopropanol was diluted with 12N Hydrochloric acid (HCl) to a final concentration of 0.04N HCl. The obtained solution was stored in refrigerator (2-8 °C), protected from light for one month.

5.3.4 Chemicals used in the experiment

The chemicals used in the experiment, are summarised in the following table:

Chemical	Supplier/Manufacturer	Lot/Batch Number	Retest/Expiry date
MTT	SIGMA-ALDRICH	MKCN5605	November 2025
Isopropanol	SIGMA-ALDRICH	STBK4193	18 March 2023
HCl	SIGMA-ALDRICH	MKCP1669	25 February 2023
10x PBS*	SIGMA-ALDRICH	SLCL4813	October 2022

* 1x PBS was prepared by appropriate diluting with ultra-pure water (prepared by Synergy Smart UV HF ASTM Type 1: F8JA80461C water purification system) in TOXI-COOP ZRT.

5.4 Test System

5.4.1 Human Skin

EpiSkin™ Small Model (EpiSkin™SM), manufactured by EPISKIN Laboratories Lyon, France, is a three-dimensional human epidermis model. Adult human-derived epidermal keratinocytes are seeded on a dermal substitute consisting of a collagen type I matrix coated with type IV collagen. A highly differentiated and stratified epidermis model is obtained after 13-day culture period comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum (Tinois *et al*., 1994). Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis, and the subsequent assessment of their effects on cell viability.

Supplier: EPISKIN Laboratories
4, rue Alexander Fleming, 69366 Lyon Cedex 07 - France
Batch No.: 22-EKIN-037
Expiry date: 19 September 2022

5.4.2 Justification for selection of the test system

The EPISKIN model has been validated for irritation testing in an international trial. After a review of scientific reports and peer reviewed publications on the EPISKIN method, it showed evidence of being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction and for being used as a replacement for the Draize Skin Irritation test (OECD TG 404 and Method B.4 of Annex V to Directive 67/548/EEC) for the purposes of distinguishing between skin irritating and no- skin irritating test substances (STATEMENT ON THE VALIDITY OF *IN-VITRO* TESTS FOR SKIN IRRITATION; ECVAM; Institute for Health & Consumer Protection; Joint Research Centre; European Commission; Ispra; 27 April 2007).

5.4.3 Demonstration of proficiency

Prior to routine use of the method TOXI-COOP ZRT. demonstrated the technical proficiency in a separate study (Study No.: 392.554.2938) using the ten Proficiency Chemicals according to OECD Test Guideline No. 439.

5.4.4 Quality Control

EpiSkin™SM kits are manufactured according to defined quality assurance procedures (certified ISO 9001). All biological components of the epidermis and the kit culture medium have been tested for the presence of viruses, bacteria and mycoplasma. A release form certifying the conformity of the batch included in the Study Report (see Appendix II).
The quality of the final product was assessed by undertaking a MTT cell viability test and a cytotoxicity test with sodium dodecyl sulphate (SDS).

5.4.5 EpiSkin™SM KIT Contents

Units: EpiSkin™SM plate containing up to 12 reconstructed epidermis units (area: 0.38 cm^2) each reconstructed epidermis was attached to the base of a tissue culture vessel with an O-ring set and maintained on nutritive agar for transport.

Plate: 12-well assay plate

Punch: EpiSkin™SM biopsy punch for easy sampling of epidermis

Medium: A flask of sterile “Maintenance Medium” for incubations.
(Batch No.: 22-MAIN3-031; Exp. Date: 21 September 2022)

A flask of sterile “Assay Medium” for use in MTT assays.
(Batch No.: 22-ESSC-031; Exp. Date: 21 September 2022)

5.4.6 Number of replicate wells

In this assay 3 replicates of test item, 3 replicates of negative control and 3 replicates of positive control were used.

5.4.7 EpiSkin™SM KIT Reception Procedure

The colour of the agar medium used for transport was checked for its pH using following criteria:

- orange colour = acceptable
- yellow or violet colour = not acceptable

The colour of the temperature indicator was inspected to verify that the kit had not been exposed to a temperature above 40 °C:

- the indicator changes from white to grey at 40 °C

The kit was found to be in good order at reception.

5.4.8 EpiSkin™SM KIT Storage Procedure

The EpiSkin™SM units were kept in their packaging at room temperature until the pre-incubation was started. The maintenance and assay medium were stored at 2-8 °C.

5.5 Indicator for potential false viability

Optical properties of the test item or its chemical action on MTT may interfere with the assay leading to a false estimate of viability. This may occur when the test item is not completely removed from the tissue by rinsing or when it penetrates the *epidermis*. If the test item acts directly on MTT (MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test item interference with the viability measurement.

5.5.1 Check-method for possible direct MTT reduction with test item

Approximately 10 mg test item was added to 2 mL MTT 0.3 mg/mL solution and mixed. The mixture was incubated for three hours at 37±1 °C in an incubator with 5±1 % CO_2, ≥ 95 % humidified atmosphere, protected from light and then any colour change observed (unaided eye assessment):

- Test items which do not interact with MTT: yellow
- Test items interacting with MTT: blue or purple

If the MTT solution colour becomes blue or purple, the test item interacts with the MTT. It is then necessary to evaluate the part of optical density (OD) due to the non-specific reduction of the MTT (i.e. by using killed epidermis).

Results of this check-test are detailed in section 10.2.

5.5.2 Check-method to detect the colouring potential of test item

Prior to treatment, chemicals are evaluated for their intrinsic colour or ability to become coloured in contact with water and/or acidified isopropanol (simulating a tissue humid environment).

Approximately 10 mg test item was added to 90 µL of water (prepared in TOXI-COOP ZRT. by Synergy UV HF ASTM Type 1: F8JA80461C water purification system) and mixed. The mixture was shaken for 15 minutes at room temperature and then the colour was checked (unaided eye assessment).

Approximately 10 mg test item was added to 90 µL of acidified isopropanol and mixed. The mixture was shaken for 15 minutes at room temperature and then the colour was evaluated (unaided eye assessment).

Results of this check are detailed in section 10.2.

5.6 Apparatus

5.6.1 Spectrophotometer

Name:	Varioskan™ LUX Type 3020
Serial number:	3020-078

<u>Absorbance/Turbidimetric measurement</u>

Light source:	Xenon flash lamp (100 Hz)
Detector:	Photodiode
Wavelength range:	200 – 1000 nm

5.6.2 Evaluation Software

Thermo Scientific™ SkanIt™ Software for Microplate Readers was used for measurements and MS Excel for further calculations.

6.0 Description of the test procedure

6.1 Pre-incubation (day [-1]-0)

The “Maintenance Medium” was pre-warmed to 37 °C. The appropriate number of assay plate wells were filled with the pre-warmed medium (2 mL per well). The epidermis units were placed with the media below them, in contact with the epidermis into each prepared well and then incubated overnight (18-24h) at 37±1 °C in an incubator with 5±1 % CO_2, ≥95 % humidified atmosphere.

6.2 Application (day 0)

Three replicates were used for the test item and positive and negative controls, respectively.

Test Item

The test item is a highly viscose material therefore exact weighing of treatment volume was not performed. The test item was applied on a plastic film (at least 10 mg) with a spatula, in such a way that the entire surface of a plastic film was covered evenly with the test item. This plastic film was applied to the tissue by forceps, in such a way that the entire surface of the epidermal surface was covered evenly with the test item.

Positive and negative control

A volume of 10 µL positive control (SDS 5 % aq.) or negative control (1x PBS) was applied on the skin surface by using a suitable pipette. Chemicals were gently spread with the pipette tip in order to cover evenly all the epidermal surface. Furthermore, the same quality of plastic film, which was used during the application of test item, was applied on the surface of the negative and positive control tissues.

6.3 Exposure (day 0)

Following applications, the plates with the treated epidermis units were incubated for the exposure time of 15 minutes (± 0.5 min) at room temperature (23.7-24.2 °C).

6.4 Rinsing (day 0)

After the incubation time the plastic films were removed from the surface of the tissues and afterward the EpiSkin^{TM}SM units were removed and rinsed thoroughly with approximately 25 mL 1x PBS solution for each tissue to remove all of the test material from the epidermal surface. The rest of the 1x PBS was removed from the epidermal surface with suitable pipette tip linked to a vacuum source (care was taken to avoid the damage of epidermis).

6.5 Post-incubation (day 0-2)

After rinsing the units were placed into the plate wells with fresh pre-warmed “maintenance medium” (2 mL/well) below them and then incubated for 42 hours (± 1 h) at 37±1 °C in an incubator with 5±1 % CO_2, ≥95 % humidified atmosphere.

6.6 MTT test after 42 hours incubation (day 2)

After the 42 hours (± 1h) incubation, the EpiSkin^{TM}SM units were transferred into the MTT solution filled wells (2 mL of 0.3 mg/mL MTT per well) and then incubated for 3 hours (± 5 min) at 37±1°C in an incubator with 5±1 % CO_2 protected from light, ≥95 % humidified atmosphere.

6.7 Formazan extraction (day 2)

At the end of incubation with MTT a formazan extraction was undertaken:

A disk of epidermis was cut from the unit (this involves the maximum area of the disk) using a biopsy punch (supplied as part of the kit). The epidermis was separated with the aid of forceps and both parts (epidermis and collagen matrix) were placed into a tube of 500 µL acidified isopropanol (one tube corresponding to one well of the tissue culture plate).

The capped tubes were thoroughly mixed by using a vortex mixer to achieve a good contact of all of the material with the acidified isopropanol then incubated for approximately four hours at room temperature, protected from light with gentle agitation (~150 rpm) for formazan extraction. At the middle and at the end of the incubation period, each tube was additionally mixed using a vortex mixer to help extraction.

6.8 Cell viability measurements (day 2)

Following the formazan extraction, 200 µL sample(s) from each tube (2×200 µL) was placed into the wells of a 96-well plate (labelled appropriately) and read Absorbance / Optical Density of the samples in the spectrophotometer at the wavelength of 570 nm using acidified isopropanol solution as the blank (6×200 µL).

7.0 Evaluation of Experimental Data

7.1 Calculations of viability percentages

7.1.1 Data calculation for normal test items

Blank:

– The mean of the 6 blank OD values was calculated

Negative control:

– Individual negative control OD values are corrected with the mean blank OD:

$OD\ Negative\ Control\ (OD_{NC}) = OD_{NCraw} - OD_{blank\ mean}$

– The corrected mean OD of the 3 negative control values was calculated: this corresponds to 100% viability

Positive control:

– Individual positive control OD values are corrected with the mean blank OD:

$OD\ Positive\ Control\ (OD_{PC}) = OD_{PCraw} - OD_{blank\ mean}$

– The corrected mean OD of the 3 positive control values was calculated
– The % viability for each positive control replicate is calculated relative to the mean negative control:

$\%\ Positive\ Control\ 1 = (OD_{PC1} / mean\ OD_{NC}) \times 100$
$\%\ Positive\ Control\ 2 = (OD_{PC2} / mean\ OD_{NC}) \times 100$
$\%\ Positive\ Control\ 3 = (OD_{PC3} / mean\ OD_{NC}) \times 100$

– The mean value of the 3 individual viability % for positive control was calculated:

$Mean\ PC\ \% = (\%PC1 + \%PC2 + \%PC3) / 3$

Test item:

– Individual test item OD values are corrected with the mean blank OD:

$OD\ Treated\ Tissue\ (OD_{TT}) = OD_{TTraw} - OD_{blank\ mean}$

– The corrected mean OD of the 3 test item values is calculated
– The % viability for each test item replicate was calculated relative to the mean negative control:

$\%\ Treated\ Tissue\ 1 = (OD_{TT1} / mean\ OD_{NC}) \times 100$
$\%\ Treated\ Tissue\ 2 = (OD_{TT2} / mean\ OD_{NC}) \times 100$
$\%\ Treated\ Tissue\ 3 = (OD_{TT3} / mean\ OD_{NC}) \times 100$

– The mean value of the 3 individual viability % for test item was calculated

$Mean\ TT\ \% = (\%TT1 + \%TT2 + \%TT3) / 3$

7.2 Assay Acceptance Criteria

- The mean OD value of the three negative control tissues should be equal to or between 0.6 and 1.5 and the standard deviation value (SD) of the % viability should be ≤ 18.
- The acceptable mean percentage viability for positive controls is < 40% and the standard deviation value (SD) of the % viability should be ≤ 18.
- For test chemicals (test item and controls), the standard deviation value (SD) of the % viability should be ≤ 18.
- The mean OD value of the blank samples (acidified isopropanol) should be < 0.1

7.3 Interpretation of test results

According to the United Nations Globally Harmonized System (UN GHS) of Classification and Labelling of Chemicals (9th revised edition; 2021) and as implemented in the Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (EU CLP), the irritancy potential of test substances is predicted for distinguishing between irritant or corrosive (Category 2 or Category 1) and non-irritant (No Category) substances.
In the present study, the irritancy potential of test item is predicted by mean tissue viability of tissues exposed to the test item. The test chemical is identified as requiring classification and labelling according to UN GHS (Category 2 or Category 1), if the mean relative viability after 15 minutes exposure and 42 hours post incubation is less or equal (≤) to 50 % of the negative control. However, this test method (OECD 439) cannot resolve between UN GHS Categories 1 and 2, further information on skin corrosion (OECD 431) will be required to decide on its final classification. In case the test chemical is found to be non-corrosive, and shows tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50 %, the test item is considered to be irritant to skin in accordance with UN GHS Category 2.
Depending on the regulatory framework in member countries, the test item may be considered as non-irritant to skin in accordance with UN GHS No Category if the tissue viability after exposure and post-treatment incubation is more than (>) 50%.
The prediction model (PM) is described below:

Criteria for *In Vitro* interpretation	**Classification**
Mean tissue viability % is ≤ 50 %	Category 2 or Category 1
Mean tissue viability % is > 50 %	No Category

8.0 Deviations from the Study Plan

No. 1:

Concerning:	Date of Draft Report
According to the Study Plan:	Not later than October 18, 2022
Deviation:	25 October 2022
Reason:	unplanned delay
Presumed effect on the study:	None

9.0 Amendment to the Study Plan

The study plan was amended once in the course of the study. Date of Amendment 1 to the Study Plan was 14 September 2022.

10.0 Results

10.1 Validity of the Test

The mean OD value of the three negative control tissues was 0.967. The mean OD value obtained for the positive control was 0.103 and this result corresponds to 11 % viability when compared to the results obtained from the negative control. Each calculated standard deviation value (SD) for the % viability was below 18. The mean OD value of the blank sample acidified isopropanol was 0.0341 below the threshold value 0.1. All validity criteria were within acceptable limits and therefore the study is considered as valid.

10.2 Indicator for potential false viability

Possible direct MTT reduction with test item:

Colour change was not observed after three hours of incubation. Therefore, the test item did not interact with the MTT and additional controls and data calculations were not necessary. A false estimation of viability can be precluded.

Colouring potential of test item:

The test item showed no ability to become coloured in contact with water and acidified isopropanol. The intrinsic colour of test item is colorless to pale yellow and therefore considered not to be able to significantly stain the tissues and lead to a false estimate of viability. Additional controls and data calculations were not necessary. A false estimation of viability can be precluded.

10.3 Cell viability

The results of the optical density (OD) measured at 570 nm of each replicate and the calculated % viability of the cells is presented below:

OD values and viability percentages of the controls and test item:

Substance		Optical Density (OD)	Viability (%)
Negative Control: 1x PBS	1	0.921	95
	2	1.017	105
	3	0.963	100
	mean	**0.967**	**100**
	standard deviation (SD)		5.01
Positive Control: SDS (5 % aq.)	1	0.108	11
	2	0.133	14
	3	0.069	7
	mean	**0.103**	**11**
	standard deviation (SD)		3.30
Test Item: Tafluprost ethyl amide	1	0.965	100
	2	1.024	106
	3	0.792	82
	mean	**0.927**	**96**
	standard deviation (SD)		12.48

Remark: Mean blank OD value was 0.0341.
Optical density means the mean value of the duplicate wells for each sample (rounded to three decimal places).

11.0 Discussion and Conclusion

Disks of EPISKIN (three units) were treated with test item and incubated for 15 minutes (± 0.5 min) at room temperature. Exposure of test material was terminated by rinsing with 1xPBS solution. Epidermis units were then incubated at 37±1 °C for 42 hours (± 1 h) in an incubator with 5±1 % CO_2, ≥95 % humidified atmosphere. The viability of each disk was assessed by incubating the tissues for 3 hours (± 5 min) with MTT solution at 37±1 °C in 5±1 % CO_2, ≥95 % humidified atmosphere, protected from light. The precipitated formazan was then extracted using acidified isopropanol and quantified spectrophotometrically.

SDS (5 % aq.) and 1×PBS treated (three units / positive and negative control) epidermis were used as positive and negative controls, respectively. For each treated tissue viability was expressed as a percentage relative to negative control.

The test item is identified as requiring classification and labelling according to UN GHS (Category 2 or Category 1), if the mean relative viability after 15 minutes exposure and 42 hours post incubation is less or equal (≤) to 50 % of the negative control.

In this *in vitro* skin irritation test using the EPISKIN model, the test item Tafluprost ethyl amide did not show significantly reduced cell viability in comparison to the negative control (mean viability: 96 %). All obtained test item viability results were above 50 % when compared to the viability values obtained from the negative control. Therefore, the test item was considered to be non-irritant to skin.

Positive and negative controls showed the expected OD and cell viability values within acceptable limits and indicated the suitability and sensitivity of the test system. Standard deviation of all calculated viability values (test item and controls) was below 18. The mean OD value of the blank sample was below 0.1. The experiment was considered to be valid.

The results obtained from this *in vitro* skin irritation test, using the EPISKIN model, indicated that the test item reveals no skin irritation potential under the utilised testing conditions. According to the current OECD Guideline No. 439, the test item Tafluprost ethyl amide is considered as non-irritant to skin. Moreover, there is no need for classification (UN GHS No Category).

12.0 References

1. OECD Guidelines for the Testing of Chemicals, Section 4, No. 439, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Methods" 14 June 2021.
2. OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998.
3. Hungarian Good Laboratory Practice Regulation: 42/2014 (VIII. 19.) EMMI decree of the Minister of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM(98)17.
4. EC (2008), REGULATION (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Official Journal of the European Union L353, 1-1355.
5. OECD (2015). OECD Guideline for Testing of Chemicals. No. 404: "Acute Dermal Irritation/Corrosion" adopted 28 July 2015.
6. Faller C., Bracher M., Dami N. and Roguet R. (2002) Predictive ability of reconstructed human epidermis equivalents for the assessment of skin irritation of cosmetics.
7. Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunological Methods* 65, 55-63.
8. Tinois E., Gaetani Q., Gayraud, B., Dupond D., Rougier A. and Pouradier-Duteil X. (1994). The Episkin model: Successful reconstruction of a human epidermis in vitro. In: Rougier, A., Goldberg M., Maibach H.I. Eds. In Vitro Skin Toxicology. Mary Ann Liebert, New York, pp. 133-140.
9. EpiSkin™ SOP, Version 1.8 (February 2009), ECVAM Skin Irritation Validation Study: Validation of the EpiSkin™ test method 15 min - 42 hours for the prediction of acute skin irritation of chemicals.
10. Commission Regulation (EU) No 2019/1390 of 31 July 2019 amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), B.46. *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method.
11. STATEMENT ON THE VALIDITY OF *IN-VITRO* TESTS FOR SKIN IRRITATION; ECVAM; Institute for Health & Consumer Protection; Joint Research Centre; European Commission; Ispra; 27 April 2007.
12. EURL ECVAM DB-ALM Protocol n° 131 (09 June 2012): EpiSkin™ Skin Irritation Test 15 min – 42 hours.
13. United Nations (UN) (2021). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Ninth revised edition, UN New York and Geneva, 2021.
14. OECD Guidelines for Testing of Chemicals, Section 4, No. 431, "*In Vitro* Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method" 18 June 2019.
15. SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21

APPENDICES

APPENDIX I

COPY OF THE GLP CERTIFICATE OF TOXI-COOP ZRT.

OGYÉI Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet

1135 Budapest, Szabolcs utca 33.
Levélcím: 1372 Postafiók 450
Tel.: +36 1 888 9300, Fax: +36 1 886 9480
E-mail: ogyei@ogyei.gov.hu
Web: www.ogyei.gov.hu

Ref. no: OGYÉI/21950-8/2022
Admin.: dr. Szaller Zoltán
Date: 11th August, 2022

GOOD LABORATORY PRACTICE (GLP) CERTIFICATE

It is hereby certified that the test facility

TOXI-COOP Toxicolgical Research Center Zrt.

H-1103 Budapest, Cserkesz u. 90.,
H-1045 Budapest, Berlini u. 47-49.,
H-8230 Balatonfüred, Arácsi u. 97-99.,
H-8230 Balatonfüred, Vasút u. 3.,
H-8230 Balatonfüred, Galamb u. 12/A ,
H-8230 Balatonfüred, Ady E. u. 12,
8354 Karmacs, hrsz 4150/2

is able to carry out

physico-chemical testing, toxicity studies, mutagenicity studies, environmental toxicity studies on aquatic and terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation studies, analytical and clinical chemistry, safety pharmacology testing, metabolism and toxico/pharmacokinetics testing, testing of toxicological properties of operative procedures and equipment, reproduction toxicological studies, tolerance studies, inhalation toxicology and in vitro studies

in compliance with the Principles of GLP (Good Laboratory Practice) and also complies with the corresponding OECD/European Community requirements.
This certificate is valid till 20th of April, 2025.

Date of the inspection: **11-14 and 19-20 April, 2022.**

El Koulali Zakariás
Deputy Director General

APPENDIX II

COPY OF THE TEST SYSTEM QUALITY CONTROL

EPISKIN

TECHNICAL DATA, SAFETY SHEET AND CERTIFICATE OF ANALYSIS

CCE-091-SM D13-5/04

NAME

EpiSkin™ Small / Human Epidermis (SM/13)

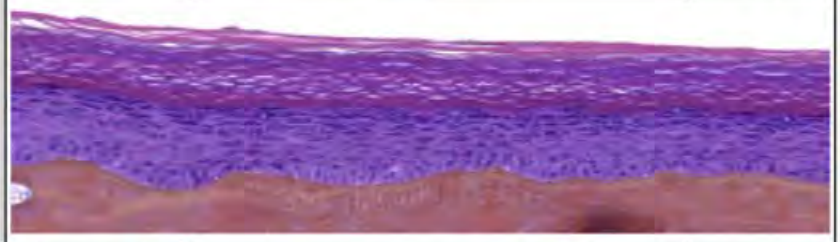
DESCRIPTION

0.38 cm^2 reconstructed epidermis of normal human keratinocytes. Cells are grown on a collagen matrix, for 13 days

BATCH : 22-EKIN-037
ORIGIN : Adult donors
USAGE : FOR SCIENTIFIC USE ONLY - PRODUCT OF HUMAN ORIGIN
STORAGE : This product was prepared and packaged using aseptic techniques. Store in an incubator at 37°C, 5% CO2 with saturated humidity

QUALITY CONTROLS

Control # E221413

	Process	Specification	Result
HISTOLOGY	HES stained paraffin section	Multi-layered, highly differentiated epidermis consisting of organized basal, spinous and granular layers, and a multilayered *stratum corneum*	Satisfactory
		Number of cell layers ≥ 4	7 cell layers
			
IC50 DETERMINATION	SDS concentration, MTT test.	1.5 mg/mL ≤ IC50 ≤ 3.0 mg/mL	2.1 mg/mL

BIOLOGICAL SAFETY:

On blood of the donors, we have verified the absence of HIV1 and 2 antibodies, hepatitis C antibodies and hepatitis B antigen HBs.
On cells from the donors, we have verified the absence of bacteria, fungus and mycoplasma.

SUGGESTED EXPIRATION DATE:

September 19, 2022

Lyon, September 13, 2022
Certified and released by **Anaïs JENSEN**, Quality Control Manager

Manufactured in accordance to the ISO9001 quality system of Episkin.

The use of this human tissue is strictly limited to *in vitro* testing. All other manipulations of this tissue such as: extraction and maintenance of single cells in culture, use of the tissue for diagnostic or therapeutic purposes and in human subjects, are strictly prohibited.

ISO 9001 Certified
4, rue Alexander Fleming - 69366 Lyon Cedex 07 - France - Tél : +33 (0)4 37 28 72 00 - Fax : +33 (0)4 37 28 72 28
S.A. au capital de 13 608 807 € - 412 127 565 R.C.S. Lyon - NAF : 7211 Z - N° TVA Intracommunautaire FR 46 412 127 565
www.episkin.com

APPENDIX III

HISTORICAL CONTROL DATA

Historical Control Data
(Period of 2011-2022 September)

	Negative Control data	**Positive Control data**	
	Phosphate Buffered Saline (1 x PBS)	Sodium Dodecyl Sulphate (SDS) 5 % aq. solution	
	Optical Density (OD)	Optical Density (OD)	Viability (% control)
Mean	0.9	0.1	13
Minimum	0.6	0.0	4
Maximum	1.5	0.3	37

TC

TOXI-COOP ZRT.

TOXI-COOP ZRT.

Address: Arácsi út 97-99. Balatonfüred, Hungary 8230
Phone: +36-30-678-2994

Final Report

In Chemico Determination of Skin Sensitisation (Direct Peptide Reactivity Assay – DPRA) of Tafluprost ethyl amide

Study number: **147-442-7156**

Date of Final Report: **October 18, 2022**

(Report including Appendices total pages 39)

Sponsor:

[redacted]

Author:

Dóra Szabóné Sági
TOXI-COOP ZRT.
Arácsi út 97-99.
8230 Balatonfüred
Hungary

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The following prints of the Report are issued:

Paper print:

Original 1 of 2	Archived at TOXI-COOP ZRT.
Original 2 of 2	Released to the Sponsor.

Electronic copy:

Electronic copy 1 of 1	An electronic copy released to the Sponsor.

The electronic file is an unaudited copy, generated after finalization of the report. The Sponsor is reminded that PDF files are not sufficiently protected against modification. Therefore, TOXI-COOP ZRT. cannot take any responsibility for the content of the electronic copy. The Sponsor is using the electronic copy on his own responsibility.

Statement of the Study Director

This study has been performed in accordance with the study plan agreed upon by the Sponsor, the principles of Good Laboratory Practice Regulations as specified by Hungarian Good Laboratory Practice Regulation: 42/2014 (VIII. 19.) EMMI decree of the Minister of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM(98)17 and followed the procedures indicated by the OECD TG 442C GUIDELINE FOR THE TESTING OF CHEMICALS, APPENDIX I: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA) (June 30, 2022); the OECD *Guideline No. 497: Defined Approaches on Skin Sensitisation*, OECD Guidelines for the Testing of Chemicals, Section 4.; the EURL ECVAM DB-ALM Protocol n°154 and the SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21.
I declare that this report constitutes a true record of the actions undertaken and the results obtained in this study.

Signature: Szabóné Sági

Dóra Szabóné Sági

Date: October 18, 2022.

Statement of the Management

According to the conditions of the research and development assignment between [redacted] (as Sponsor) and **TOXI-COOP ZRT.** (as Test Facility) "*In Chemico* Determination of Skin Sensitisation (Direct Peptide Reactivity Assay – DPRA) of Tafluprost ethyl amide" has been performed in laboratory of **TOXI-COOP ZRT**. as GLP study.

Signature: _____

Dr. Gábor Hirka

Date: 04 18, 2022

Quality Assurance Statement

Study Number: **147-442-7156**

Study Title: ***In Chemico* Determination of Skin Sensitisation (Direct Peptide Reactivity Assay – DPRA) of Tafluprost ethyl amide**

Test Item: **Tafluprost ethyl amide**

This study has been inspected and this report audited by the Quality Assurance in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in writing to the study director and to management. The dates of such inspections and of the report audit are given below:

Date	Inspection/Audit	Date of report to Management	Date of report to Study Director
September 19, 2022	Study Plan	September 19, 2022	September 19, 2022
September 21, 2022	Handling of Test Item, Formulation	September 21, 2022	September 21, 2022
October 04, 2022	Draft Report	October 04, 2022	October 04, 2022
October 18, 2022	Final Report	October 18, 2022	October 18, 2022

Signature: _____ Date: 18 October 2022

Katalin Böröczki
Quality Assurance

General Statements and Responsibilities

Study title:	***In Chemico* Determination of Skin Sensitisation (Direct Peptide Reactivity Assay – DPRA) of Tafluprost ethyl amide**
Study number:	**147-442-7156**
Sponsor:	[redacted]
Sponsor representative:	**Dr. Karsten Schilling** IRSC - International Regulatory & Scientific Consulting Paul-Lincke-Str. 36 D-67304 Eisenberg Germany
Test facility:	**TOXI-COOP ZRT.** 8230 Balatonfüred, Arácsi út 97-99. Hungary Phone: +36-30-678-2994
Test facility management:	**Dr. Gábor Hirka**
Study director:	**Dóra Szabóné Sági**
Quality Assurance:	**Ildikó Hermann** **Katalin Böröczki**

Study Schedule

Date of Study Plan:	September 19, 2022
Non-GLP solubility trial:	August 18, 2022
Start of experimental phase:	September 21, 2022
End of experimental phase:	September 23, 2022
Date of Draft Report:	October 06, 2022
Date of Final Report:	October 18, 2022

1.0 Summary

In the course of this study the skin sensitisation potential of the test item **Tafluprost ethyl amide** was studied using the Direct Peptide Reactivity Assay (DPRA).

For the test item in order to derive a prediction, one valid test was evaluated with cysteine peptide.

Means of the peak areas versus the concentrations of cysteine peptide showed good linearity, covering the concentration range from 0.0167 mM to 0.534 mM.

Peptide depletion resulting from the positive control cinnamaldehyde was within the expected percentage range and confirmed the suitability and sensitivity of the test system.

The mean back-calculated peptide concentrations of the reference control replicates were within the expected molarity concentration range and the CV % values for the nine reference controls B and C in acetonitrile were acceptable.

The mean cysteine peptide depletion value of the test item was 5.61 % ± 0.44 % indicative for negative DPRA prediction.

All validity criteria were met, confirming the validity of the study.

Based on these results and the cysteine 1:10 prediction model, the test item Tafluprost ethyl amide showed no or minimal reactivity under the experimental conditions of the *in chemico* Direct Peptide Reactivity Assay (DPRA) method and was therefore concluded to be negative according to the prediction criteria.

2.0 Study Objective and Introduction

The purpose of this study was the *In Chemico* determination of skin sensitisation potential (Direct Peptide Reactivity Assay – DPRA) with high-performance liquid chromatography (HPLC) analysis. The DPRA is proposed to address the molecular initiating event of the skin sensitisation Adverse Outcome Pathway (AOP), namely protein reactivity, by quantifying the reactivity of the test chemical towards model synthetic peptides containing either cysteine or lysine. Cysteine and/or lysine peptide depletion values will be then used to categorize the test substance in one of four classes of reactivity for supporting the discrimination between skin sensitisers and non-sensitisers.

3.0 Regulatory Guidelines and Test Methods

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD 442C Guideline for the Testing of Chemicals, APP. I: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA) (June 30, 2022) [2]
- DB-ALM (INVITTOX) Protocol 154: Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing (October 21, 2021) [3]
- OECD 497 Guideline on Defined Approaches for Skin Sensitisation, Annex 1. Prediction model for the individual *in chemico/in vitro* tests with multiple runs for use in 2o3 DA (June 14, 2021) [9]
- SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21 [10]

4.0 Archiving

The study documents and samples as listed below will be archived according to the OECD GLP and to the TOXI-COOP ZRT. SOPs in the archives of TOXI-COOP ZRT. (H-8230 Balatonfüred, Galamb u. 12/A):

- Study Plan (15 years)
- All raw data (15 years)
- Retained sample of the test item and reference item (5 years)
- Correspondence (15 years)
- Study report and any amendments (15 years).

For the first 5 years archiving is included, thereafter archiving occurs at additional costs of the Sponsor. After this period, the Sponsor will be notified to decide on further archiving to comply with current legal requirements. After the retention time all the archived materials listed above will be returned to the Sponsor or retained for a further period if agreed by a contract or destroyed on their behalf.
None of the above cited documents or material will be discarded without the explicit written consent of the Sponsor. At the end of the study, any remaining test item will be returned to the Sponsor or will be discarded, unless otherwise instructed by the Sponsor.

5.0 Materials and Methods

5.1 Test Item

5.1.1 Name and Data of the Test Item

Name:	Tafluprost ethyl amide
Batch No.:	0652603-2
Expiry date:	24 June 2023
CAS number:	1185851-52-8
Molecular weight:	437.5 g/mol
Purity (HPLC):	98.5 %
Appearance:	colourless to pale yellow, oily consistency
Storage:	refrigerator (5±3 °C)
Safety precautions:	According to the SDS

Test item information is based on written information given by the Sponsor.

5.1.2 Identification, Receipt of the Test Item

The test item of a suitable chemical purity was supplied by the Sponsor. All precautions required in the handling and disposal of the test item were outlined. These documents are part of the raw data. Identification of the test item was performed in the Laboratory of TOXI-COOP ZRT. on the basis of the information included in the test item documentation supplied by the Sponsor.

5.1.3 Formulation of the Test Item

The solubility of the test item was tested in a non-GLP preliminary solubility test as follows: the solubility of the test item was evaluated at the concentration of 100 mM. Acetonitrile dissolved the test item completely. Homogenous and clear solution was formed after vortexing. Therefore the chosen vehicle is acetonitrile, because it is the first preferred vehicle according to the OECD 442C guideline.
The behaviour of the formulation of the test item in acetonitrile was determined in the buffers of the test system (phosphate and acetate buffer) in a ratio corresponding to the reaction sample assembly (for the cysteine run 50 µL of 100 mM test item solution+200 µL acetonitrile+750 µL phosphate buffer; for the lysine run 250 µL of 100 mM test item solution+750 µL acetate buffer). The compatibility of the formulation with phosphate buffer was proven, no precipitate was observed in case of phosphate buffer (for cysteine analysis) after vortexing and homogenous, clear solution was obtained. In case of acetate buffer (for lysine analysis) opalescent formulation was obtained and precipitation was observed. This formulation was not suitable for the HPLC analysis. Because of the opalescent formulation lysine run was not performed, only cysteine run and the evaluation was performed according to cysteine 1:10 prediction model.
The pre-experiments on solubility of the test item were not performed in compliance with the GLP-Regulations and are excluded from the Statement of Compliance in the final report, but the raw data of these tests will be archived under the study number of present study.

5.2 Positive Control

Name:	**Cinnamaldehyde - natural, ≥95 %, FG**
Supplier:	Sigma-Aldrich
Batch No.:	SHBN2484
Expiry date:	March 31, 2026
Storage:	Room temperature
Purity:	98.7 %
CAS number:	104-55-2
Molecular weight:	132.16 g/mol
Formula:	C_9H_8O
Appearance:	Light yellow liquid

5.2.1 Identification, Receipt of the Positive Control

The positive control of a suitable chemical purity was supplied by the supplier. All precautions required in the handling and disposal of the positive control were outlined by the supplier. These documents are part of the raw data.
Identification of the positive control was performed in the TOXI-COOP ZRT., on the basis of the information included in the cover documentation that was supplied by the supplier.

5.3 Additional Materials

5.3.1 Synthetic Peptide

Cysteine peptide

Name:	Cysteine peptide
Batch No.:	111016HS-MHeW0522-01
Storage:	at -20°C or below
Purity:	94.09 %
Molecular weight:	750.88 g/mol
Sequence:	Ac-RFAACAA-OH
Expiry date:	November 19, 2022
Supplier:	JPT

5.3.2 Vehicle

Name:	ACETONITRILE RS PLUS – For HPLC Gradient- ACS-Reag.Ph.Eur.- Reag.USP.
Lot No.:	P0G107290G
Appearance:	colorless liquid
Expiring date:	July 2024
Assay:	100.00 %
Molecular formula:	H_3CCN
Storage:	Room temperature
Supplier:	CARLO ERBA

Since according to the guideline [2] and protocol [3], some acetonitrile batches had negative impact on cysteine peptide stability, this acetonitrile batch is validated to have no negative impact on cysteine peptide stability by performing an autosampler stability test according to the recommendations of the protocol (TOXI-COOP Study number.: 392-442-5971).

5.3.3 Additional Chemicals Used in the Experiment

Table 1. Characteristics of additional chemicals used in the experiment

Name	Grade	Supplier/ Manufacturer	Expiry date/ Retest date	Batch/Lot number
Acetonitrile - ACS – Reag.Ph.Eur.-Reag. USP	RS Plus for HPLC gradient	Carlo Erba	July, 2024	P0G107290G
Trifluoroacetic acid	>= 99%	Thermo Fisher	August 15, 2025	2196207
Sodium dihydrogen phosphate monohydrate	Reag. Ph. Eur.	Carlo Erba	October, 2026	V0D003230L
Sodium phosphate dibasic heptahydrate	ACS reagent	Sigma-Aldrich	April, 2024	SLCJ8741
Ultrapure water	ASTM Type I	freshly prepared by Millipore Direct Q5		

5.4 Apparatus

5.4.1 HPLC System and Conditions

HPLC:	Shimadzu LC-2030i Prominence
Serial number:	L21445402951AE
Detector:	D2 lamp (220 nm)*
Column:	Zorbax SB-C18 (2.1 x 100 mm, 3.5 µm)
Serial number:	USRY003976
Column temperature:	30°C
Sample temperature:	25°C
Injection volume:	7 µL
System equilibration:	running mobile phase A and mobile phase B in a ratio of 1:1 for 2 hours at 30°C column temperature and running the gradient twice before injecting the first sample
Run time:	20 min
Flow conditions:	gradient flow

*Data acquisition was performed at 258 nm too, but the evaluation was not necessary, as no co-elution occurred.

Mobile phases for HPLC:
Mobile Phase A – 0.100 % (v/v) trifluoroacetic acid in ultrapure water
Mobile Phase B – 0.085 % (v/v) trifluoroacetic acid in acetonitrile

Table 2. Gradient flow conditions

Time	Flow	A phase (%)	B phase (%)
0 min	0.35 mL / min	90	10
10 min		75	25
11 min		10	90
13 min		10	90
13.5 min		90	10
20 min		gradient ends	

5.4.2 Other Equipments

Balances:	BP 210D, Sartorius, No.: 60602907 BL 120S, Sartorius, No.: 15307011 PG203-S, Mettler Toledo, No: 1122082152
Vortex mixer:	Velp, No.: 425653
Ultrasonic bath:	Bransonic M5800H-E, No.: BIB121662770B
pH meter:	inoLab pH Level2, No.: 03260014

5.5 Preparation of Solutions

5.5.1 Buffer Solution

Phosphate buffer (pH 7.5 ± 0.05):

- 100 mM Sodium Phosphate Dibasic solution: 2.680 g Sodium phosphate dibasic heptahydrate was dissolved in 100 mL ultrapure water.
- 100 mM Sodium Phosphate Monobasic solution: 1.380 g Sodium phosphate monobasic monohydrate was dissolved in 100 mL ultrapure water.
- 18 mL of 100 mM Sodium Phosphate Monobasic solution and 82 mL 100 mM Sodium Phosphate Dibasic solution were mixed and pH was adjusted to 7.498 with ~7 mL of 100 mM Sodium Phosphate Dibasic solution.

5.5.2 Peptide Stock Solution

Cysteine peptide stock solution, 0.667 mM, 0.501 mg/mL:
0.01048 g of the cysteine peptide was measured into a glass beaker. It was dissolved in 19.691 mL of phosphate buffer and thoroughly mixed with ultrasonic bath. Peptide was pre-weighed but buffer was not added before beginning the assay.

$$\text{estimated mL of phosphate buffer} \times \frac{0.501\ \text{mg/mL}}{\text{purity of the peptide}} = \text{peptide target weight (mg)}$$

$$\text{estimated mL of phosphate buffer} \times \frac{\text{peptide actual weight (mg)}}{\text{peptide target weight (mg)}} = \text{actual volume of buffer (mL)}$$

5.5.3 Calibration Solutions

Six calibration standard points were prepared by serial dilution of the peptide stock solution with the following nominal molarities: STD 1 = 0.534 mM, STD 2 = 0.267 mM, STD 3 = 0.1335 mM, STD 4 = 0.0668 mM, STD 5 = 0.0334 mM and STD 6 = 0.0167 mM. As dilution buffer a 20% acetonitrile:phosphate buffer solution was used. For the zero standard point (STD 7 = 0 mM) dilution buffer was used.

5.5.4 Positive Control Stock Solution

100 mM solution of the positive control chemical in acetonitrile was prepared just before use. Into a 5 mL volumetric glass 0.0701 g cinnamaldehyde was weighed for the positive control stock solution during cysteine peptide depletion determination, dissolved in acetonitrile and thoroughly mixed.

$$\frac{\text{molecular weight}}{\%\ \text{purity}} \times 50 = \text{target weight of cinnamaldehyde (mg)}$$

5.5.5 Test Item Stock Solution

100 mM solution of the test item in the appropriate solvent was prepared just before use. The needed amount of test item was calculated based on the molecular weight and purity of the test item. Into a 5 mL volumetric glass 0.2223 g test item was weighed for the stock solution used for the cysteine peptide depletion determination, dissolved in acetonitrile and thoroughly mixed.

$$\frac{\text{molecular weight}}{\%\ \text{purity}} \times 50 = \text{target weight of test item (mg)}$$

5.5.6 Control Samples

Reference control A: Peptide stock solution was combined with acetonitrile (see Table 3). System suitability was checked by the use of the three replicates of reference control A.

Reference control B: Peptide stock solution was combined with acetonitrile (see Table 3). Stability of the peptide was checked by the use of the three replicates of reference control B, measured before and after the reaction samples.

Reference control C: Peptide stock solution was combined with acetonitrile for the test item and the positive control (see Table 3). Three replicates of reference control C were used as a solvent control to which the peptide concentration/depletion of the reaction samples was compared.

Positive control: Peptide stock solution was combined with positive control stock solution (100 mM) and acetonitrile (see Table 3). The three replicates of positive control sample were measured with the reaction samples.

Co-elution controls: Test item stock solution and acetonitrile was combined with phosphate buffer (see Table 3). Three replicates of co-elution controls were used to check for test item and peptide co-elution.

5.5.7 Reaction Samples

Peptide stock solution was combined with test item stock solution and acetonitrile (see Table 3). The three replicates of reaction samples were measured with the positive control samples and respective reference control C samples.

6.0 Description of the Test

6.1 Principle of the DPRA Method

The reactivity of a test chemical and synthetic Cysteine or Lysine containing peptides is evaluated by combining the test chemical with a solution of the peptide and monitoring the remaining concentration of the peptide following 24 hours of interaction time at room temperature.

The peptide is a custom material containing phenylalanine to aid the detection and either Cysteine ("C") or Lysine ("K") as the reactive centre.

Relative concentrations of the peptides following the 24 hour incubation are determined by high performance liquid chromatography with gradient elution and UV detection at 220 nm. Reaction samples, reference controls A, B and C, co-elution controls and positive controls are prepared and analysed in triplicates. Batches of up to 26 chemicals are measured, in order not to exceed the maximum 30 hours analysis time (including controls).

6.2 Steps of the DPRA Method Done in Chronological Order

- Solubility assessment of test item – acetonitrile was used as a solvent
- Preparation of buffer solution
- Pre-weighing of test item and positive control
- Pre-weighing of cysteine peptide for stock solution
- Test item and positive control solution preparation
- Peptide stock solution preparation
- Standard preparation by serial dilution using dilution buffer
- Assembling of reaction samples, positive controls, reference controls (A, B and C) and co-elution controls. For each set of control/sample replicates, the triplicate vials are prepared individually but from the same solutions.
- Incubation at 25 °C, for 24±2 hours
- Preparation of HPLC system (column equilibration)
- HPLC analysis
- Data evaluation

Table 3. Assembly of reaction samples and controls

1:10 ratio cysteine peptide 0.5 mM peptide, 5 mM test chemical
750 µL cysteine peptide stock solution (or phosphate buffer for the co-elution control)
200 µL acetonitrile
50 µL 100 mM test item solution (or solvent for the reference controls A,B,C or 100 mM positive control solution for the positive control)

The autosampler vials were capped, vortexed to mix and placed into the HPLC autosampler tray for incubation at 25 °C in the dark. HPLC analysis of the reaction samples was started 24±2 hours after the test item was added to the peptide solution. The samples were clear and homogenous after the incubation period. System equilibration was applied with the eluents of 50% phase A and 50% phase B for 2 hours and running the gradient twice before injecting the first sample. The temperature of the column oven was 30 °C and that of the autosampler was 25 °C. The batch consisted of 2 parts: one part with the A reference controls and the first sampling of the calibration standards. These samples were run before the 24 hours incubation time ended. The other part contained the B and C reference controls, the positive controls, the reaction samples, the other samplings of the calibration standards and the co-elution controls. These samples were run after the 24 hours incubation time had elapsed. The total HPLC analysis time was less than 30 hours.

7.0 Demonstration of Proficiency

Prior to routine use of the method, TOXI-COOP ZRT. demonstrated technical proficiency in a separate study (Study number.: 392-442-2996) by correctly obtaining the expected DPRA prediction for 10 proficiency substances as recommended in the OECD TG 442C guideline.

8.0 Evaluation Method of Experimental Data

8.1 Percent Peptide Depletion

The concentration of the peptide is determined in each sample and control, from absorbance at 220 nm measuring the peak area of the appropriate peaks and calculating the concentration of the peptide using the linear calibration curve derived from the calibration standards.

The percent peptide depletion is determined in each reaction sample and positive control calculating the quotient of the peak area and the mean respective reference control C peak area, according to the formula described below.

$$\text{peptide percent depletion } = [1 - (\frac{\text{peak area of the reaction sample}^*}{\text{mean peak area of reference controls C}})] \times 100$$

*or positive control

8.2 Presence of Precipitate

If precipitation occurs it is recorded and caution is used in interpreting data. Samples can be centrifuged to settle and remove the precipitate to avoid clogging the HPLC. Centrifugation at low speed (max. 400 x g) is recorded as well and data interpretation is done with caution.

In this study the samples were clear and homogenous, no precipitation occurred thus no centrifugation was needed.

8.3 Co-elution

In cases where the test item co-elutes with the cysteine peptide and the peptide peak cannot be integrated, a determination of reactivity cannot be made and the data is reported as “inconclusive”.

8.3.1 Co-elution Controls

If the test item (Co-elution Control) absorbs at 220 nm and has a similar retention time as the peptide (Reference Control), the peaks are overlapping, then co-elution of the test item with the peptide is reported. In order to assure that baseline noise is not being called interference, the “interfering” test item peak has to have a peak area that is > 10 % of the mean peptide peak area in the appropriate Reference Control. If co-elution occurs, proper integration and calculation of Percent Peptide Depletion is not possible.

8.3.2 Negative Depletion Values

If the Percent Peptide Depletion is negative, it is considered that this may be a situation of co-elution, inaccurate peptide addition to the reaction mixture or just baseline "noise." If this happens, the co-elution controls are carefully analyzed. If the peptide peak appears at the proper retention time and has the appropriate peak shape and the negative value is smaller than - 10 %, the peak can be integrated, but the calculated %-depletion should be reported as an "estimate".

If the peak does not have the proper shape due to complete overlap in retention time of the test item and peptide and cannot be integrated, calculation of Percent Peptide Depletion is not possible and the data is reported as "inconclusive".

9.0 Acceptance Criteria

The following criteria should be met for a run to be considered valid:

- the standard calibration curve should have an $r^2 > 0.99$
- the mean peptide concentration of reference controls A should be 0.50 ± 0.05 mM and the coefficient of variation (CV) of peptide peak areas for the nine reference controls B and C in acetonitrile should be < 15.0 %.
- the mean percent peptide depletion value of the three replicates for the positive control cinnamaldehyde should be between 60.8 % and 100 % and the maximum standard deviation (SD) for the positive control replicates should be < 14.9 %
- the maximum standard deviation for the test item replicates should be smaller than 14.9 % for the percent cysteine depletion
- the mean peptide concentration of the three reference controls C in the appropriate solvent should be 0.50 ± 0.05 mM.

If one or more of these criteria is not met the run should be repeated.

10.0 Deviations from the Study Plan/Testing Guideline

There was no deviation from the Study Plan/Testing Guidelines.

11.0 Amendment to the Study Plan

There was no amendment to the Study Plan.

12.0 Evaluation and Interpretation of Results

The mean percent cysteine depletion value is calculated for each test chemical. Negative depletion (> -10 %) is considered as "0" when calculating the mean.

Before applying the cysteine 1:10 prediction model, the experimental data regarding possible co-elution are evaluated.

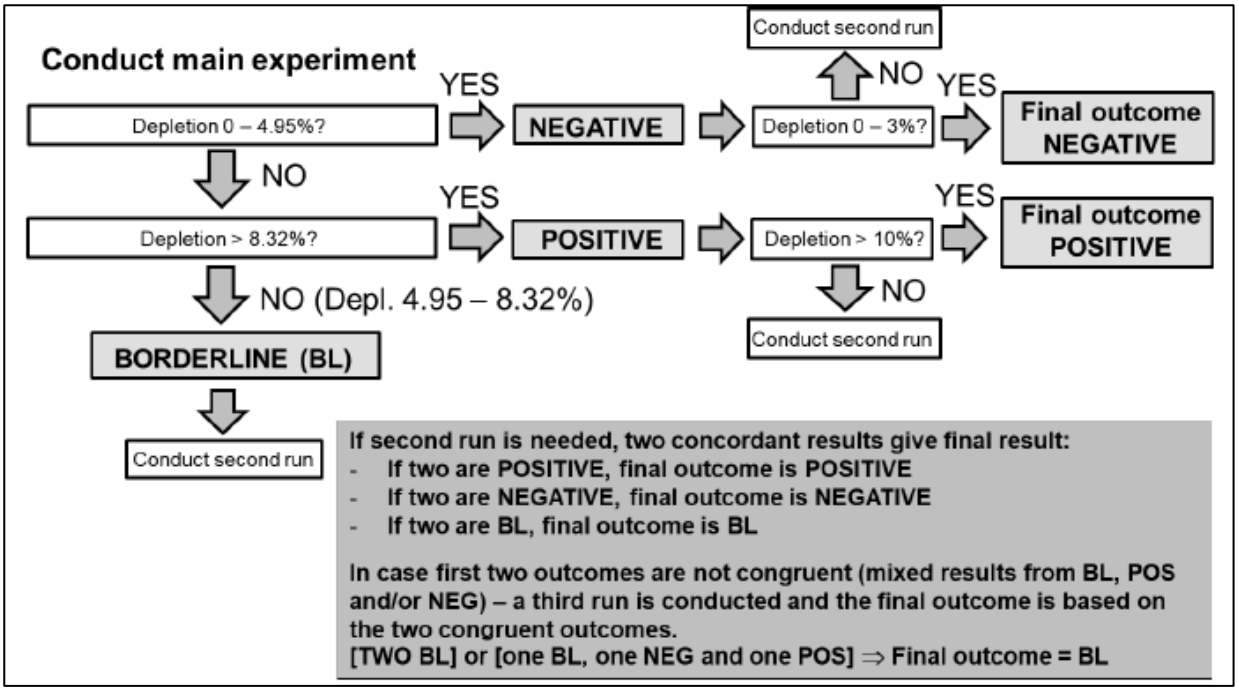
Note: A single HPLC analysis for the cysteine peptide should be sufficient for a test chemical when the result is unequivocal. However, in cases of results close to the threshold used to discriminate between positive and negative results (cysteine percent depletion falls in the range of 9 % to 17 %), additional testing is recommended. In particular, in case of negative results in these ranges (9 % to 13.89 %), a second run should be conducted, as well as a third one in case of discordant results between the first two runs.

Application of the prediction model assigns a test chemical to a reactivity class (no or minimal, low, moderate or high reactivity). Chemicals assigned to the no or minimal reactivity category should be classified as non-sensitisers whereas chemicals assigned to the low, moderate or high reactivity categories should be classified as sensitisers.

Table 4. Cysteine 1:10 prediction model

Mean percent cysteine depletion	Reactivity class	DPRA prediction
0 % ≤ % depletion ≤ 13.89 %	no or minimal reactivity	negative
13.89 % < % depletion ≤ 23.09 %	low reactivity	positive
23.09 % < % depletion ≤ 98.24 %	moderate reactivity	
98.24 % < % depletion ≤ 100 %	high reactivity	

Figure 1. Decision tree for borderline values based on OECD 497 TG



The same flowchart applies to the cysteine-only prediction model, whereby the following thresholds apply: 9 % instead of 3 %, >17 % instead of >10 %, 10.56 % instead of 4.95 % and > 18.47 % instead of >8.32 %.

13.0 Results

The chromatograms were evaluated with the help of "LabSolutions" software and the calculations were carried out using "Microsoft Office Excel".

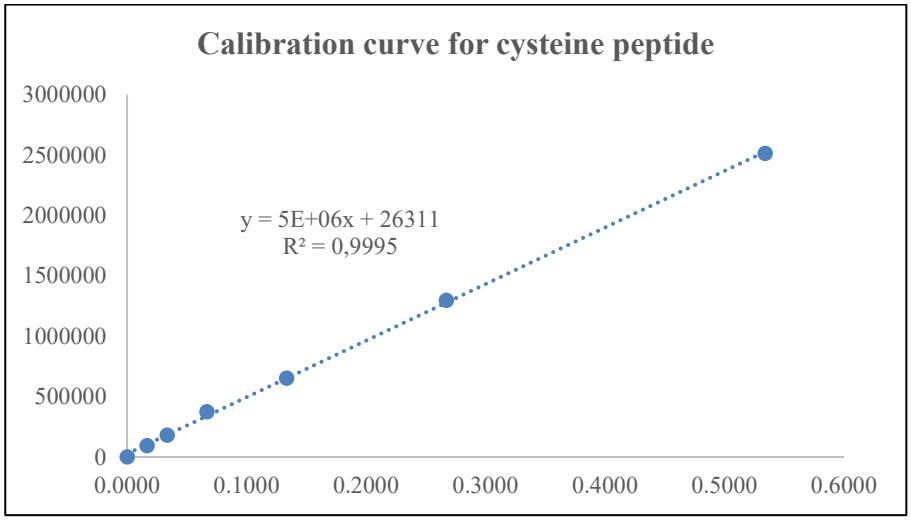
13.1 Calibration Graph for Peptide

A standard calibration curve was generated for cysteine peptide using serial dilutions from the peptide stock solution. Calibration standard points were analyzed by linear regression.

Means of the peak areas versus the concentrations of cysteine peptide showed good linearity with $r^2 = 0.9995$, covering the concentration range from 0.0167 mM to 0.534 mM.

The graphical representation of the cysteine peptide calibration line is given as Figure 2.

Figure 2. Graphical representation of the calibration curve for cysteine peptide



13.2 System Suitability

Reference control A replicates were included in the HPLC run sequence to verify the HPLC system suitability prior analysis. The mean peptide concentration of reference control A sample replicates was 0.51 mM. Data for the reference control A sample replicates are presented in Table 5.

Table 5. Reference control A replicates for cysteine peptide

Sample	Peptide conc. calculated (mM)	Mean peptide conc. (mM)	Peptide peak area CV %
ref A, rep I	0.51	0.51	1
ref A, rep II	0.51		
ref A, rep III	0.51		

13.3 Analysis Sequences

Reference control B replicates were included in the sequence to verify the stability of the peptide over time and reference control C replicates were used to verify that the solvent of the test item did not impact the percent peptide depletion. Moreover the CV % for the peak areas of the nine reference control B and C replicates in acetonitrile (acn) were smaller than 15 % for cysteine peptide, since it was 2 %. The mean peptide concentration of reference control B sample replicates was 0.49 mM. The mean peptide concentration of reference control C (acn) sample replicates was 0.49 mM.
Data for the reference control B and C (acn) sample replicates are presented in Table 6.

Table 6. Reference control B and C (acn) replicates for cysteine peptide

Sample	Peptide conc. calculated (mM)	Mean peptide conc. (mM)	Peptide peak area CV %
ref B, rep I	0.50	0.49	2
ref B, rep II	0.50		
ref B, rep III	0.50		
ref B, rep I / 2	0.49		
ref B, rep II / 2	0.48		
ref B, rep III / 2	0.49		
ref C acn, rep I	0.48	0.49	
ref C acn, rep II	0.49		
ref C acn, rep III	0.51		

13.4 Cysteine Depletion of the Test Item and Positive Control

The acceptance criteria was met for the positive control with a cysteine peptide depletion value of 70.67 % ± 0.69 %.
The mean cysteine peptide depletion value obtained with the test item was 5.61 % ± 0.44 %.
The SD for the test item was within the acceptance range.
Data for the positive control and the test item sample replicates are presented in Table 7.

Table 7. Cysteine peptide depletion values for the positive control and the test item

Sample	Peptide conc. calculated (mM)	Mean peptide conc. (mM)	Peptide depletion (%)	Mean Peptide depletion (%)	Standard deviation (SD) for peptide depletion (%)
positive control, rep I	0.14	0.14	69.88	70.67	0.69
positive control, rep II	0.14		70.99		
positive control, rep III	0.14		71.14		
test item, rep I	0.47	0.47	5.11	5.61	0.44
test item, rep II	0.46		5.93		
test item, rep III	0.47		5.79		

13.5 Assigning the Test Chemical to a Reactivity Class and Category

No co-elution was observed with the peptide. Cysteine 1:10 prediction model was used for the discrimination between sensitiser and non-sensitiser. The mean cysteine peptide depletion of the test item was 5.61 %, which is under the 13.89 % threshold of the applicable prediction model and classified as **negative with no or minimal reactivity**.

14.0 Conclusion

In the course of this study the skin sensitisation potential of the test item **Tafluprost ethyl amide** was studied using the Direct Peptide Reactivity Assay (DPRA).

For the test item in order to derive a prediction, one valid test was evaluated with cysteine peptide.

Means of the peak areas versus the concentrations of cysteine peptide showed good linearity, covering the concentration range from 0.0167 mM to 0.534 mM.

Peptide depletion resulting from the positive control cinnamaldehyde was within the expected percentage range and confirmed the suitability and sensitivity of the test system.

The mean back-calculated peptide concentrations of the reference control replicates were within the expected molarity concentration range and the CV % values for the nine reference controls B and C in acetonitrile were acceptable.

The mean cysteine peptide depletion value of the test item was 5.61 % ± 0.44 % indicative for negative DPRA prediction.

All validity criteria were met, confirming the validity of the study.

Based on these results and the cysteine 1:10 prediction model, the test item Tafluprost ethyl amide showed no or minimal reactivity under the experimental conditions of the *in chemico* Direct Peptide Reactivity Assay (DPRA) method and was therefore concluded to be negative according to the prediction criteria.

15.0 References

[1] OECD (2014). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168, OECD, Paris.

[2] 442C OECD GUIDELINE FOR THE TESTING OF CHEMICALS, APPENDIX I: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA) (June 30, 2022)

[3] DB-ALM (INVITTOX) Protocol 154: Direct Peptide Reactivity assay (DPRA) for skin sensitisation testing 23pp.

[4] Hungarian Good Laboratory Practice Regulation: 42/2014 (VIII. 19.) EMMI decree of the Minister of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM(98)17

[5] OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998

[6] EC EURL ECVAM (January 2012). Direct Peptide Reactivity Assay (DPRA) Validation Study Report pp. 1-74.

[7] EURL-ECVAM, "Addendum to the Direct Peptide Reactivity Assay (DPRA) ECVAM Validation Study Report," pp. 1–4.

[8] EC EURL-ECVAM (2013). Recommendation on the Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing.

[9] OECD (2021), *Guideline No. 497: Defined Approaches on Skin Sensitisation*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.

[10] SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21

APPENDICES

APPENDIX I

COPY OF THE GLP CERTIFICATE OF TOXI-COOP ZRT.

OGYÉI Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet

1135 Budapest, Szabolcs utca 33.
Levélcím: 1372 Postafiók 450
Tel.: +36 1 886 9300, Fax: +36 1 886 9460
E-mail: ogyei@ogyei.gov.hu
Web: www.ogyei.gov.hu

Ref. no: OGYÉI/21950-8/2022
Admin.: dr. Szaller Zoltán
Date: 11th August, 2022

GOOD LABORATORY PRACTICE (GLP) CERTIFICATE

It is hereby certified that the test facility

TOXI-COOP Toxicolgical Research Center Zrt.

H-1103 Budapest, Cserkesz u. 90.,
H-1045 Budapest, Berlini u. 47-49.,
H-8230 Balatonfüred, Arácsi u. 97-99.,
H-8230 Balatonfüred, Vasút u. 3.,
H-8230 Balatonfüred, Galamb u. 12/A ,
H-8230 Balatonfüred, Ady E. u. 12,
8354 Karmacs, hrsz 4150/2

is able to carry out

physico-chemical testing, toxicity studies, mutagenicity studies, environmental toxicity studies on aquatic and terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation studies, analytical and clinical chemistry, safety pharmacology testing, metabolism and toxico/pharmacokinetics testing, testing of toxicological properties of operative procedures and equipment, reproduction toxicological studies, tolerance studies, inhalation toxicology and in vitro studies

in compliance with the Principles of GLP (Good Laboratory Practice) and also complies with the corresponding OECD/European Community requirements.
This certificate is valid till 20th of April, 2025.

Date of the inspection: **11-14 and 19-20 April, 2022.**

El Koulali Zakariás
Digitálisan aláírta: El Koulali Zakariás
Dátum: 2022.08.11 15:42:41 +02'00'
Deputy Director General

APPENDIX II

COPY OF THE CERTIFICATE OF ANALYSIS OF THE TEST ITEM

CERTIFICATE of ANALYSIS [redacted]

Tafluprost ethyl amide

N-ethyl-9α,11α-dihydroxy-15,15-difluoro-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide

Item No. 9000843 • Batch No. 0652603

Purity Specification: ≥98%
Molecular Formula.: C24H33F2NO4
CAS Number: 1185851-52-8 Formula Weight: 437.5 Expiry date: 24JUN2023

Overview

Tests	Results
HPLC	Purity: 98.5 %
Mass spec	M+Na: 460.8 MH+: 438.4
TLC	Purity: 100 %

Reviewed and approved by: [redacted]

WARNING
THIS PRODUCT IS FOR RESEARCH USE ONLY. NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE. IT IS THE RESPONSIBILITY OF THE PURCHASER TO DETERMINE SUITABILITY FOR OTHER APPLICATIONS.

SAFETY DATA
This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent via email to your institution.

WARRANTY AND LIMITATION OF REMEDY
Buyer agrees to purchase the material subject t[redacted]rms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

[redacted]

APPENDIX III

CHROMATOGRAMS

SHIMADZU LabSolutions **Analysis Report**

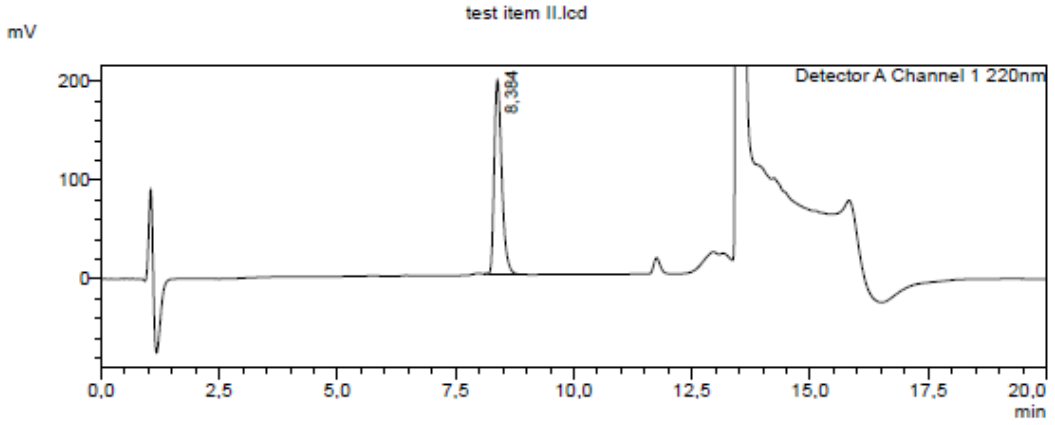
<Sample Information>

test item II.lcd

Sample Name	: test item II
Sample ID	:test item II
Data Filename	: test item II.lcd
Method Filename	: cysteine analysis 20 min.lcm
Batch Filename	: 20220922.lcb
Vial #	: 1-23
Injection Volume	: 7 uL
Date Acquired	: 2022.09.22. 16:07:45
Acquired by	: Szabóné Sági Dóra

<Chromatogram>

test item II.lcd



<Peak Table>

test item II.lcd

Detector A Channel 1 220nm

Peak#	Ret. Time	Area	Name	Mark
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AuditTrail Information test item II.lcd

<<Data File>>

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Batch File	:D:\Data\DPRA studies\147-442-7156\cysteine_20220922\20220922.lcb
Report Format File	:D:\Data\DPRA studies\cysteine report format.lsr
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Original Method File	:D:\Data\DPRA studies\cysteine analysis 20 min.lcm
Original Batch File	:D:\Data\DPRA studies\147-442-7156\cysteine_20220922\20220922.lcb
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Acquired by	:Szabóné Sági Dóra
Date Created	:2022.09.22. 16:07:26
Created by	:Szabóné Sági Dóra
Date Modified	:2022.09.23. 9:56:10
Modified by	:Szabóné Sági Dóra

<<AuditTrail Information>>

Change History # :0

Change History Detail

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CHROMATOGRAM 1.

Chromatogram of the test item

SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

reference C I acn.lcd

Sample Name	: reference C I acn
Sample ID	:ref C I acn
Data Filename	: reference C I acn.lcd
Method Filename	: cysteine analysis 20 min.lcm
Batch Filename	: 20220922.lcb
Vial #	: 1-54
Injection Volume	: 7 uL
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Acquired by	: Szabóné Sági Dóra

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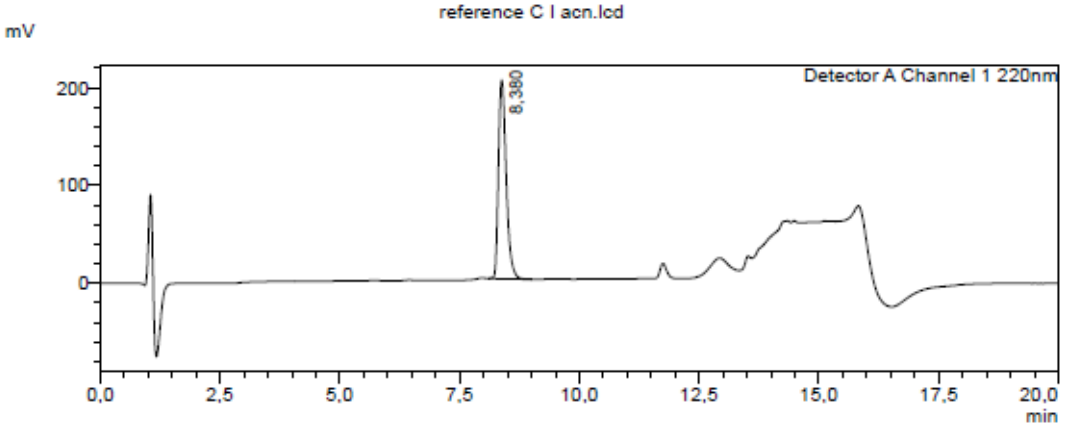
reference C I acn.lcd



<Peak Table>

reference C I acn.lcd

Detector A Channel 1 220nm

Peak#	Ret. Time	Area	Name	Mark
1	8,380	2288587	Cysteine	

AuditTrail Information reference C I acn.lcd

<<Data File>>

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Acquired by	:Szabóné Sági Dóra
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Created by	:Szabóné Sági Dóra
Date Modified	:2022.09.23. 9:56:03
Modified by	:Szabóné Sági Dóra

<<AuditTrail Information>>

Change History #	:0
Change History Detail	

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CHROMATOGRAM 2.

Chromatogram of Reference control C acn

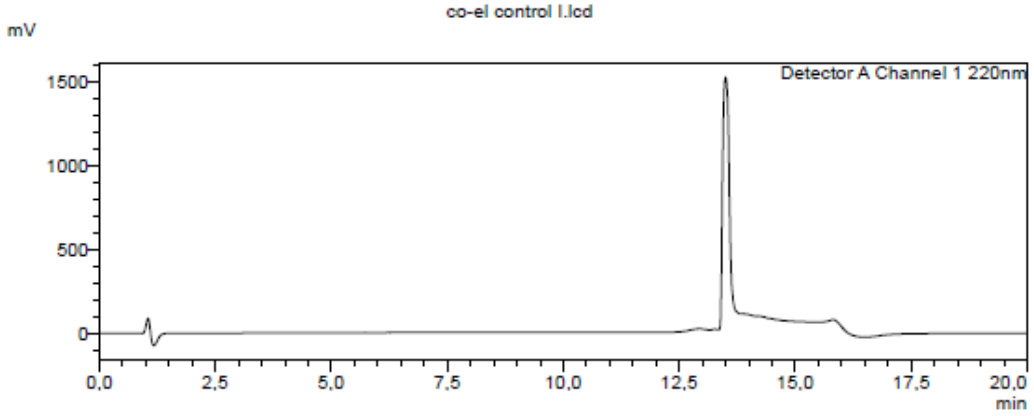
SHIMADZU LabSolutions **Analysis Report**

<Sample Information>

co-el control I.lcd

Sample Name	: co-elution control I
Sample ID	:co-el control I
Data Filename	: co-el control I.lcd
Method Filename	: cysteine analysis 20 min.lcm
Batch Filename	: 20220922.lcb
Vial #	: 1-21
Injection Volume	: 7 uL
Date Acquired	: 2022.09.22. 23:15:01
Acquired by	: Szabóné Sági Dóra

<Chromatogram>



<Peak Table>

co-el control I.lcd

Detector A Channel 1 220nm

AuditTrail Information co-el control I.lcd

<<Data File>>

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Batch File	:D:\Data\DPRA studies\147-442-7156\cysteine_20220922\20220922.lcb
Report Format File	:D:\Data\DPRA studies\cysteine report format.lsr
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Original Method File	:D:\Data\DPRA studies\cysteine analysis 20 min.lcm
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Acquired by	:Szabóné Sági Dóra
Date Created	:2022.09.22. 23:14:43
Created by	:Szabóné Sági Dóra
Date Modified	:2022.09.23. 9:56:44
Modified by	:Szabóné Sági Dóra

<<AuditTrail Information>>

Change History #	:0
Change History Detail	

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CHROMATOGRAM 3.

Chromatogram of the co-elution control

APPENDIX IV

EVALUATION EXCEL SHEETS

Table 14: Results of cysteine run I.

	Peak Area	Peak Area Mean	Peak Area CV%	Peptide Concentration (mM)
STD 1	2614401	2514458	3%	0.5311
	2460291			
	2468683			
STD 2	1362764	1296206	4%	0.2711
	1264599			
	1261255			
STD 3	700179	652156	6%	0.1336
	628461			
	627828			
STD 4	384790	373590	3%	0.0741
	367229			
	368750			
STD 5	187162	179616	4%	0.0327
	176173			
	175514			
STD 6	95016	93153	2%	0.0143
	92468			
	91976			
STD 7	0	0	-	-
	0			
	0			

Table 15: Results of cysteine run II.

	Peak Area	Peak Area Mean	Peak Area CV	Peptide Concentration (mM)	Mean peptide Concentration (mM)	Peptide Concentration CV	Peptide Concentration SD	Peptide depletion (%)	
reference control A	2400301	2416473	1%	0.51	0.51	1%	0.003	-	
	2422555			0.51				-	
	2426564			0.51				-	
reference control B	2389306	2344052	2%	0.50	0.49	2%	0.01	-	Ref. B and C acn
	2391955			0.50				-	peak area SD:
	2380622			0.50				-	48272
	2315154			0.49				-	
	2291869			0.48				-	
	2300018			0.49				-	
reference control C acn	2288587			0.48	0.49	3%	0.01	-	Ref C acn
	2330142			0.49				-	peak area mean:
	2408817			0.51				-	2342515
positive control	705569	687065	2%	0.14	0.14	2%	0.003	69.88%	
	679508			0.14				70.99%	
	676118			0.14				71.14%	
test item	2222791	2211107	0.5%	0.47	0.47	0.5%	0.002	5.11%	
	2203597			0.46				5.93%	
	2206932			0.47				5.79%	
co-elution control	0	0	-	-	-	-	-	-	
	0			-	-	-	-	-	
	0			-	-	-	-	-	

	Mean cysteine peptide depletion	SD	CV
Test item	5.61%	0.44%	7.80%
Positive control	70.67%	0.69%	0.97%

Final outcome: **Negative (no or minimal reactivity)**

Final Report

Original 1 of 1

Determination of the potential of
Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
(Neat Oil) to react with synthetic model Cys- and Lys-peptides
using the *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)
according to OECD 442C and EU-Method B.59

Study No.: 22120103G875

Sponsor:

[redacted]

Monitor:

ToxMinds BVBA
Dr. Thomas Petry
Avenue de Broqueville, 116
1200 Brussels
Belgium

Test Facility:

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler
Germany

Study Director:

Diana Brandt

1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following documents:

- OECD Principles of Good Laboratory Practice and Compliance Monitoring, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998 and subsequent advisory/consensus OECD GLP documents (where appropriate).
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemicals Act of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 from 28. Aug. 2013, published in Federal Law Gazette, Germany (BGBl) No. 55/2013 as of 06. Sep. 2013, and further revisions.

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

This report contains the following parameter which was not performed under GLP conditions: Determination of the proficiency chemicals and stability test of cysteine protein in acetonitrile.

15 JUN 2023

Diana Brandt
Study Director

Date

Information on Study Organisation:

Study Director	Diana Brandt
Deputy Study Director	Dr. Jörg Johannes
Study Plan dated	31. Jan. 2023
Experimental Starting Date	08. Feb. 2023
Experimental Completion Date	10. Feb. 2023

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice.

All phases of the study (Study plan, performance of the study and Final report) were checked by the quality assurance. Dates of inspections are given below. Findings are reported to the Study Director and Test Facility Management.

The inspection of the performance of short-term studies (duration less than four weeks) may be carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the Final report was written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study plan (if any) were acknowledged and assessed by the Study Director and included in the Final report.

The reported results reflect the raw data of the study.

Phases of Study	Inspected on	Findings reported on	Audit Report No.
Study plan	25. Jan. 2023	25. Jan. 2023	230125-03
Performance of study	08. Feb. 2023	08. Feb. 2023	230208-12
Final report	31. May 2023	31. May 2023	230531-05

Schedler

Dr. Anette Schedler
Quality Assurance

14 JUN 2023

Date

Table of Contents

3 SUMMARY

Title of Study: Determination of the potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) to react with synthetic model Cys- and Lys-peptides using the *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA) according to OECD 442C and EU Method B.59.

Findings and Results:

This *in chemico* study evaluates the skin sensitization potential of the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil), according to OECD Guideline 442C and EU Method B.59, in compliance with GLP. The direct peptide reactivity assay (DPRA) addresses the first molecular key event (KE1) of the adverse outcome pathway (AOP) of skin sensitisation assessing the reactivity of the test substancetowards cysteine (Cys-) and lysine (Lys-) containing peptides.

The calculated peptide depletion could be used to support the discrimination between skin sensitisers and non-sensitisers. The DPRA is part of a tiered strategy for the evaluation of skin sensitisation potential in the context of an integrated approach to testing and assessment (IATA).

The test item was incubated for 22 h at 25 °C together with Cys- and Lys-peptides, respectively. The peptide concentration after the incubation period was measured using HPLC-UV.

Three replicates were prepared using 1:10 and 1:50 molar ratio of the test item with the Cys- and Lys-peptides, respectively. Triplicate samples of the solvent without test item were incubated and measured simultaneously.

One valid experiment was performed.

The peptide depletion values after incubation are shown in in the following table.

Table 3 Results

Cys-Peptide Depletion [%]	Lys-Peptide Depletion [%]	Mean Peptide Depletion [%]
3.20	0.08	1.64

The DPRA prediction is “negative” according to the Cysteine 1:10/Lysine 1:50 prediction model. However, the test item showed precipitates in the Lys-peptide-assays and therefore, a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result and therefore, no firm conclusions on the lack of reactivity is possible under the conditions of the study. It is, however, important to note that the mean peptide depletion in the Cys-peptide assay, which shows no precipitation for test item, was 3.2 %. This is well below 6.38 % or 13.89 % suggesting no or minimal reactivity. The results of the Cys-peptide assay are considered as an alternative for interpretation of assays where co-elution occurs. As the test item does not show co-elution, the interpretation from Cys-peptide assay cannot be used for the final conclusion. It, nevertheless, supports no or minimal reactivity.

4 PURPOSE AND PRINCIPLE OF THE STUDY

The adverse outcome pathway (AOP) for skin sensitisation is initiated by a first molecular key event 1 (KE1) corresponding to covalent interaction with skin proteins, which is followed sequentially by: (KE2) keratinocyte activation, (KE3) dendritic cell activation, and (KE4) proliferation of antigen-specific T cells. Currently, none of the assays addressing the different KEs is accepted as stand-alone test method as it may not be sufficient to conclude on the presence or absence of skin sensitisation potential of chemicals. However, it supports the discrimination between skin sensitisers and non-sensitisers in combination with other complementary data.
The direct peptide reactivity assay (DPRA) addresses the first molecular key event (KE1) of the AOP of skin sensitisation. It is an *in chemico* assay addressing epidermal protein binding and reactivity towards proteins, by mimicking the reaction with artificial peptides. The principle of the assay is based on the quantification of the depletion of synthetic model peptides caused by known amounts of the test item measured by HPLC.

In this study the reactivity of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) towards cysteine and lysine containing peptides was evaluated. The peptide depletion compared to the solvent controls is calculated and leads to a DPRA prediction (reactive/positive or non-reactive/negative) which could be used to support the discrimination between skin sensitisers and non-sensitisers. Additionally, an assignment to one of four reactivity classes could be made in order to possibly support a potency assessment.

The DPRA is part of a tiered strategy for the evaluation of the skin sensitisation potential. Hence, all data generated with the present Test Guideline OECD 442C and EU-Method B.59 should be used in the context of an integrated approach to testing and assessment (IATA).

5 LITERATURE

The study was conducted in compliance with the following guideline(s):

- OECD Guidelines for the Testing of Chemicals, Method No. 442C, adopted 30. Jun. 2022: “Key Event-Based Test Guideline for *in chemico* skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins“
- EURL ECVAM (European Union Reference Laboratory for alternatives to animal testing): “DB-ALM Protocol n° 154: Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing.”, 21. Oct. 2021
- Commission Regulation (EU) 2017/735, EU-Method B.59 adopted 14 February 2017 “In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)”

Corresponding SOP of LAUS GmbH:

- SOP 118 00 875 edition 2, valid from 04. Nov. 2019, „Durchführung des DPRA-Tests nach OECD 442C“

6 MATERIALS AND METHODS

6.1 Test Item

Designation in Test Facility:	22120103G
Date of Receipt:	01. Dec. 2022
Condition at Receipt:	cooled, in proper conditions

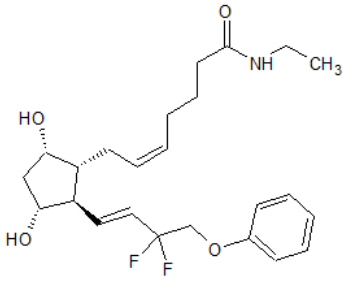
6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Batch no.	TAF-10-1122-01
CAS no.	1185851-52-8
EC no.	867-521-0
Composition	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
Storage	fridge (2 - 8 °C); keep under inert gas
Expiry date	23. Nov. 2026
Stability	stable under storage conditions
Appearance	clear, colorless to light yellow liquid
Purity	99.78 %
Homogeneity	homogeneous
Production date	18. Nov. 2022
EC no.	867-521-0
Molecular formula	$C_{24}H_{33}F_2NO_4$
Molecular weight	437.52 g/mol
Vapour pressure	unknown
Solubility in solvents	water: not stated; ethanol: >1g/L; acetone: not stated; acetonitrile: not stated; DMSO: >1g/L; methanol: >1g/L; DMF: 0.1-1g/L
Stability in solvents	water: not stated; ethanol: not stated; acetone: not stated; acetonitrile: not stated; DMSO: not stated; methanol: not stated; DMF: not stated

A certificate of analysis is provided by the sponsor and attached (in copy) in Annex 6.

6.1.2 Structural Formula



O[C@@H]2C[C@H](O)[C@H](C/C=C\CCCC(=O)NCC)[C@H]2/C=C/C(F)(F)COc1ccccc1

6.1.3 Storage in the Test Facility

The test item was stored in a closed vessel in the fridge (2 - 8 °C); kept under inert gas.

6.1.4 Preparation of Test Item

The concentration of the test item to be used was determined based on the molecular weight (MW) 437.52 g/mol and the purity 99.78 %.

The target weight (± 10 %) of the test item was calculated using this equation:

$$\textbf{Weight [mg]} = 3\ \text{mL} \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{100\ \text{mmol}}{\text{L}} \times \text{MW}\left[\frac{\text{mg}}{\text{mmol}}\right] \times \frac{100}{\%\ \text{Purity}} = \frac{\textbf{MW}}{\textbf{\% Purity}} \times \textbf{30}$$

In a non-GLP pre-test the solubility of 132.1 mg test item (corresponding to 100 mM) was determined in 3 mL:

- Acetonitrile

Based on this non-GLP pre-test, the 100 mM test item solution was prepared by dissolving 131.6 mg and 132.0 mg test item in 3 mL acetonitrile for the Cys-peptide and Lys-peptide assay, respectively. The solution was vortexed until it was dissolved completely.

6.2 Test System

6.2.1 Analytical Instrument

The details of the HPLC system which was used are listed below.

Designation:	HPLC_4
Components:	Degasser G1322A
	Quaternary pump G1311A
	Autosampler G1313A
	Column compartment G1316A
	UV/VIS-Detector DAD G1315A
Manufacturer:	Agilent Technologies
Software:	CHROMELEON 6.80 SR15b Build 4981

Usage and calibration followed the corresponding SOP 114 00 526, edition 2 valid from 04. Mar. 2022.

6.2.2 Column

An ACE Excel SuperC18 150 x 3 mm column with 3 µm particles and pre-column Phenomenex SecurityGuard C18, 4 x 3 mm was used. This column was selected because it delivers substantially better peak shape for the peptides than the Agilent Zorbax SB-C18 column recommended in the guideline OECD 442C.

6.2.3 HPLC Program

Eluent D	H_2O + 0.1 % TFA		
Eluent B	Acetonitrile + 0.085 % TFA		
Gradient	time (min)	% D	% B
	0	90	10
	10	75	25
	10.5	10	90
	12	10	90
	13	90	10
	20	90	10
Flow rate	0.55 mL/min		
Injection volume	7 µL		
Column temperature	30 °C		
Wavelength 1	220 nm		
Wavelength 2	258 nm		

6.2.4 Synthetic Peptides

Peptides with ≥ 95 % purity, synthesized by RS synthesis, (Louisville, KY, USA), were used.

Cys-Peptide (Cysteine)
Sequence: Ac-RFAA**C**AA-COOH (MW = 750.9 g/mol)
Batch no.: 220314-P013439
Purity: 98.06 %
Expiration date: Nov. 2023
Quality assurance system: not stated

Lys-Peptide (Lysine)
Sequence: Ac-RFAA**K**AA-COOH (MW = 775.9 g/mol)
Batch no.: 220314-P013440
Purity: 99.32 %
Expiration date: Nov. 2023
Quality assurance system: not stated

6.3 Instruments and Devices

The instruments and devices that were used in the test are listed in the following table.

Table 6 Instruments and Devices

Device	Device Name	Manufacturer
Repeater pipette	AutoRep E	Rainin ®, Mettler Toledo
Heating chamber	IN 110	Memmert
Glass thermometer	Glass thermometer 20151201-1	LW
Fridge	LKv 3913 Index 21A/001	Liebherr
Pipette 200 – 2000 µL	L-1000XLS	Rainin ®, Mettler Toledo
Ultrasonic bath	Sonorex	Bandelin
Vortexer	IKA Vortex 2 S000	IKA
pH-meter	3310	wtw
Analytical scale	XSR205 Dual Range	Mettler Toledo
Precision scale	ME5002T/M00	Mettler Toledo

Usage and, if applicable, calibration of all instruments followed the corresponding SOP in the current edition.

Standard laboratory material (glassware) was also used in the performance of the study.

6.4 Chemicals

- Water for chromatography
  H_2O, avantor™, LC/MS grade, batch no.: 22J124006, CAS no.: 7732-18-5, expiry date: 06. Oct. 2024
- Acetonitrile for chromatography
  CH_3CN, ACN, Honeywell, HPLC grade, p. a., batch no.: L1650S, CAS no.: 75-05-8, expiry date: 04. Jun. 2023, used only for eluents
  CH_3CN, ACN, Carl Roth, 99.98 %, batch no.: 1398801, CAS no.: 75-05-8, expiry date: 23. Nov. 2023
- Trifluoroacetic Acid for protein sequencing
  TFA, Sigma-Aldrich, 99 %, batch no.: STBK3572, CAS no.: 76-05-1, expiry date: 01. May 2023
- 25 % Ammonia Solution
  NH_3, Carl Roth, ≥ 25 %, p. a., batch no.: 462327278, CAS no.: 1336-21-6, expiry date: 03. Aug. 2026
- Ammonium Acetate
  CH_3COONH_4, AppliChem, p. a., batch no.: 0001925413, CAS no.: 631-61-8, expiry date: 30. Sep. 2026
- Sodium Dihydrogen Phosphate Monohydrate
  NaH_2PO_4 * 1 H_2O, Sigma-Aldrich, 98 % – 102 %, p. a., ACS, batch no.: BCCD9705, CAS no.: 10049-21-5, expiry date: 01. Sep. 2023
- Disodium Hydrogen Phosphate Heptahydrate
  Na_2HPO_4 * 7 H_2O, Sigma-Aldrich, 98 % – 102 %, p. a., ACS, batch no.: SLCG9636, CAS no.: 7782-85-6, expiry date: 01. Aug. 2023

6.5 Buffers

6.5.1 100 mM Phosphate Buffer

Mix out of solution A + B, batch no.: T20230207

Solution A: 1.38 g sodium dihydrogen phosphate monohydrate (monobasic) were dissolved in 100 mL HPLC grade water.

Solution B: 6.70 g disodium hydrogen phosphate heptahydrate (dibasic) were dissolved in 250 mL HPLC grade water.

Final 100 mM phosphate buffer was mixed out of 18 mL of solution A and 82 mL of solution B. The pH was adjusted to 7.501 with solution A.

6.5.2 100 mM Ammonium Acetate Buffer

Batch no.: T20230207

1.5418 g ammonium acetate (anhydrous) were dissolved in 200 mL HPLC grade water, pH was adjusted to 10.200 with 25 % ammonia solution.

6.6 Controls

6.6.1 Positive Control

Positive controls were treated identically as the test item. The following positive controls were used:

- Cinnamaldehyde (Sigma-Aldrich, ≥ 98.6 %, batch no.: MKCJ4653, CAS no.: 104-55-2, expiry date: 01. Feb. 2024) was used as 100 mM (± 10 %) solution in acetonitrile for the Cys-peptide.
- 2,3-Butanedione (Sigma-Aldrich, ≥ 99 %, batch no.: SHBL0314, CAS no.: 431-03-8, expiry date: 13. Oct. 2023) was used as 100 mM (± 10 %) solution in acetonitrile for the Lys-peptide

As cinnamaldehyde mixed with the Lys-peptide turned turbid in all experiments performed during the implementation phase, it was considered unsuitable as positive control. Instead, the proficiency chemical 2,3-Butanedione was used as positive control showing mid-range depletion for the Lys-peptide.

6.6.2 Reference Controls

For both peptides, four sets of reference controls using acetonitrile instead of test item stock solution were prepared in triplicate (sets A, B1, B2 and C, total 12 samples per peptide). Set A was analysed together with the peptide calibration standards, sets B1 and B2 were analysed at the start and end of the analysis sequence and were used as stability control for the peptide over the total analysis time. Set C was incubated and analysed together with the samples and was used for calculation of the peptide depletion.

6.6.3 Co-Elution Control

Sample prepared from the respective peptide buffer and the test item, but without peptide.

7 PERFORMANCE OF THE STUDY

7.1 Test Solutions

7.1.1 Dilution Buffers

- 2 mL Acetonitrile were mixed with 8 mL phosphate buffer, pH 7.5 (Peptide dilution buffer C)
- 2 mL Acetonitrile were mixed with 8 mL ammonium acetate buffer, pH 10.2 (Peptide dilution buffer K)

7.1.2 Peptide Stock Solutions

The peptide stock solutions were freshly prepared for each assay.

- 0.667 mM Cys-Peptide solution was prepared by dissolving 15.0 mg of the peptide in 30 mL phosphate buffer, pH 7.5. (batch no.: T20230209)
- 0.667 mM Lys-Peptide solution was prepared by dissolving 15.4 mg of the peptide in 30 mL ammonium acetate buffer, pH 10.2. (batch no.: T20230208)

7.1.3 Peptide Calibration Standards

From each peptide stock solution, the following calibration standards were prepared in the appropriate dilution buffer (see chapter 7.1.1): 0.534 / 0.267 / 0.1335 / 0.0667 / 0.0334 / 0.0167 mM Peptide.

Calibration samples were analysed before the samples containing the test item. Blank dilution buffer was also measured.

7.1.4 Test Item Samples

Samples were prepared in triplicate for each peptide. The Cys-peptide samples were prepared in 1:10 molar ratio (0.5 mM peptide: 5 mM test item solution), the Lys-peptide samples in 1:50 molar ratio (0.5 mM peptide and 25 mM test item solution) using the stock solutions described in chapter 7.1.2. A final volume of 1 mL per sample was prepared for each sample.

7.2 Incubation

The positive control, solvent control sets C, and test item samples were incubated in closed amber glass HPLC vials in an incubation chamber at 25.0 ± 2.5 °C for 22 h.

All three replicates for the Lys-peptide were turbid before incubation. After incubation, the undiluted test item precipitated at the bottom of the vials (phase separation). The clear supernatant was taken from the vials and used for the measurement (see chapter 13 Deviations)

All replicates for the Cys-peptide were clear and without precipitation before and after incubation.

8 FINDINGS

Measurements were performed using the HPLC method described in chapter 6.2. Results are shown in chapter 9.

8.1 Measurements Cys-Peptide

The measurements for the Cys-peptide are stated in the following table.

Table 8.1 Measurements Cys-Peptide

Sample Name	Peak Area 220 nm [mAU*min]	Peak Area 258 nm [mAU*min]	Area Ratio 220 nm/258 nm [dimension-less]
Blank	n. a.	n. a.	n. a.
Standard 0.0167 mM	0.5873	n. a.	n. c.
Standard 0.0334 mM	1.1979	0.0327	36.60
Standard 0.0667 mM	2.4552	0.0638	38.49
Standard 0.1335 mM	5.0027	0.1302	38.43
Standard 0.267 mM	9.9731	0.2562	38.93
Standard 0.534 mM	18.8567	0.4957	38.04
Reference A Rep. 1	18.4693	0.4856	38.04
Reference A Rep. 2	18.3300	0.4771	38.42
Reference A Rep. 3	18.4495	0.4846	38.08
Co-elution control positive control	n. a.	n. a.	n. a.
Co-elution control test item	n. a.	n. a.	n. a.
Reference B Rep. 1	18.2793	0.4772	38.31
Reference B Rep. 2	18.5181	0.4846	38.22
Reference B Rep. 3	18.5024	0.4847	38.17
Reference C ACN Rep. 1	18.6993	0.4872	38.38
Positive control Rep. 1	3.2979	0.0871	37.86
Test item Rep. 1	17.9647	0.4674	38.43
Reference C ACN Rep. 2	18.3691	0.4797	38.29
Positive control Rep. 2	3.2498	0.0852	38.16
Test item Rep. 2	17.9097	0.4667	38.37
Reference C ACN Rep. 3	18.4636	0.4791	38.54
Positive control Rep. 3	3.2144	0.0827	38.86
Test item Rep. 3	17.8778	0.4678	38.21
Reference B Rep. 4	18.7082	0.4906	38.14
Reference B Rep. 5	18.5414	0.4877	38.02
Reference B Rep. 6	18.6185	0.4892	38.06
Mean peak area ratio of reference controls A, B and C (ACN)[1]			**38.22**

n. a. = no peak detected
n. c. = not calculable
[1] Used as reference for calculation of peak purity

8.2 Measurements Lys-Peptide

The measurements for the Lys-peptide are stated in the following table.

Table 8.2 Measurements Lys-Peptide

Sample Name	Peak Area 220 nm [mAU*min]	Peak Area 258 nm [mAU*min]	Area Ratio 220 nm/258 nm [dimension-less]
Blank	n. a.	n. a.	n. a.
Standard 0.0167 mM	0.6722	0.0172	38.98
Standard 0.0334 mM	1.3543	0.0352	38.43
Standard 0.0667 mM	2.6886	0.0698	38.49
Standard 0.1335 mM	5.3468	0.1391	38.43
Standard 0.267 mM	10.7331	0.2856	37.58
Standard 0.534 mM	21.4826	0.5807	36.99
Reference A Rep. 1	20.0567	0.5461	36.73
Reference A Rep. 2	20.0253	0.5393	37.13
Reference A Rep. 3	20.0229	0.5456	36.70
Co-elution control positive control	n. a.	n. a.	n. a.
Co-elution control test item	n. a.	n. a.	n. a.
Reference B Rep. 1	20.1556	0.5455	36.95
Reference B Rep. 2	20.0818	0.5409	37.13
Reference B Rep. 3	20.0905	0.5421	37.06
Reference C ACN Rep. 1	20.0793	0.5456	36.80
Positive control Rep. 1	15.7684	0.4496	35.07
Test item Rep. 1	20.0971	0.5478	36.69
Reference C ACN Rep. 2	20.0202	0.5413	36.98
Positive control Rep. 2	15.3826	0.4375	35.16
Test item Rep. 2	20.0936	0.5470	36.73
Reference C ACN Rep. 3	20.1543	0.5495	36.67
Positive control Rep. 3	15.5648	0.4504	34.56
Test item Rep. 3	20.0367	0.5465	36.66
Reference B Rep. 4	20.0537	0.5444	36.84
Reference B Rep. 5	20.0048	0.5443	36.75
Reference B Rep. 6	20.0658	0.5460	36.75
Mean peak area ratio of reference controls A, B and C (ACN)[1]			**36.87**

n. a. = no peak detected
[1] Used as reference for calculation of peak purity

9 RESULTS

9.1 Calibration Curve

9.1.1 Determination

From the peak areas of the peptide calibration standards detected at 220 nm, linear calibration curves in the form y = b*x + a were calculated for both peptides using validated Microsoft Excel® spreadsheets, with

y = Measured peak area [mAU*min]

b = Slope [mAU*min / mM]

a = Intercept [mAU*min]

x = Standard concentration [mM]

Table 9.1 Linear Calibration Curves

	Cys-Peptide	Lys-Peptide
Slope b	35.43929	40.21987
Intercept a	0.13593	-0.00092
r²	0.99904	1.00000

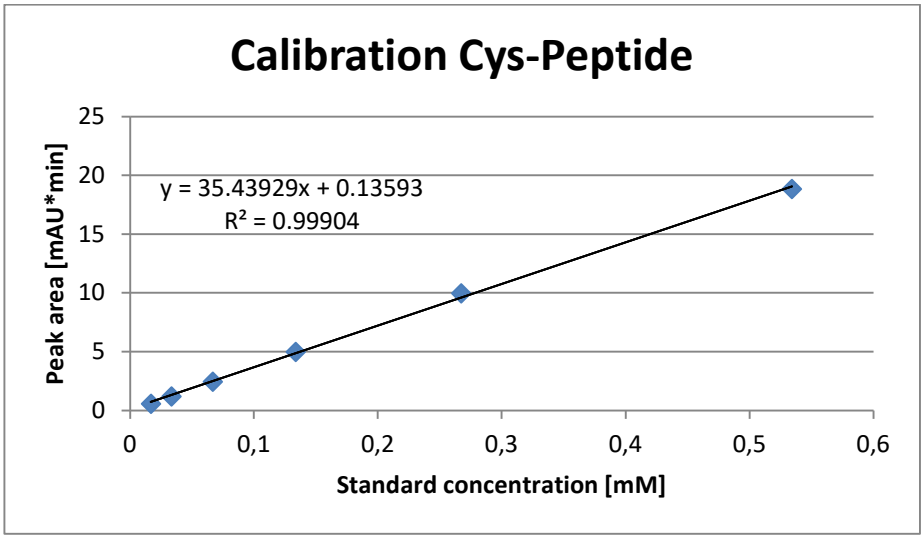


Figure 9.1–a Calibration Curve of the Cys-Peptide

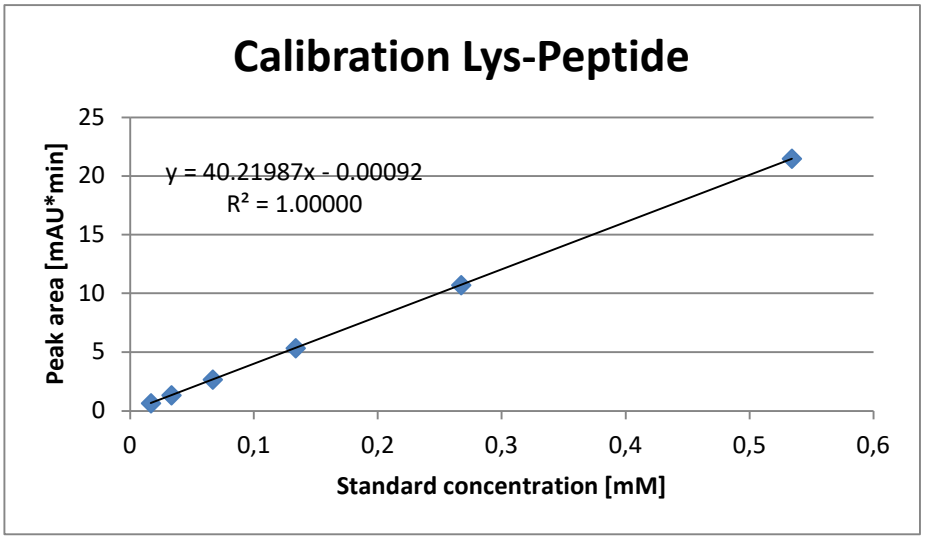


Figure 9.1–b Calibration Curve of the Lys-Peptide

9.1.2 Acceptance Criteria

The r² of linear calibration should be > 0.99.

9.1.3 Assessment

The calibration curve was linear with acceptable coefficient of determination 0.99904 for the Cys-peptide and 1.00000 for the Lys-peptide, respectively.

9.2 Solvent Controls

9.2.1 Determination

The peptide concentrations in the solvent controls were calculated using the linear regression (a = intercept, b = slope).

$$\text{Peptide concentration [mM]} = \frac{\text{Peak area [mAU*min]} - a}{b}$$

Table 9.2 Calculated Peptide Concentration for Solvent Controls

Sample Name	Cys-Peptide Concentration [mM]	Lys-Peptide Concentration [mM]
Reference A Rep. 1	0.517	0.499
Reference A Rep. 2	0.513	0.498
Reference A Rep. 3	0.517	0.498
Reference B Rep. 1	0.512	0.501
Reference B Rep. 2	0.519	0.499
Reference B Rep. 3	0.518	0.500
Reference B Rep. 4	0.524	0.499
Reference B Rep. 5	0.519	0.497
Reference B Rep. 6	0.522	0.499
Reference C (ACN) Rep. 1	0.524	0.499
Reference C (ACN) Rep. 2	0.514	0.498
Reference C (ACN) Rep. 3	0.517	0.501
Mean concentration of Reference controls A and C [mM]	**A: 0.52 C: 0.52**	**A: 0.50 C: 0.50**
Variation coefficient (RSD) of Reference controls B and C (ACN) [%]	**0.8**	**0.3**

9.2.2 Acceptance Criteria

a) The mean peptide concentration of solvent control samples of sets A and C (ACN) should be 0.50 ± 0.05 mM
b) The variation coefficient (relative standard deviation, RSD) of measured values of the nine samples from sets B1, B2 and C should be < 15 %

9.2.3 Assessment

a) The mean peptide concentration of all solvent controls (Reference A and Reference C) were with 0.52 mM and 0.52 mM for the Cys-peptide and 0.50 mM and 0.50 mM for the Lys-peptide in the acceptable range of 0.50 ± 0.05 mM.
b) The variation coefficients (RSD) of the measured values of Reference controls B and C in acetonitrile were in the acceptable range (< 15 %) with 0.8 % for the Cys-peptide and 0.3 % for the Lys-peptide, respectively.

10 EVALUATION AND RESULTS

10.1 Calculations

10.1.1 Calibration Curve

From the peak areas of the peptide calibration standards detected at 220 nm, a linear calibration curve is calculated using a Microsoft Excel® spreadsheet.

The peptide concentration in all other samples is calculated using the linear regression (a = intercept, b = slope).

$$\text{Peptide concentration [mM]} = \frac{\text{Peak area [mAU*min]} - a}{b}$$

As a peak purity criterion, the percent peak area ratio 220/258 nm should be constant over all analyzed samples (100 ± 10 %). For small peaks, this calculation may not be possible.

10.1.2 Peptide Depletion

The peptide depletion is calculated for each individual sample using the following equation in a first step (equations are shown for Cys-peptide, Lys-peptide is calculated analogously):

$$\text{Peptide depletion}_{Cys,i} = \left(1 - \frac{\text{measured peptide peak area in sample}}{\text{mean peptide peak area in Solvent controls C}}\right) \times 100\ \%$$

The mean peptide depletion of the Cys-peptide is calculated as follows:

$$\text{Peptide depletion}_{Cys} = \frac{\sum_{C,i=1,2,3} \text{peptide depletion}_i}{3}$$

The mean peptide depletion of the test item is calculated using the following equation:

$$\text{Mean peptide depletion [\%]} = \frac{\text{peptide depletion}_{Cys}\ [\%] + \text{peptide depletion}_{Lys}\ [\%]}{2}$$

10.2 Results

The results are stated in the following tables.

Table 10.2-a Calculated Peptide Depletion Values for the Cys-Peptide

Sample name	Depletion [%]		
	Single	Mean	SD
Positive control Rep. 1	82.18	82.42	0.23
Positive control Rep. 2	82.44		
Positive control Rep. 3	82.63		
Test item Rep. 1	2.95	3.20	0.24
Test item Rep. 2	3.25		
Test item Rep. 3	3.42		

SD = standard deviation

Table 10.2-b Calculated Peptide Depletion Values for the Lys-Peptide

Sample name	Depletion [%]		
	Single	Mean	SD
Positive control Rep. 1	21.49	22.47	0.96
Positive control Rep. 2	23.41		
Positive control Rep. 3	22.50		
Test item Rep. 1	0 (-0.06) *	0.08	0.14
Test item Rep. 2	0 (-0.04) *		
Test item Rep. 3	0.24		

*** Note: Negative depletion values were considered as "zero" when calculating the mean.**
SD = standard deviation

Mean depletion of both peptides after incubation with the test item: 1.64 %

10.2.1 Acceptance criteria

a) The mean peptide depletion value for the positive control cinnamaldehyde should be 60.8 % - 100.0 % with a maximum standard deviation (SD) of < 14.9 % for the Cys-peptide.
b) The mean peptide depletion value for the positive control 2,3-Butanedione should be 10.0 % - 45.0 % with a maximum standard deviation < 11.6 % for the Lys-peptide.
c) The standard deviation for the test item replicates should be < 14.9 % for the percent cysteine depletion and < 11.6 % for the percent lysine depletion.

10.2.2 Assessment

a) The mean peptide depletion with 82.42 % and a standard deviation of 0.23 % of the three replicates of the positive control cinnamaldehyde were in the acceptable range of 60.8 – 100.0 % and < 14.9 %, respectively, for the Cys-peptide.
b) The mean peptide depletion with 22.47 % and a standard deviation of 0.96 % of three replicates of the positive control 2,3-Butanedione were in the acceptable range of 10.0 – 45.0 % and < 11.6 %, respectively, for the Lys-peptide.
c) The standard deviation for the test item replicates with 0.24 % was < 14.9 % for the percent cysteine depletion for the test item.
   The standard deviation for the test item replicates with 0.14 % was < 11.6 % for the percent lysine depletion for the test item.

10.3 Prediction Model

According to the test guideline OECD 442C and EU-Method B.59, the reactivity is classified as “high”, “moderate”, “low” or “minimal” using the Cysteine 1:10/Lysine 1:50 prediction model shown in the following table.

Table 10.3 Evaluation of Results According to the Cysteine 1:10/Lysine 1:50 Prediction Model

Mean peptide depletion [%]	Reactivity Class	DPRA Prediction
0 – ≤ 6.38	No or Minimal	Negative
> 6.38 – ≤ 22.62	Low	Positive
> 22.62 – ≤ 42.47	Moderate	
> 42.47 - ≤ 100	High	

The mean peptide depletion in the Cys-peptide and Lys-peptide assay was 1.64 %.

The DPRA prediction is “negative” according to the Cysteine 1:10/Lysine 1:50 prediction model. However, the test item shows precipitates in Lys-peptide-assay and therefore, a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result and no “negative” prediction is possible under the conditions of the study.

It is, however, important to note that the mean peptide depletion in the Cys-peptide assay, which shows no precipitation for test item, was 3.2 %. This is well below 6.38 % or 13.89 % suggesting no or minimal reactivity. The results of the Cys-peptide assay are considered as an alternative for interpretation of assays where co-elution occurs. As the test item does not show co-elution, the interpretation from Cys-peptide assay cannot be used for the final conclusion. It, nevertheless, supports no or minimal reactivity. In addition, the result in the Cys-peptide assays is not close to the threshold for positive and negative results.

10.4 Proficiency Chemicals

The ten proficiency chemicals listed in the guideline were tested using the analysis method described in chapter 6.2.

All ten proficiency chemicals showed the expected DPRA prediction for both peptide assays. For the Cys-peptide assay nine out of the ten chemicals and for the Lys-peptide assay all ten chemicals showed depletion values consistent with the classification reported in the OECD guideline (LAUS in-house study, see chapter 18).

11 VALIDITY

The criteria of the calibration curve ($r^2 > 0.99$) and reference controls (mean peptide concentration 0.50 ± 0.05 mM) were fulfilled.

The mean peptide depletion of the positive control cinnamaldehyde was within the range 60.8 % - 100.0 %, the peptide depletion of the positive control 2,3-Butanedione was within 10.0 % - 45.0 %. The standard deviation of the replicates of the positive control and test item was < 14.9 % in the Cys-peptide assay and < 11.6 % in the Lys-peptide assay respectively. For detail see chapter 10.2.

All acceptance criteria were fulfilled; therefore, the study was considered valid.

12 DISCUSSION

One valid experiment was performed.

The test item was incubated for 22 h at 25 °C together with Cys- and Lys-peptides, respectively. The peptide concentration after the incubation period was measured using HPLC-UV.

Three replicates were prepared using 1:10 and 1:50 molar ratio of the test item with the Cys- and Lys-peptides, respectively. Triplicate samples of the solvent without test item were incubated and measured simultaneously.

The test item showed turbidity right after mixing the test item solution with buffer and the Lys-peptide-solution. After the incubation period, precipitation was observed and therefore only the supernatant was used for measurement.

The test item “Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)” shows minimal reactivity towards the Cys-peptide (mean depletion 3.20) and very low reactivity was observed towards the Lys-peptide (mean depletion 0.08). Therefore, the DPRA prediction would be “negative” with “no or minimal” reactivity.

According to the guideline OECD 442C, in samples with observed precipitation or phase separation, the peptide depletion may be underestimated and a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result, as one cannot be sure how much test item remains in solution to react with the peptides.

Therefore, it is no firm conclusions on the lack of reactivity is possible under the conditions of the study. It is, however, important to note that the mean peptide depletion in the Cys-peptide assay, which shows no precipitation for test item, was 3.2 %. This is well below 6.38 % or 13.89 % suggesting no or minimal reactivity. The results of the Cys-peptide assay are considered as an alternative for interpretation of assays where co-elution occurs. As the test item does not show co-elution, the interpretation from Cys-peptide assay cannot be used for the final conclusion. It, nevertheless, supports no or minimal reactivity.

No observations arousing doubts concerning the accuracy of the results and the validity of the study were made.

13 DEVIATIONS

13.1 Deviations from the Study Plan

The following deviation was observed:

- The supernatant after the incubation of the test item with the Lys-peptide, was used without centrifugation. As the supernatant was clear, no centrifugation was needed and this deviation can be stated as uncritical.

The deviation was assessed and signed by the study director on 28. Feb. 2023.

13.2 Deviations from the Guidelines

No deviations were ascertained.

14 RECORDING AND ARCHIVING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP-Document-Archive of the test facility for 15 years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP-Substance Archive for 15 years and then discarded.

Number of originals of the final report to be sent to the sponsor: 0, PDF-file only

15 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6, 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien / toxicological in vitro studies on mammalian cells and bacteria)

Datum der Inspektion / Date of Inspection
(Tag.Monat.Jahr / day.month.year)
28. und 29.04.2021

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist spätestens drei Jahre nach der letzten Inspektion zu beantragen. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for not later than three years after the last inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Landesamt für Umwelt Rheinland-Pfalz

Unterschrift, Datum / Signature, Date Mainz, 21.06.21

Sabine Riewenherm - Präsidentin -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

16 ANNEX 2: CHROMATOGRAMS

16.1 Cys-Peptide Assay

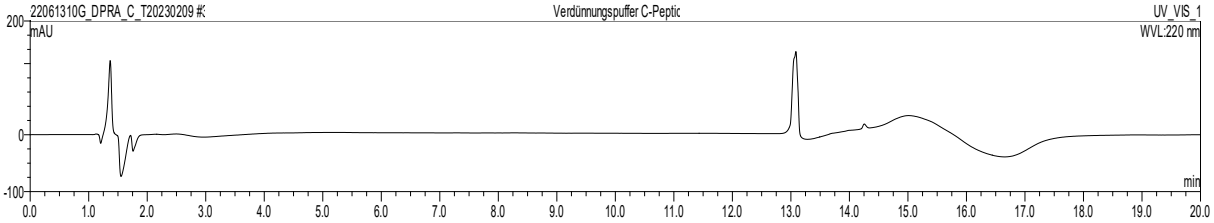


Figure 16.1–a Blank Dilution Buffer

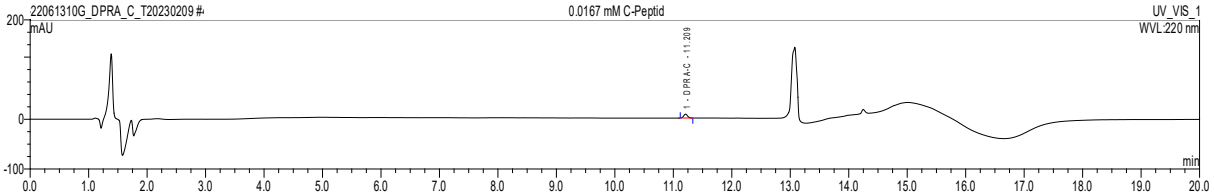


Figure 16.1–b Calibration Standard 0.0167 mM Cys-Peptide

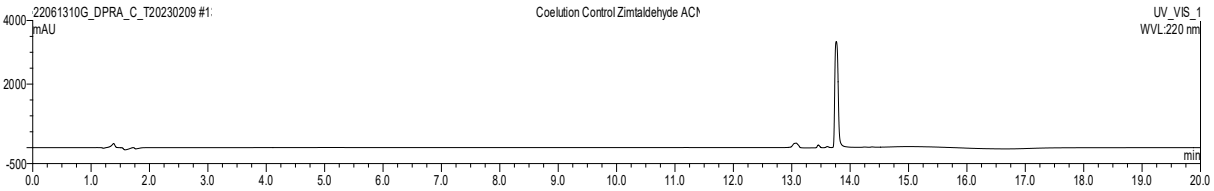


Figure 16.1–c Co-Elution control 100 mM Cinnamaldehyde in Acetonitrile

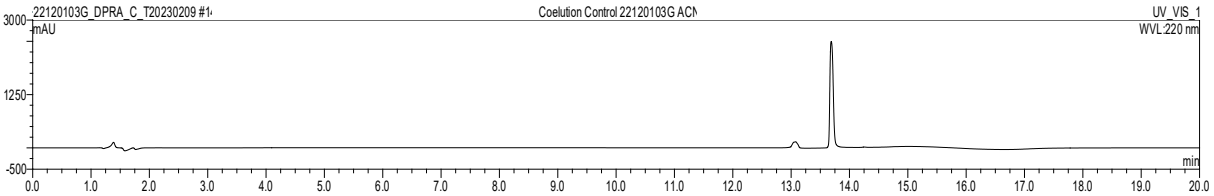


Figure 16.1–d Co-Elution Control 100 mM Test Item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in Acetonitrile

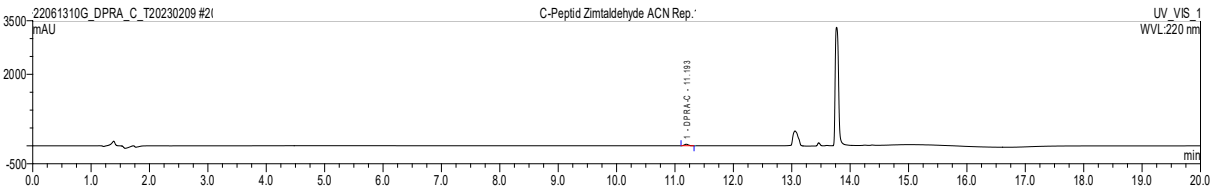


Figure 16.1–e Cys-Peptide after Incubation with 100 mM Positive Control Cinnamaldehyde in Acetonitrile

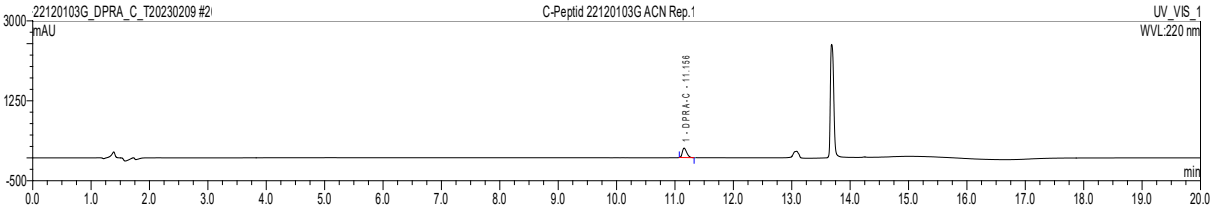


Figure 16.1–f Cys-Peptide after Incubation with 100 mM Test Item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in Acetonitrile

16.2 Lys-Peptide Assay

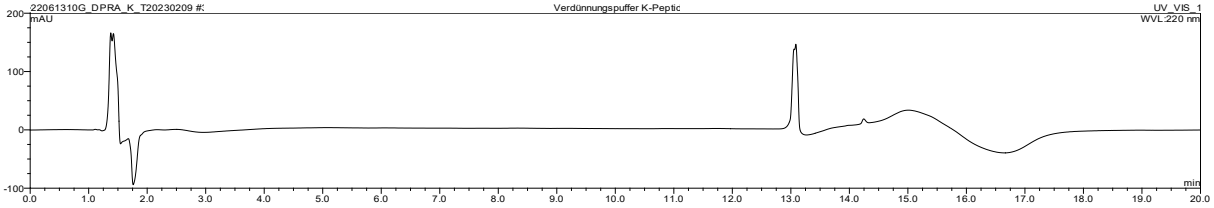


Figure 16.2–a Blank Dilution Buffer

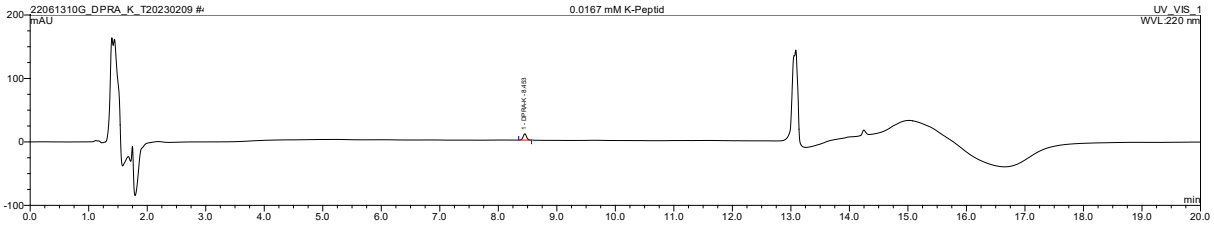


Figure 16.2–b Calibration Standard 0.0167 mM Lys-Peptide

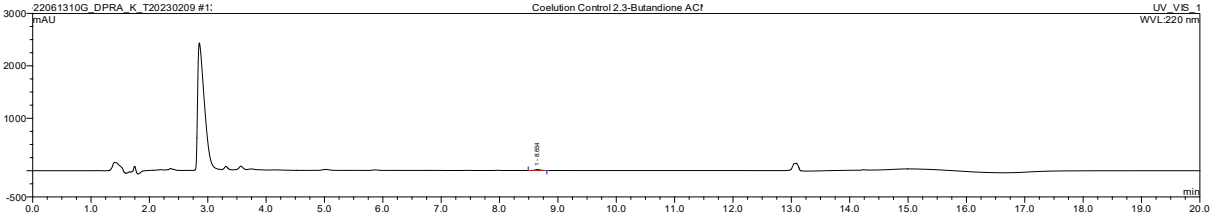


Figure 16.2–c Co-Elution Control 100 mM 2,3-Butanedione in Acetonitrile

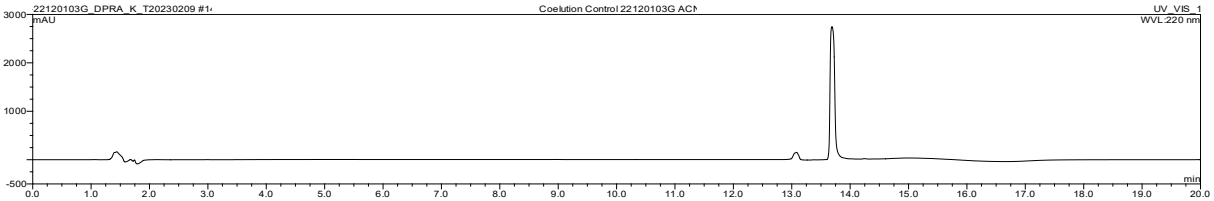


Figure 16.2–d Co-Elution Control 100 mM Test Item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in Acetonitrile

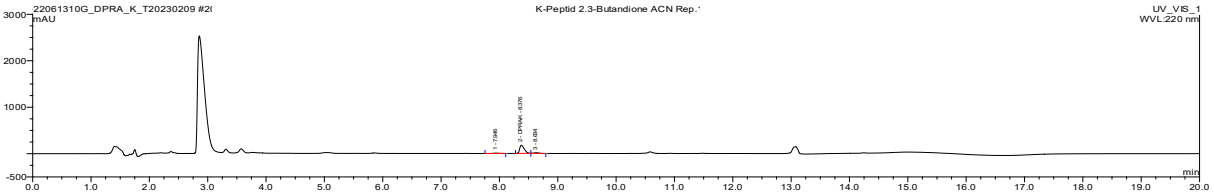


Figure 16.2–e Lys-Peptide after Incubation with 100 mM Positive Control 2,3-Butanedione in Acetonitrile

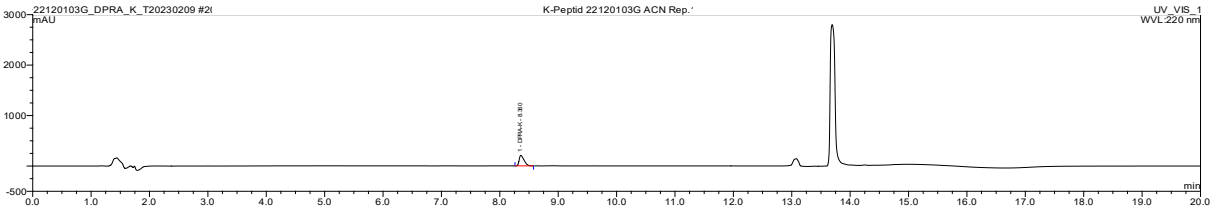


Figure 16.2–f Lys-Peptide after Incubation with 100 mM Test Item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) in Acetonitrile

17 ANNEX 3: COMPARISON WITH HISTORICAL DATA

In the following table, the means of the positive controls of all valid experiments up to 30. Nov. 2022 (Cys-peptide) resp. 01. Dec. 2022 (Lys-peptide) are stated and compared with the values which were found in this study.

Table 17 Historical Data

Parameter	**Depletion [%]**	
Peptide	**Cys-Peptide**	**Lys-Peptide**
Mean	80.16	25.75
Standard Deviation	0.91	1.71
Range Mean ± 2 SD	65.04 – 95.28	12.81 – 36.68
Study 22120103G875	**82.42**	**22.47**

SD = Standard Deviation

18 ANNEX 4: PROFICIENCY CHEMICALS

The demonstration of proficiency was performed under non-GLP conditions but within the GLP-environment at LAUS GmbH.

Cited from OECD 442C:

“Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected DPRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining cysteine and lysine depletion values that fall within the respective reference range for 8 out of the 10 proficiency substances for each peptide.”

In the following table the outcome of the proficiency chemicals testing is stated.

9 out of 10 proficiency chemicals were correctly predicted.

For the Cys-peptide the depletion values for 8 out of the 10 substances and for the Lys-peptide the depletion values for 9 out of the 10 substances lay within the respective reference range.

Table 18 Results of the Tests for Demonstration of Proficiency

Proficiency Substance in Acetonitrile	CAS No.	DPRA Prediction OECD 442C	Findings LAUS GmbH	% Peptide Depletion inside Reference Range	
				Cys-Peptide	Lys-Peptide
2,4-Dinitrochlorobenzene	97-00-7	Positive	-- *	No *	Yes
Oxazolone	15646-46-5	Positive	Positive	Yes	No
Formaldehyde	50-00-0	Positive	Positive	No	Yes
Benzylidenacetone (in acetone)	122-57-6	Positive	Positive	Yes	Yes
Farnesal	19317-11-4	Positive	Positive	Yes	Yes
2,3-Butanedione	431-03-8	Positive	Positive	Yes	Yes
1-Butanol	71-36-3	Negative	Negative	Yes	Yes
6-Methylcoumarin	92-48-8	Negative	Negative	Yes	Yes
Lactic Acid	50-21-5	Negative	Negative	Yes	Yes
4-Methoxyacetophenone	100-06-1	Negative	Negative	Yes	Yes

*** The Measurement of the cys-peptide depletion was not possible. Therefore, for this substance no prediction can be made.**

19 ANNEX 5: RESULTS OF STABILITY TEST IN ACETONITRILE (NON-GLP)

As some supplies of acetonitrile have had a negative impact on peptide stability (particularly cysteine), a stability test is performed when starting a new batch of acetonitrile.

A small amount of 0.501 mg/mL cysteine peptide solution in phosphate buffer was prepared and 750 µL of this peptide solution were mixed with 250 µL acetonitrile. After incubation for 24 h at 25 °C, a HPLC run was started by using the conditions described in chapter 6.2.

The sample was injected every 2 – 3 h for approximately 48 h. Afterwards the peak areas were compared for each injection and CV was calculated. The following table shows the results, the test is passed if the CV is < 15.

Batch of acetonitrile used in this study: 1398801 (Carl Roth)

Date of test performance: 26. Nov. 2022

Table 19 Measurement of Peak Area of Cys-Peptide

Sample No.	Peak Area [mAU*min]
1	16.4185
2	16.4962
3	16.5423
4	16.5536
5	16.5621
6	16.5904
7	16.6135
8	16.6621
9	16.6457
10	16.5979
11	16.6383
12	16.6351
13	16.5914
14	16.6174
15	16.6680
16	16.6364
17	16.6372
18	16.6838
19	16.6491
20	16.6353
21	16.6587
22	16.6330
23	16.6653
24	16.6850
Mean	**16.61**
SD	0.06
CV	**0.38**

SD = standard deviation
CV= coefficient of variation or rel. standard deviation (RSD)

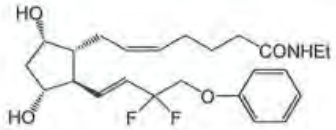
20 ANNEX 6: COPY OF CERTIFICATE OF ANALYSIS

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{33}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications.

Quality Control: [redacted] *Date:* 23NOV2022

Approved: [redacted] *Date:* 23 Nov 2022

Quality Control Laboratory
[redacted]

Final Report

Original 1 of 1

Determination of the potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) to activate the Nrf2 transcription factor in KeratinoSens™ cells with "The ARE-Nrf2 Luciferase KeratinoSens™ Test Method" according to OECD Guideline 442D "*In Vitro* Skin Sensitisation assays addressing the AOP Key Event on Keratinocyte activation"

Study No.: 22120103G881

Sponsor:

[redacted]

Monitor:
ToxMinds BVBA
Dr. Thomas Petry
Avenue de Broqueville, 116
1200 Brussels
Belgium

Test Facility:
LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler
Germany

Study Director:
Dr. Anne Frühmesser

1 GLP-COMPLIANCE STATEMENT

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following documents:

- OECD Principles of Good Laboratory Practice and Compliance Monitoring, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998 and subsequent advisory/consensus OECD GLP documents (where appropriate).
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemicals Act of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 from 28. Aug. 2013, published in Federal Law Gazette, Germany (BGBl) No. 55/2013 as of 06. Sep. 2013, and further revisions.

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

This report contains the following data which were performed under non-GLP conditions: pre- test for solubility of the test item.

I herewith accept responsibility for the data presented within this report.

There were no circumstances that may have affected the quality or integrity of the study.

Dr. Anne Frühmesser
Study Director

05 JUL 2023
Date

Information on Study Organisation:

Study Director	Dr. Anne Frühmesser
Deputy Study Director	Caroline Przewalla
Study Plan dated	09. Feb. 2023
Experimental Starting Date	14. Feb. 2023
Experimental Completion Date	03. Mar. 2023

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice.

All phases of the study (Study plan, performance of the study and Final report) were checked by the quality assurance. Dates of inspections are given below. Findings are reported to the Study Director and Test Facility Management.

The inspection of the performance of short-term studies (duration less than four weeks) may be carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the Final report was written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study plan (if any) were acknowledged and assessed by the Study Director and included in the Final report.

The reported results reflect the raw data of the study.

Phases of Study	Inspected on	Findings reported on	Audit report no.
Study plan	06. Feb. 2023	06. Feb. 2023	230206-11
Study plan Amendment No. 1	24. Feb. 2023	24. Feb. 2023	230224-04
Performance of study	01. Mar. 2023	01. Mar. 2023	230301-01
Final report	06. Jun. 2023	06. Jun. 2023	230606-09

Schedler

Dr. Anette Schedler
Quality Assurance

04 JUL 2023

Date

Table of Contents

3 SUMMARY

Title of Study: Determination of the potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) to activate the Nrf2 transcription factor in KeratinoSens™ cells with "The ARE-Nrf2 Luciferase KeratinoSens™ Test Method" according to OECD Guideline 442D "*In Vitro* Skin Sensitisation assays addressing the AOP Key Event on Keratinocyte activation"

Findings and Results:

This *in vitro* study evaluates the skin sensitization potential of the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil), according to OECD Guideline 442D, in compliance with GLP. The KeratinoSens™ test addresses the second molecular key event (KE2) of the adverse outcome pathway (AOP) for skin sensitization assessing the induction of the Nrf2 transcription factor by the test substance in keratinocytes.

The assay included one experiment, consisting of two independent repetitions (repetition I and II) with a treatment period of 48 h.

In the experiment (repetition I and II), the highest nominal applied concentration was 2000 µM. In addition, a geometric series of eleven dilutions (factor 2) was tested.

Precipitation of the test item was not visible in any of the repetitions.

DMSO (final concentration: 1 %) was used as solvent control and cinnamic aldehyde (5 concentrations ranging from 4 to 64 µM) as positive control.

The evaluated experimental points and the results are summarised in chapter 8.

A statistically significant increase in luciferase induction ≥ 1.5 fold in was observed in both repetitions at one non-cytotoxic concentration (250 µM). However, since no clear dose-response was observed, the result of both repetitions has to be considered as "inconclusive" according to the criteria of OECD 442D (see chapter 9.2).

As an additional information, an evaluation of the results of the test item in accordance to OECD 497 (see chapter 9.3) was also performed. Based on this guideline, the result of repetition I has to be declared as "inconclusive" due to the missing dose-response. For repetition II the result was considered as "Borderline". Since the results of the two repetitions do not match, a final assessment would lead to an inconclusive or borderline conclusion irrespective of the outcome of repetition III according to OECD 497. Therefore, in agreement with the sponsor no further repetition was performed.

Conclusion:

Under the experimental conditions of this study and due to the lack of clear dose response, the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil), was "inconclusive" in the KeratinoSens™ Test according to OECD 442D. No clear assessment of the potential to activate the Nrf2 transcription factor can be made.

4 PURPOSE AND PRINCIPLE OF THE STUDY

The adverse outcome pathway (AOP) for skin sensitisation is initiated by a first molecular key event 1 (KE1) corresponding to covalent interaction with skin proteins, which is followed sequentially by: (KE2) keratinocyte activation, (KE3) dendritic cell activation, and (KE4) proliferation of antigen-specific T cells. Currently, none of the assays addressing the different KEs is accepted as stand-alone test method as it may not be sufficient to conclude on the presence or absence of skin sensitisation potential of chemicals. However, it supports the discrimination between skin sensitisers and non-sensitisers in combination with other complementary data.

The KeratinoSens™ test addresses the second molecular key event of the AOP for skin sensitization assessing the induction of cyto-protective signaling pathways like the Keap1/Nrf2/ARE pathway in keratinocytes in responds to electrophiles and oxidative stress.

This study was performed to assess the potential of the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil), to activate the Nrf2 transcription factor by using the genetically modified keratinocyte cell-line “KeratinoSens™”.

It employs the use of a luciferase reporter gene placed under the control of the antioxidant response element (ARE) and hence monitors Nrf2 transcription factor activity. The measured endpoint is the up-regulation of the luciferase activity after 48 h of incubation with the test substance at different concentrations. This up-regulation is an indicator for the activation of the Keap1/Nrf2/ARE signaling pathway. In order to conclude on the Nrf2 transcription factor activity of the test substance, at least two independent and valid repetitions were performed.

The assay is used for supporting the discrimination between skin sensitizers (i.e. UN GHS Category 1) and non-sensitizers in accordance with the UN GHS. A categorization in the sub-categories 1 A and 1 B is not possible.

5 LITERATURE

The study is conducted in accordance with the following guidelines:

- OECD Test Guideline No. 442D: "*In Vitro* Skin Sensitization ARE-Nrf2 Luciferase Test Method" (adopted on 30 Jun 2022)
- OECD, Guideline No. 497: Defined Approaches on Skin Sensitisation. OECD Guidelines for the Testing of Chemicals, Section 4, adopted on 14. Jun. 2021)
- EU-Method B.60 of the Commission Regulation (EU) No. 2017/735 adapted 14. Feb. 2017: "In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method"

Corresponding SOP of LAUS GmbH:

- SOP 118 00 881, "Durchführung des KeratinoSens™ Tests"- edition 1 valid from 14. Nov. 2022"

Additional information was taken from:

- DB-ALM (INVITTOX) (2022) Protocol 155: KeratinoSens™., 17 pp. Available: [http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LA-TEST/online/DBALM_docs/155_P_%20KeratinoSens.pdf].
- Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. 2013 May 1 [Updated 2016 Jul 1]. In: Markossian S, Grossman A, Brimacombe K, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK144065/
- Präbst K, Engelhardt H, Ringgeler S, Hübner H. Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. Methods Mol Biol. 2017;1601:1-17.
-

6 MATERIALS AND METHODS

6.1 Test Item

Designation in Test Facility:	22120103G
Date of Receipt:	01. Dec. 2022
Condition at Receipt:	cooled, in proper conditions

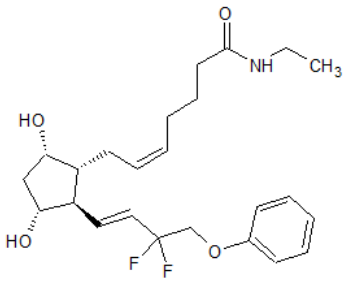
6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Batch no.	TAF-10-1122-01
CAS no.	1185851-52-8
EC no.	867-521-0
Composition	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
Storage	fridge (2 - 8 °C); keep under inert gas
Expiry date	23. Nov. 2026
Stability	stable under storage conditions
Appearance	clear, colorless to light yellow liquid
Purity	99.78 %
Homogeneity	homogeneous
Production date	18. Nov. 2022
Molecular formula	$C_{24}H_{33}F_2NO_4$
Molecular weight	437.52 g/mol
Vapour pressure	unknown
Solubility in solvents	water: not stated; ethanol: >1g/L; acetone: not stated; acetonitrile: not stated; DMSO: >1g/L; methanol: >1g/L; DMF: 0.1-1g/L
Stability in solvents	water: not stated; ethanol: not stated; acetone: not stated; acetonitrile: not stated; DMSO: not stated; methanol: not stated; DMF: not stated

See also copy of Certificate of Analysis in Annex 8.

6.1.2 Structural Formula



O[C@@H]2C[C@H](O)[C@H](C/C=C\CCCC(=O)NCC)[C@H]2/C=C/C(F)(F)COc1ccccc1

6.1.3 Storage

The test item was stored in the test facility in the fridge (2 - 8 °C) under inert gas.

6.1.4 Solubility Test

The solubility of the test item was determined in a non-GLP pre-test according to the specifications of the OECD 442D: Since the test item has a defined molecular weight, the solubility was tested at 200 mM (corresponding to 87.5 mg/mL) in dimethyl sulfoxide (DMSO) and medium (RPMI 1640). The test item was insoluble in medium but soluble in DMSO at 200 mM after approximately 5 minutes vortexing. Therefore, DMSO was used as solvent in this study.

6.1.5 Preparation

The highest test item concentration in the KeratinoSens™ test is 2000 µM. Since the final concentration of the solvent during treatment is limited to 1 %, a stock solution containing 200 mM test item in DMSO was prepared. Subsequent dilution to 1 % finally yielded a maximum concentration of 2000 µM in the experiment.

For that, the stock solution was first used to prepare a geometric series of solutions (1:2) on a 100 x DMSO Master Plate. Afterwards all concentrations were further diluted (1:25) in medium no. 3 on a 4 x Master Plate. Another 1:4 dilution was achieved by adding 50 µL of each concentration of the 4 x Master Plate to the corresponding wells of the Assay Plates (Viability Assay Plate and Luciferase Assay Plates) containing the cells as well as 150 µL medium no. 3. In the end, the total dilution factor was 1:100. The stock solution as well as the dilutions were freshly prepared on the day of treatment.

6.2 Controls

6.2.1 Positive Control

Name	Cinnamic aldehyde
CAS no.	14371-10-9
Solvent	DMSO
Supplier:	Sigma-Aldrich
Purity:	> 99%
Lot no.:	STBJ1063
Expiry Date:	01. Jul. 2023
Final concentrations:	64 µM, 32 µM, 16 µM, 8 µM, 4 µM
Storage:	2 - 8 °C
Stability:	Stable under specified storage conditions

The solution was freshly prepared on the day of treatment.

6.2.2 Solvent Control

Name	DMSO
CAS no.	67-68-5
Supplier:	Carl Roth
Purity:	99.5 %
Lot no.:	411310628
Expiry Date:	10. Jun. 2024
Final concentration:	1 % (in medium no. 3)
Storage:	Room temperature
Stability:	Stable under specified storage conditions

6.3 Test System

6.3.1 Reasons for the Choice of the KeratinoSens™ Cell Line

The KeratinoSens™ transgenic cell line are immortalised adherent human keratinocytes (HaCaT) which were stably transfected with a selectable plasmid (insertion of the luciferase reporter gene under the control of the ARE-element) by Givaudan Schweiz AG (Switzerland). The cell line was specially designed for this test system.

6.3.2 Cell Cultures

The KeratinoSens™ cell line was obtained from the acCELLerate GmbH (Hamburg, Germany). For mycoplasma contamination screened stocks of KeratinoSens™ cells are stored in liquid nitrogen in the cell bank of LAUS GmbH to allow a continuous stock of cells (mycoplasma contamination free), which guarantees similar parameters of the experiment and reproducible characteristics of the cells.

After thawing the cells were cultivated in DMEM containing FBS and Geneticin G418 (to allow maintaining the gene) in cell culture flasks at 37 ± 1 °C in a humidified atmosphere with 5.0 ± 0.5 % CO_2. The day before seeding medium was removed and the cells were cultivated in medium without Geneticin G418 to avoid any kind of stress to the cells.

For the repetitions, cells of passage 13 and 17 were used.

6.4 Test vessels

All vessels used were made of glass or sterilizable plastic. In case of non-sterilization by the manufacturer, they were sterilized before usage in a heating chamber or autoclave. The test was performed in 96-well plates. For the transfer of the culture medium, pipettes were used. Glass measuring flasks and cylinders with conformity sign and standard laboratory material were also used.

6.5 Chemicals and Media

The purity of the chemicals which were used was either “analytical grade” or “for microbiological purposes”.

Note: The given volumes are exemplary for the composition of the media/solutions. The real volumes/weights are stated in the raw data.

6.5.1 Medium

Culture base medium DMEM, Supplier PAN-Biotech, 94501 Aidenbach, Germany serving as base for medium no. 1, 2 and 3:
Batch number 2487282, 2561391, 2505790, 2487282

6.5.2 Medium no. 1 (medium for cell culture one day before seeding)

DMEM	500 mL
FBS (final concentration 10 %)	50 mL

Batch number T20230206 with expiry date 06. Mar. 2023
Batch number T20230227 with expiry date 27. Mar. 2023
Prepared at LAUS GmbH

6.5.3 Medium no. 2 (medium for cell culture)

DMEM	500 mL
FBS (final concentration 9.1 %)	50 mL
G418 (final concentration 500 µg/mL)	5.5 mL

Batch number T20230102 with expiry date 30. Jan. 2023
Batch number T20230118 with expiry date 15. Feb. 2023
Batch number T20230207 with expiry date 07. Mar. 2023
Prepared at LAUS GmbH

6.5.4 Medium no. 3 (medium for treatment)

DMEM	500 mL
FBS (final concentration 1 %)	5 mL

Batch number T20230207, expiry date 07. Mar. 2023
Batch number T20230227, expiry date 27. Mar. 2023
Prepared at LAUS GmbH

6.5.5 FBS (Fetal Bovine Serum) Superior

Ready to use, Supplier Merck KGaA, 64293 Darmstadt, Germany

Batch number: 0001636679, 0001647079

6.5.6 PBS

Phosphate buffered saline, ready to use, Supplier PAN-Biotech, 94501 Aidenbach, Germany
Batch number 4840321

6.5.7 Trypsin/EDTA

Ready to use, Supplier Biochrom AG, 12247 Berlin, Germany

Batch number 2457723

6.5.8 CASYton

Isotonic solution used for the dilution of cell suspensions for cell counting, ready to use, Supplier OLS OMNI Life Science, 28359 Bremen, Germany

6.5.9 EDTA Solution 0.5 M

Ready to use, VWR, 64295 Darmstadt, Germany
Batch number 22G1256055

6.5.10 PBS + 0,5M EDTA

PBS	997 mL
EDTA Solution (0.5M) Batch number 22G1256055	3 mL

Batch number T20230103, expiry date 03. Jul. 2023, prepared at LAUS GmbH

6.5.11 MTT (3-(4,5-Dimethyl thiazole 2-yl)-2,5-diphenyltetrazolium-bromide) Solution (5 mg/mL)

MTT reagent (Supplier: Roth, Karlsruhe, Germany)	100 mg
PBS	20 mL

6.5.12 MTT-Working Solution

MTT Stock Solution (5 mg/mL)	2.7 mL
Medium No. 3	20 mL

Batch Number: T20230303, T20230217; immediate use, prepared at LAUS GmbH

6.5.13 Steady-Glo® Reagent

Steady-Glo® Substrate (lyophilized)	
Batch: 0000520472	full volume of 1 vial (≙ 1 mL)
Steady-Glo® Buffer	
Batch: 0000522648	full volume of 1 vial (≙ 10 mL)

Immediate use
Supplier: Promega, Mannheim, Germany

6.6 Demonstration of proficiency

Prior to routine use, the validity of the KeratinoSens™ test at LAUS GmbH was demonstrated in a proficiency study. 10 proficiency chemicals indicated by the OECD 442 D (version: 30. June 2022) were tested. According to OECD 442D the prediction of at least 8 out of 10 proficiency chemicals has to be correct.
The reference range ($EC_{1.5}$) was correctly obtained for 10 out of 10 substances. The reference range (IC_{50}) was correctly obtained for 9 out of 10 substances whereby at one proficiency chemical the IC_{50} value was only slightly below the threshold in only one of the two individual runs.
In conclusion, the proficiency of the KeratinoSens™ test was demonstrated.
All technicians performing the KeratinoSens™ test at LAUS GmbH have successfully completed the first training experiment as prescribed by OECD 442D.
For the positive control cinnamic aldehyde, historical data are available (chapter 17) which demonstrates the reliability and the validity of this substance.

6.7 Instruments and Devices

The instruments and devices that were used in the test are listed in the following table.

Table 6-a Instruments and devices

Device	Device name	Manufacturer
Clean bench, category II	Mars 1800	Scanlaf
Cell counter	CASY Cell Counter & Analyzer	OMNI Life Science
Liquid dispenser	Ceramus 2 – 10 mL	Hirschmann
Inverse microscope	Axio Vert.A1	Zeiss
Heating chamber	Inkubator CB210 (E3)	Binder
Fridge	LKexv 3910-24B - 001	Liebherr
Pipette 20 – 200 µL, 100 - 1000 µL, 1 - 10 mL	Pipet-lite XLS	Mettler Toledo
Pipette 20 – 200 µL, 100 - 1000 µL,	Pipet-lite XLS+	Mettler Toledo
Multi-channel pipette 2 - 20 µL, 20 - 200 µL	Pipet-lite XLS	Mettler Toledo
Multi-channel pipette 20 - 200 µL, 100 - 1200 µL	E4 XLS	Mettler Toledo
Pipetting device	Pipetboy, Pipetgirl	Integra
Analytical scale	XS205 Dual Range	Mettler Toledo
Orbital shaker	Schüttelapparat SSM5	IKA Labortechnik
pH meter	3310	wtw
Photometer	Anthos Reader 2010-Flexi	Anthos Mikrosysteme GmbH
Luminometer	GloMax® Discover System GM 3000	Promega

Usage and, if applicable, calibration of all instruments followed the corresponding SOP in the current edition.

7 PERFORMANCE OF THE STUDY

7.1 Dose Selection for the Experiment (repetition I and II)

In accordance with the OECD guideline 442D, the maximum final test item concentration of 2000 µM was used..

The following 12 nominal concentrations were tested in repetition I and II:

0.98 µM, 1.95 µM, 3.91 µM, 7.81 µM, 15.63 µM, 31.25 µM, 62.5 µM, 125 µM, 250 µM, 500 µM, 1000 µM, 2000 µM

The real test item concentrations (under consideration of the real weighing) are given in chapter 18.

A test item concentration inducing a viability below 70 % is considered as cytotoxic and is not allowed to be evaluated for luciferase induction.

7.2 Experimental Parameters of Repetition I and II

7.2.1 Experimental Performance

Repetition I and II were performed in the same way. The exposure dates were 15. Feb. 2023 and 01. Mar. 2023.

At the time of seeding the cells were 80 % (repetition I) and 90 % (repetition II) confluent. The cells were washed twice with PBS (without Ca^{2+}/Mg^{2+}) containing 0.05% EDTA. Afterwards the cells were trypsinized by adding Trypsin/EDTA until the cells detached. To stop this reaction, medium no. 1 was added. After quantification the cell suspension was adjusted to 80 000 (± 10 %) cells per mL. 125 µL of the cell suspension (≙ approximately 10 000 cells) were seeded in all wells except well H12 (blank) of one clear flat bottom 96 well plate as well as three white flat bottom 96 well plates. Afterwards, the cells were left in the clean bench for 30 min in order that the cells could attach evenly distributed. All four plates were incubated at 37 ± 1 °C and 5.0 ± 0.5 % CO_2 in a humidified atmosphere for 24 h.

<u>The treatment procedure was performed on all 96 well plates identically:</u>
After the incubation time the medium was removed from the cells in all four plates and 150 µL medium no. 3 was added to each well. Afterwards 50 µL of each single test item concentration and the controls of the 4 x master plate (see chapter 6.1.5) were added to the corresponding wells of the four test plates containing the cells and the medium. Six wells were used for the solvent control, five wells for positive control and one well for blank. The plates were sealed with breathable tape to avoid evaporation of volatile compounds and to avoid cross contamination between wells. Then the plates were incubated for 48 h at 37 ± 1 °C in a humidified atmosphere containing 5.0 ± 0.5 % CO_2.

For the evaluation of the viability, the clear flat bottom 96 well plate was used:

All solutions were removed from the wells and 200 µL MTT working solution (see chapter 6.5.12) were added to each well. The plate was incubated for 4 h (repetition I) and 2 h (repetition II) at 37 ± 1 °C and 5.0 ± 0.5 % CO_2 in a humidified atmosphere. Afterwards the solution was removed and 50 µL isopropanol was added to each well. The plate was agitated for 30 min before it was measured at 570 nm with photometer.

The cell viability was measured by the reduction of the tetrazolium dye MTT (3-(4,5-Dimethyl thiazole 2-yl)-2,5-diphenyltetrazolium-bromide) (yellow color) to its insoluble formazan (purple color in combination with culture medium) in living cells and therefore indicates the amount of living cells. After the measurement of the color change, the values were transferred in a validated spreadsheet for the calculation of the viability (see chapter 7.2.2).

For the evaluation of the Luciferase induction, the three white 96 well plates were used:

For the evaluation of the Luciferase expression all solutions were removed from the wells and the cells were washed with 200 µL PBS. Afterwards 100 µL Medium no. 3 and 100 µL Steady-Glo® reagent were added to each well and the plates were shaken again slowly for 5 min at room temperature. Afterwards the luminescence was measured using a luminometer.

For calculation of the luciferase induction (see chapter 7.2.2), a validated Microsoft Excel® spreadsheet was used.

7.2.2 Data Evaluation

For calculation of the result a validated Microsoft Excel® spreadsheet was used.

Calculation of Luciferase fold induction I_{max}

The fold luciferase activity induction was calculated by equation 1 and the overall maximal fold induction (I_{max}) was calculated as the average of the individual repetitions.

Equation 1:

Fold induction = $[(L_{sample} - L_{blank}) / (L_{solvent} - L_{blank})]$

L_{sample} = luminescence reading in the test item well
L_{blank} = luminescence reading in blank well containing no cells and no treatment
$L_{solvent}$ = average luminescence reading in the wells containing cells and solvent control

Afterwards the arithmetic mean of the single replicates was calculated.

For each concentration, statistical significance ($p < 0.05$) was determined by using a two-tailed Student's t-test by comparing the luminescence values of the three replicate samples with the luminescence values in the solvent control wells.

Calculation of $EC_{1.5}$ Value

$EC_{1.5}$ was calculated by linear interpolation according to equation 2, and the overall $EC_{1.5}$ is calculated as the geometric mean of the individual repetitions.

Equation 2:

$EC_{1,5} = (C_b – C_a) * [(1.5 – I_a) / (I_b - I_a)] + C_a$

where
C_a = lowest concentration in µM with > 1.5 fold induction
C_b = highest concentration in µM with < 1.5 fold induction
I_a = fold induction at the lowest concentration with > 1.5 fold induction (mean of three replicate wells)
I_b = fold induction at the highest concentration with < 1.5 fold induction (mean of three replicate wells)

Calculation of Relative Viability

The calculation of the relative Viability [%] was performed as follows:

Equation 3:

relative viabilität [%] = $[(V_{sample} - V_{blank}) / (V_{solvent} - V_{blank})] * 100$

where
V_{sample} = MTT-absorbance reading in the test chemical well
V_{blank} = MTT-absorbance reading in the blank well
$V_{solvent}$ = average MTT-absorbance reading in the wells containing the solvent control

Calculation of IC_{50} and IC_{30}

IC_{50} and IC_{30} were calculated by linear interpolation according to Equation 4, and the overall IC50 and IC30 are calculated as the geometric mean of the individual repetitions.

Equation 4:

$IC_x = (C_b - C_a) * [((100 – x) – V_a) / V_b – V_a] + C_a$

where
X = % reduction at the concentration to be calculated (50 and 30 for IC_{50} and IC_{30})
C_a = lowest concentration in µM with > x% reduction in viability
C_b = highest concentration in µM with < x% reduction in viability
V_a = % viability at the lowest concentration with > x% reduction in viability
V_b = % viability at the highest concentration with < x% reduction in viability

8 RESULTS

8.1 Results of Repetition I

Table 8-a Results of Controls in Repetition I

	SC	SC	SC	SC	SC	SC	PC 4 µM	PC 8 µM	PC 16 µM	PC 32 µM	PC 64 µM
Viability [%]	**86**	**100**	**103**	**101**	**105**	**105**	**101**	**111**	**107**	**106**	**94**
Induction [Fold]	**0.9**	**1.0**	**0.9**	**1.0**	**1.1**	**1.0**	**1.1**	**1.2**	**1.4**	**1.8**	**2.7**
Standard Deviation	0.01	0.04	0.03	0.01	0.05	0.05	0.04	0.10	0.18	0.12	0.13
p-value	-	-	-	-	-	-	0.18	0.07	0.05	0.00	0.00

Abbreviations: SC = Solvent Control, PC = Positive Control Table 8-b Results of Test Item Concentrations in Repetition I

Conc [µM]	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	*500*	*1000*	*2000*
Precipitates	-	-	-	-	-	-	-	-	-	-	-	-
Viability [%]	**95**	**104**	**96**	**100**	**103**	**106**	**106**	**106**	**81**	***2***	***2***	***1***
Induction [Fold]	**1.0**	**1.1**	**1.1**	**1.1**	**1.1**	**1.2**	**1.1**	**1.0**	**2.1**	***0.0***	***0.0***	***0.0***
Standard Deviation	0.02	0.06	0.08	0.05	0.03	0.15	0.11	0.16	0.36	*0.00*	*0.00*	*0.00*
p-value	0.67	0.48	0.20	0.09	0.08	0.19	0.48	0.80	0.01	*0.00*	*0.00*	*0.00*

Note: The top three concentrations (written in italics) were not used for the final evaluation due to <70% viability.

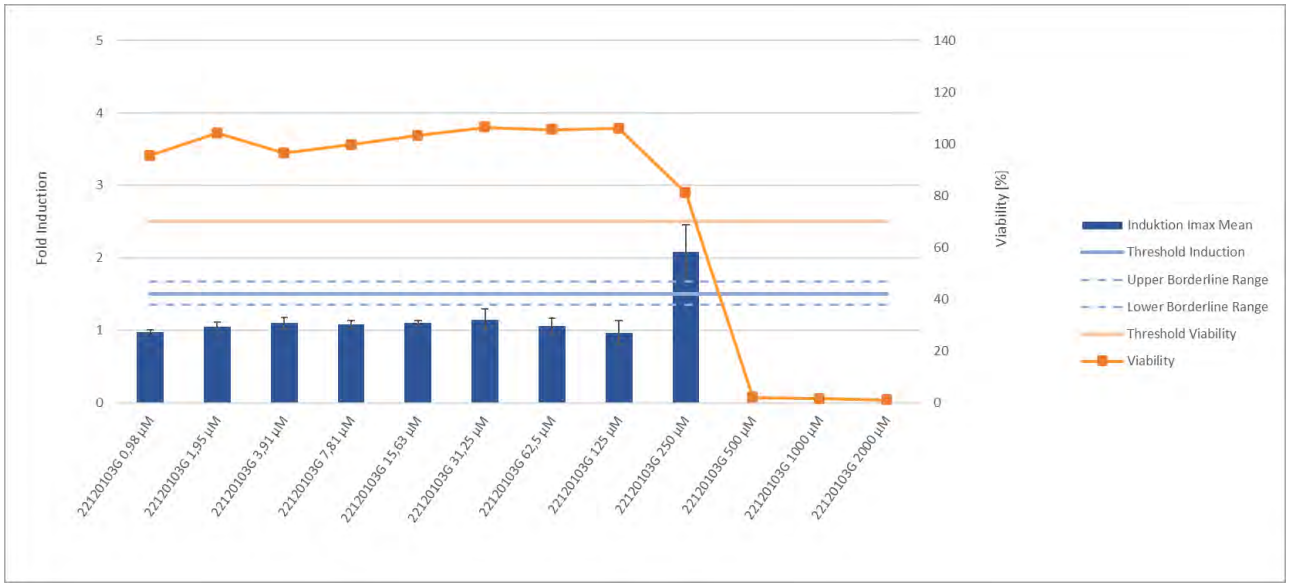


Figure 8-a Graphical Presentation of the Results of the Test Item Concentrations in Repetition I.

8.2 Results of Repetition II

Table 8-c Results of Controls in Repetition II

	SC	SC	SC	SC	SC	SC	PC 4 µM	PC 8 µM	PC 16 µM	PC 32 µM	PC 64 µM
Viability [%]	**77**	**94**	**109**	**106**	**110**	**104**	**104**	**109**	**124**	**111**	**110**
Induction [Fold]	**1.0**	**1.0**	**0.9**	**1.0**	**0.9**	**1.1**	**1.1**	**1.1**	**1.3**	**1.6**	**2.2**
Standard Deviation	0.06	0.09	0.03	0.08	0.03	0.20	0.10	0.14	0.20	0.28	0.32
p-value	-	-	-	-	-	-	0.45	0.50	0.18	0.01	0.00

Abbreviations: SC = Solvent Control, PC = Positive Control

Table 8-d Results of Test Item Concentrations in Repetition II

Conc [µM]	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500	1000	2000
Precipi-tates	-	-	-	-	-	-	-	-	-	-	-	-
Viability [%]	**89**	**91**	**92**	**94**	**103**	**109**	**115**	**120**	**108**	**-4**	**-4**	**-3**
Induction [Fold]	**0.9**	**1.0**	**0.9**	**0.9**	**0.9**	**0.9**	**1.0**	**0.8**	**1.5**	**0.0**	**0.0**	**0.0**
Standard Devia-tion	0.08	0.07	0.08	0.08	0.12	0.11	0.20	0.02	0.18	0.00	0.00	0.00
p-value	0.49	0.54	0.52	0.40	0.36	0.19	0.81	0.14	0.00	0.00	0.00	0.00

Note: The top three concentrations (written in italics) were not used for the final evaluation due to <70% viability.

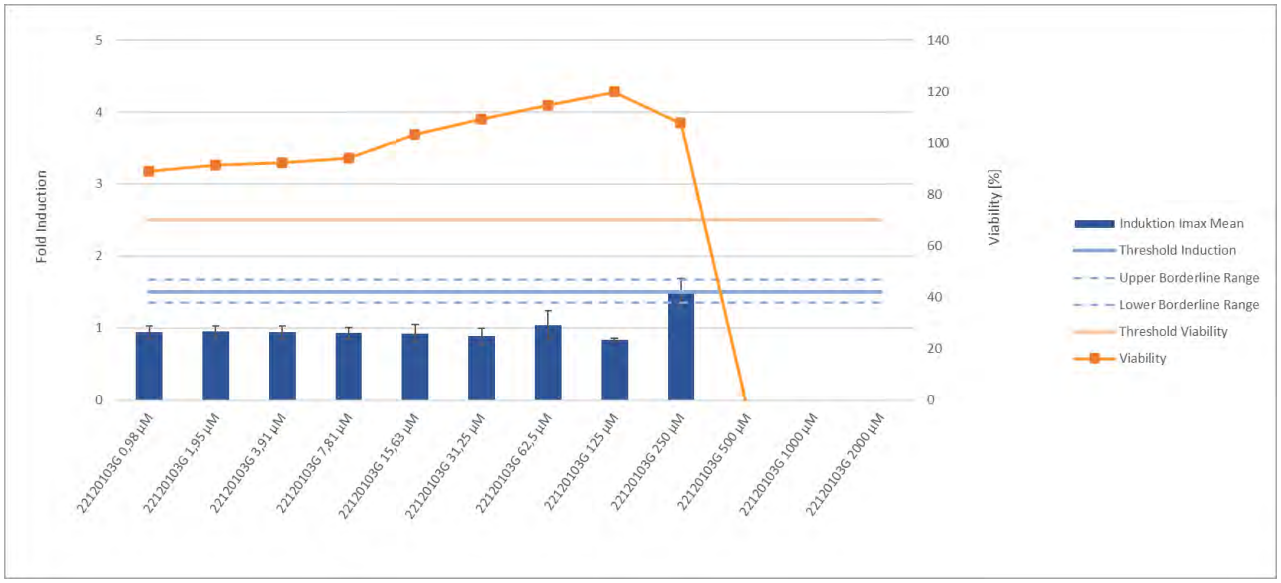


Figure 8-b Graphical Presentation of the Results of the Test Item Concentrations in Repetition II.

8.3 Summary of Results of the Repetitions

Table 8-e Summary of Results of the Repetitions

	Imax	EC1.5	IC50	IC30
	[Fold]	[µM]	[µM]	[µM]
Value of Repetition I	2.1	184.2	348.4	285.2
Value of Repetition II	1.5	247.1	379.0	334.2
Mean	1.8	213.3	363.4	308.7

9 EVALUATION

9.1 Acceptability

In the following table the criteria for acceptability as well as the corresponding results in repetition I and II are given.

Table 9-a Acceptability of Repetition I and II

Criteria	Found in repetition I	Found in repetition II
The luciferase activity induction obtained with the positive control should be statistically significantly increased (e.g. using a t-test) in at least one of the tested concentrations (4 to 64 µM) with an induction above (>) 1.5fold.	Luciferase induction is statistically significantly increased at the concentrations 32 µM and 64 µM.	Luciferase induction is statistically significantly increased at the concentrations 32 µM and 64 µM.
The EC1.5 value of the positive control should be within two standard deviations (95.5 % control limit) of the historical mean of the testing facility and between 7 µM and 30 µM based on the validation dataset.	Within the 95.5 % control range.	Within the 95.5 % control range.
	$EC_{1.5}$: 20.1 µM	$EC_{1.5}$: 26.5 µM
The average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.	2.7	2.2
The average coefficient of variation of the luminescence reading for the solvent/vehicle control (i.e. DMSO) should be below 20% in each repetition.	10.2 %*	15.9 %

*** Note: Potential outlier removal of solvent control in accordance to OECD 442D (Stated in the DB-ALM Protocol 155 (2022): The data of one well of the solvent control at replicate 1 was removed as outlier because the value was > 25% higher than the average of the other 5 values (see table 14-a on page 31). Since the outlier value is from the well directly next to the well of the positive control, and is greatly elevated compared to all other solvent control values, it can be assumed that the positive control may have had an effect on the solvent control at this well.**

All validity criteria were met. Therefore, the study is valid.

9.2 Prediction Model according to OECD 442D without Borderline Ranges

Each valid experiment (i.e. meeting all acceptance criteria, according to the procedure described above) is interpreted as follows:

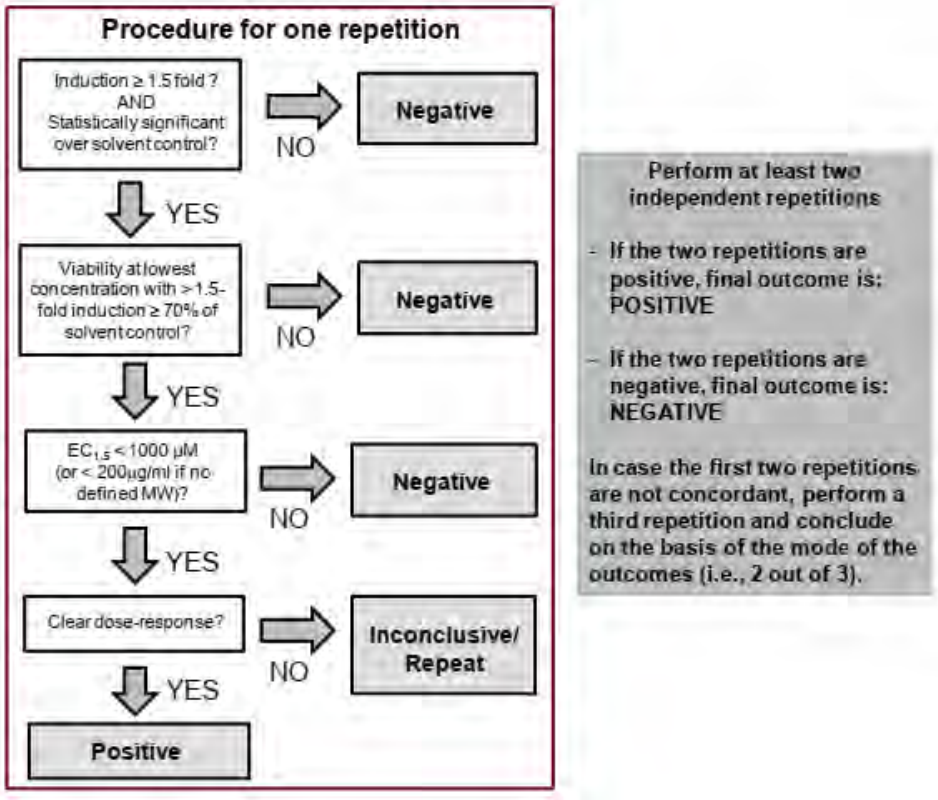


Figure 9-a: Prediction model of the KeratinoSens™ assay without taking Borderline outcomes into account as described in Figure 1 of OECD TG 442D. Source: OECD Test Guideline No. 442D: "*In Vitro* Skin Sensitization ARE-Nrf2 Luciferase Test Method" (adopted on 30 Jun 2022).

If, in a repetition, the first three conditions are met but no clear dose-dependent increase in luciferase induction can be observed, the result of this repetition should be considered "inconclusive" and a further repetition should be performed.
At least two consecutive concentrations should have a viability higher than (>) 70%. Otherwise, a further repetition should be performed and the concentration range should be adjusted.
If a test item can only be tested in concentrations < 1000 µM or < 200 µg/mL due to low solubility, only a positive result can be detected. In this case, a negative result can only be detected if a cytotoxic effect (viability < 70 %) is present in the tested concentrations, otherwise it is classified as "inconclusive".
In cases when test item induces the luciferase activity very close to the cytotoxic levels, they can be positive in some repetitions at non-cytotoxic levels (i.e. $EC_{1.5}$ determining concentration below (<) the IC_{30}), and in other repetitions only at cytotoxic levels (i.e. EC1.5 determining concentration above (>) the IC_{30}). Such test chemicals shall be retested with more narrow dose-response analysis using a lower dilution factor (e.g. 1.33 or $\sqrt{2}$ (=1.41) fold dilution between wells), to determine if induction has occurred at cytotoxic levels or not.

9.3 Prediction Model taking Borderline Outcomes into Account as Implemented in OECD TG 497

Since 2021 defined areas where lower confidence in a test method exist were defined by the OECD TG 497. These areas were defined as borderline ranges (BRs) in which the significance of the test system is low.
For the KeratinoSens™ the borderline range was defined for a maximal induction in the range of 1.35-fold – 1.67-fold with > 70% cell viability. For the assessment of the individual runs and the final assessment a modified prediction model was applied by the DB-ALM protocol n° 155.
This prediction model introduces a third outcome (borderline) to be used within the 2 out of 3 defined approach. For the interpretation of the individual runs, the following prediction model (figure 9-b) which was indicated in the DB-ALM protocol n°155 (2022) was used.

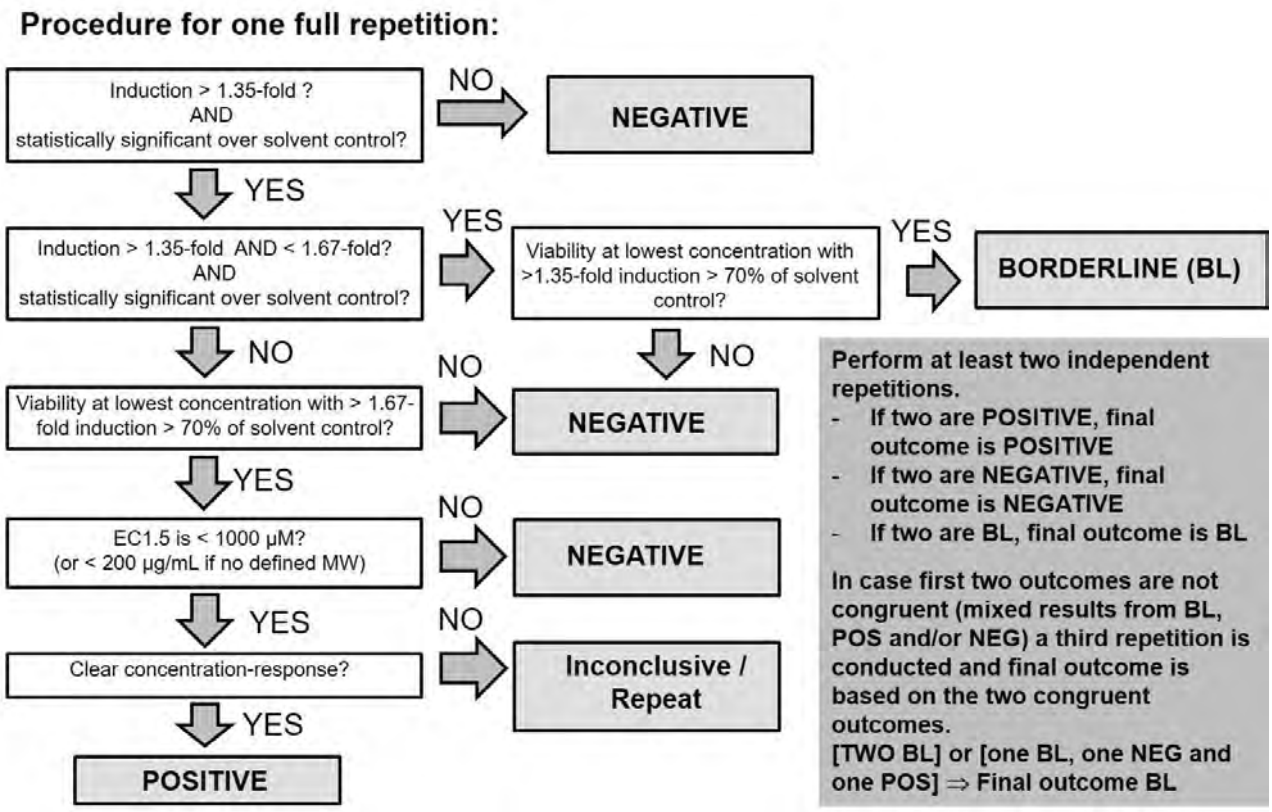


Figure 9-b: Prediction model of the KeratinoSens™ assay taking borderline outcomes into account as described in annex 1, figure 1.2 of OECD TG 497 and assessing multiple runs to conclude on borderline results within the 2 out of 3 defined approach. The original threshold for a positive classification is 1.5-fold induction, and the statistically derived borderline range around this threshold is 1.35 – 1.67-fold. Adapted from DB-ALM (INVITTOX) (2022) protocol 155: KeratinoSens™. POS = positive, NEG = negative, BL = borderline

10 DISCUSSION AND CONCLUSION

This *in vitro* study was performed to investigate the potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) to activate the Nrf2 transcription factor, by using the KeratinoSens™ cell line.

A detailed listing of all measured and calculated values of the assay is given in Annex 2 (values of repetition I) and Annex 3 (values of repetition II). In addition, the final results of both repetitions are summarized in table 8-a and 8-b and graphically illustrated in figure 8-a and 8-b.

The assay was performed in two independent repetitions (I and II). 12 concentrations of the test item were evaluated. The exposure time was 48 h. The following nominal concentrations of the test item were investigated in repetition I and II:

0.98 µM, 1.95 µM, 3.91 µM, 7.81 µM, 15.63 µM, 31.25 µM, 62.5 µM, 125 µM, 250 µM, 500 µM, 1000 µM, 2000 µM

Precipitation of the test item was not visible in any of the repetitions.

None of the real treatment concentrations (under consideration of the real weighing) in all repetitions deviated more than 10 % from the nominal concentration.

The coefficient of variation of the luminescence reading for the solvent control (i.e. DMSO) was below 20% in each repetition (after potential outlier removal of solvent control).

In addition, the positive control cinnamic aldehyde was tested in a series of 5 concentrations ranging from 4 to 64 µM and fulfils all acceptability criteria. Furthermore, the luciferase induction values remained well within the historical control range.

Therefore, all acceptability criteria were met and the study is valid.

In repetition I and II a cytotoxic effect was observed at the concentrations 2000 µM, 1000 µM and 500 µM. For that reason, the three highest test item concentrations were excluded from the evaluation of the luciferase induction in both repetitions. At the next lower test item concentration (250 µM) the viability rises sharply to a viability above 80 %.

Finally, the following test item concentrations showed a viability ≥ 70 % and could therefore be evaluated for luciferase induction in repetition I and II:

0.98 µM, 1.95 µM, 3.91 µM, 7.81 µM, 15.63 µM, 31.25 µM, 62.5 µM, 125 µM, 250 µM

In repetition I a statistically significant increase in luciferase induction > 1.5 fold was observed at the test item concentration 250 µM. All lower concentrations showed induction values in the range of the solvent control. Since no dose-dependence was detected, this repetition was considered as “inconclusive” and was repeated.

In repetition II again, a statistically significant increase in luciferase induction to exactly 1.5 fold was observed at the test item concentration 250 µM. As in repetition I the induction

values at the lower test item concentrations were all in the range of the solvent control. For that reason, again no clear dose-dependent effect was observed and the result has to be considered as inconclusive according to the criteria of OECD 442D.

Since the missing does-response in repetition I could be verified in repetition II, no further repetition is necessary and the final result of the study is “inconclusive” under the experimental conditions of this study.

The following values were calculated as the final results:

I_{max}: **1.8 fold** (average of the two values of replicate I and II)

$EC_{1.5}$: **213.3 µM** (geometric mean of the two values of replicate I and II)

IC_{50}: **363.4 µM** (geometric mean of the two values of replicate I and II)

IC_{30}: **308.7 µM** (geometric mean of the two values of replicate I and II)

As an additional information, an evaluation of the results of the test item in accordance to OECD 497 (see chapter 9.3) was also performed. Based on this guideline, the result of repetition I has to be declared as “inconclusive” due to the missing dose-response. For repetition II the result was considered as “Borderline”. Since the results of the two repetitions do not match, a final assessment would lead to an inconclusive or borderline conclusion irrespective of the outcome of repetition III according to OECD 497. Therefore, in agreement with the sponsor no further repetition was performed.

In conclusion, it can be stated that under the experimental conditions of this study, no clear assessment of the potential to activate the Nrf2 transcription factor can be made for the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil), due to the lack of a clear dose response. The result of the KeratinoSens^{TM} Test is therefore “inconclusive”.

11 DEVIATIONS

11.1 Deviations from the Study Plan

- The following deviation from the Study Plan was observed:
-
- To stop the trypsin reaction, medium no. 1 was used instead of medium no. 3. The deviation is uncritical as only the serum present in the medium is required to stop the reaction. Since the serum content of medium no. 1 is higher (9.1 %) than that of medium no. 3 (1 %), this reaction can take place even faster and better with medium no. 1 than with medium no. 3. This is a typing error in the study plan. According to OECD 442D, medium no. 3 is also used at this point.

The deviation was assessed and signed by the study director on 19. Jun. 2023.

11.2 Deviations from the Guideline

No deviations from the guideline were ascertained.

11.3 Deviations from SOP 118 00 881

The following deviation from the SOP 118 00 881 was observed:

Any kind of liquid (medium, buffer etc.) that has to be removed from the wells in the 96 well plates was removed by carefully turning the plates and not by aspiration. The deviation is uncritical since this alternatively performance offers various advantages and thus leads to a better study performance as it is ensured that the entire volume of liquid has always been removed and the cells are covered with liquid again more quickly due to the faster process. The deviation is only due to a missing information in the standard operation procedure and the SOP will be adjusted in this respect.

The deviation was assessed and signed by the study director on 31. Mar. 2023.

12 RECORDING AND ARCHIVING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP Document Archive of the test facility for 15 years. After that, the sponsor’s instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP Substance Archive for 15 years; then, the retain sample will be discarded.

Number of originals of the final report which will be sent to the sponsor: 0, pdf file only

13 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6, 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien / toxicological in vitro studies on mammalian cells and bacteria)

Datum der Inspektion / Date of Inspection
(Tag.Monat.Jahr / day.month.year)
28. und 29.04.2021

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist spätestens drei Jahre nach der letzten Inspektion zu beantragen. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for not later than three years after the last inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Unterschrift, Datum / Signature, Date Mainz, 21.06.21

Sabine Riewenherm - Präsidentin -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

14 ANNEX 2: REPETITION I - DETAILED DATA

Table 14-a RLU Values of the Single Replicates in Repetition I

	Test Item concentrations [µM]											
	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500	1000	2000
Rep 1	4838	5426	5532	5139	5388	5524	5048	4696	9114	17	13	8
Rep 2	4507	4421	4613	4954	4921	4628	4393	3707	8648	13	9	11
Rep 3	4466	5018	5452	5151	5246	6062	5432	5278	11530	17	10	11
	SC	SC	SC	SC	SC	SC	PC	PC	PC	PC	PC	Blank
	1 %	1 %	1 %	1 %	1 %	1 %	4 µM	8 µM	16 µM	32 µM	64 µM	-
Rep 1	4633	4962	4461	5134	5821	8669*	5767	6016	7279	9332	14360	10
Rep 2	4054	4221	4267	4672	4897	4708	4892	4947	5263	7615	11710	11
Rep 3	4263	4243	4294	4842	5471	4533	4992	6024	6967	8942	12430	6

Note: SC = Solvent control; PC = Positive Control

*** value is declared as an outlier and excluded from evaluation since it is > 25 % higher than the average of the other 5 wells which is 5002 see Annex 7.**

Table 14-b OD_{570} Values in Repetition I

Test Item concentrations [µM]											
0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500	1000	2000
0.771	0.830	0.777	0.800	0.824	0.846	0.840	0.843	0.673	0.132	0.129	0.126
SC	SC	SC	SC	SC	SC	PC	PC	PC	PC	PC	Blank
1 %	1 %	1 %	1 %	1 %	1 %	4 µM	8 µM	16 µM	32 µM	64 µM	-
0.708	0.805	0.824	0.806	0.835	0.834	0.810	0.876	0.848	0.844	0.764	0.118

Note: SC = Solvent control; PC = Positive Control

15 ANNEX 3: REPETITION II - DETAILED DATA

Table 15-a RLU Values of the Single Replicates in Repetition II

	Test Item concentrations [µM]											
	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500	1000	2000
Rep 1	3282	3200	3091	3081	3375	3026	3425	2691	5326	12	3	7
Rep 2	3163	3385	3498	3366	3071	3246	4188	2931	5292	10	8	5
Rep 3	3568	3531	3466	3423	3359	3101	3351	3307	5393	12	13	6
	SC	SC	SC	SC	SC	SC	PC	PC	PC	PC	PC	Blank
	1 %	1 %	1 %	1 %	1 %	1 %	4 µM	8 µM	16 µM	32 µM	64 µM	-
Rep 1	3025	3106	3042	3235	2952	3651	3788	3912	4241	6037	7603	5
Rep 2	3665	3952	3266	3398	3267	3117	3589	3376	3920	5076	7951	3
Rep 3	4236	4018	3681	3561	3595	5278	4047	4178	6204	5578	7310	12

Note: SC = Solvent control; PC = Positive Control

Table 15-b OD_{570} Values in Repetition II

Test Item concentrations [µM]											
0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500	1000	2000
0.421	0.428	0.431	0.436	0.464	0.482	0.498	0.514	0.477	0.141	0.141	0.145
SC	SC	SC	SC	SC	SC	PC	PC	PC	PC	PC	Blank
1 %	1 %	1 %	1 %	1 %	1 %	4 µM	8 µM	16 µM	32 µM	64 µM	-
0.386	0.437	0.482	0.471	0.484	0.465	0.465	0.482	0.526	0.487	0.485	0.153

Note: SC = Solvent control; PC = Positive Control

16 ANNEX 4: GLOSSARY

Table 16-a List of Abbreviations

Abbreviation	Full name
AOP	Adverse Outcome Pathway
ARE	Antioxidant response element
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle Medium
OD	Optical density
PC	Positive control
PBS	Dulbecco’s Phosphate Buffered Saline
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
RLU	Relative Light Unit
SC	Solvent control

17 ANNEX 5: HISTORICAL DATA

Table 17-a Historical Data of the Positive Control Cinnamic Aldehyde (status: 10. Feb. 2023)

Conc.	mean Induc-tion	SD	range	95.5% con-trol range	∑ of data	Study No.: 22120103G881	
[µM]						Repetition I	Repetition II
64	2.8	0.513	2.1-3.5	1.8-3.8	8	2.7	2.2
32	1.8	0.213	1.5-2.1	1.4-2.3	8	1.8	1.6
16	1.4	0.076	1.3-1.5	1.2-1.6	8	1.4	1.3
8	1.3	0.079	1.2-1.4	1.1-1.4	8	1.2	1.1
4	1.2	0.052	1.1-1.2	1.1-1.3	8	1.1	1.1
$EC_{1.5}$	20.3	4.479	15.0-28.9	11.4-29.3	8	20.1	26.5

Note: The data of this study are not included in the historical data.
Abbreviations: Conc. = Concentration; SD = Standard deviation

18 ANNEX 6: NOMINAL AND REAL TEST ITEM CONCENTRATIONS

Table 18-a Nominal and Real Test Item Concentrations

Repetition I		Repetition II	
nominal concentration [µM]	**real concentration [µM]**	**nominal concentration [µM]**	**real concentration [µM]**
0.98	0.97	0.98	0.98
1.95	1.95	1.95	1.96
3.91	3.90	3.91	3.91
7.81	7.79	7.81	7.82
15.63	15.59	15.63	15.65
31.25	31.17	31.25	31.30
62.5	62.3	62.5	62.6
125	125	125	125
250	249	250	250
500	499	500	501
1000	997	1000	1002
2000	1995	2000	2003

Note: Partially rounded values.

19 ANNEX 7: OUTLIER REMOVAL

In accordance to DB-ALM Protocol 155 in repetition 1, the data for one well of plate 1 in repetition I was removed as outlier since this value was > 25 % higher than the average of the other 5 wells. In the following table the outlier removal is shown.

Table 19-a Calculation of Outlier Removal of Solvent Control in Repetition I

Row	1	2	3	4	5	6
Measured value of solvent control on plate 1*	4633	4962	4461	5134	5821	8669
Calculated average excluding the value from row	1	2	3	4	5	6
	5809.4	5743.6	5843.8	5709.2	5571.8	5002.2
Standard deviation of measured value in comparison to average value excluding the value from row	1	2	3	4	5	6
	80	86	76	90	104	**173**

*see table 14-a in Annex 2.

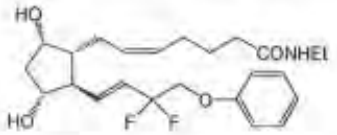
20 ANNEX 8: COPY OF CERTIFICATE OF ANALYSIS

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{33}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications

Quality Control: [redacted] *Date:* 23NOV 2022

Approved: [redacted] *Date:* 23 Nov 2022

Quality Control Laboratory
[redacted]

TC

TOXI-COOP ZRT.

TOXI-COOP ZRT.

Address: Balatonfüred, Arácsi út 97-99. 8230 Hungary
Phone: +36-30-678-2994

Final Report

In vitro determination of the Skin Sensitisation potential (ARE-Nrf2 Luciferase Test Method - KeratinoSens™) of Tafluprost ethyl amide

Study Number: **147-442-7157**

Date of Final Report: **December 14, 2022**

(Report including Appendices total pages 46)

Sponsor:

[redacted]

Author:

Viktória Hummel-Kocsi
TOXI-COOP ZRT.
Arácsi út 97-99.
8230 Balatonfüred
Hungary

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The following prints of the Report are issued:

Paper print:

Original 1 of 2	Archived at TOXI-COOP ZRT.
Original 2 of 2	Released to the Sponsor.

Electronic copy:

Electronic copy 1 of 1	Released to the Sponsor.

The electronic file is an unaudited copy, generated after finalisation of the report. The Sponsor is reminded that PDF files are not sufficiently protected against modification. Therefore, TOXI-COOP ZRT. cannot take any responsibility for the content of the electronic copy. The Sponsor is using the electronic copy on his own responsibility.

Study Number: 147-442-7157

Statement of the Study Director

This study has been performed in accordance with the principles of Good Laboratory Practice Regulations as specified by Hungarian Good Laboratory Practice Regulation: 42/2014 (VIII. 19.) EMMI decree of the Minister of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM(98)17 and with the OECD/OCDE KEY EVENT BASED TEST GUIDELINE NO. 442D – Appendix IA: *In vitro* Skin Sensitisation: The ARE-Nrf2 Luciferase KeratinoSens™ Test Method (30 June 2022), OECD Guideline No. 497: Defined Approaches for Skin Sensitisation - Annex I. Prediction model for the individual *in chemico/in vitro* tests with multiple runs for use in 2o3 DA (14 June 2021) and the EURL EVCAM DB-ALM (INVITTOX) Protocol n° 155: KeratinoSens™ (2018). There were no circumstances that may have affected the quality or integrity of the study.
I declare that this report constitutes a true record of the actions undertaken and the results obtained in this study.

Signature: _____ Date: December 14, 2022

Viktória Hummel-Kocsi

Statement of the Management

According to the conditions of the research and development assignment between **Walberg GmbH** (as Sponsor) and **TOXI-COOP ZRT.** (as Test Facility) the "*In vitro* determination of the Skin Sensitisation potential (ARE-Nrf2 Luciferase Test Method - KeratinoSens™) of **Tafluprost ethyl amide**" has been performed in accordance with the GLP requirement.

Signature: _____ Date: Dec 14, 2022

Dr. Gábor Hirka

Quality Assurance Statement

Study Number: **147-442-7157**

Study Title: ***In vitro* determination of the Skin Sensitisation potential (ARE-Nrf2 Luciferase Test Method - KeratinoSens™) of Tafluprost ethyl amide**

Test Item: **Tafluprost ethyl amide**

This study has been inspected and this report audited by the Quality Assurance in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in writing to the study director and to management. The dates of such inspections and of the report audit are given below:

Date	Inspection/Audit	Date of report to Management	Date of report to Study Director
October 06, 2022	Study Plan	October 06, 2022	October 06, 2022
October 27, 2022	Sample preparation	October 27, 2022	October 27, 2022
November 25, 2022	Draft Report	November 25, 2022	November 25, 2022
December 14, 2022	Final Report	December 14, 2022	December 14, 2022

Signature: _____ Date: Dec 14, 2022

Vanda Gyimesi
Quality Assurance

General Statements and Responsibilities

Study title:	***In vitro* determination of the Skin Sensitisation potential (ARE-Nrf2 Luciferase Test Method - KeratinoSens™) of Tafluprost ethyl amide**
Study number:	**147-442-7157**
Sponsor:	[redacted]
Sponsor representative:	**Dr. Karsten Schilling** IRSC - International Regulatory & Scientific Consulting Paul-Lincke-Str. 36 D-67304 Eisenberg Germany
Test facility:	**TOXI-COOP ZRT.** 8230 Balatonfüred, Arácsi út 97-99. Hungary Phone: +36-30-678-2994
Test facility management:	**Dr. Gábor Hirka**
Study director:	**Viktória Hummel-Kocsi**
Leader Assistant:	**Éva Steinbach Kőváriné**
Assistant:	**Aranka Kiss**
Quality Assurance:	**Ildikó Hermann** **Vanda Gyimesi**

Study Schedule

Date of Study Plan:	**October 06, 2022**
Start of Experimental phase:	October 24, 2022
First test:	from October 24, 2022 to October 27, 2022
Second test:	from November 01, 2022 to November 04, 2022
Third test:	from November 07, 2022 to November 10, 2022
End of Experimental phase:	November 10, 2022
Date of Draft Report:	**November 28, 2022**
Date of Final Report:	**December 14, 2022**

1.0 Summary

In the course of this study the skin sensitisation potential of the test item "**Tafluprost ethyl amide**" was studied using the KeratinoSens™ method (ARE-Nrf2 Luciferase Test Method).

In order to derive a prediction for the test item the results of three independent tests were used. Since the results of the two tests were not concordant, a third was needed in order to derive a conclusion.

The luciferase activity induction obtained with the positive control, Trans-Cinnamaldehyde, was dose-dependent and statistically significant above the threshold of 1.5-fold in all tests and met the acceptance criteria. The solvent DMSO used as negative control reacted as expected and revealed no sensitising potential. Therefore, the sensitivity and suitability of the test system was confirmed.

For the test item, twelve doses ranging from 2000.00 µM to 0.98 µM and 2-fold dilution factor were used in the first test. In order to be able to determine IC_{30} and IC_{50} values more precisely (since strong cytotoxicity was observed at the higher tested concentrations) and to investigate the possible positive effect of the test item, lower top concentration and narrower dilution factor was used in the second and third tests. Thus, twelve doses ranging from 1000.00 µM to 55.80 µM and 1.3-fold dilution factor were used in the second test, while twelve doses ranging from 500.00 µM to 67.29 µM and 1.2-fold dilution factor were used in the third test.

The test item induced cytotoxicity in KeratinoSens™ cells compared to the solvent/vehicle control in all tests at the higher tested concentrations (2000.00 – 500.00 µM in the first test, 1000.00 – 350.13 µM in the second test and 500.00 – 289.35 µM in the third test). Thus, IC_{30} and IC_{50} values could be determined for each independent test. The overall IC_{30} was determined as 278.51 µM, while the overall IC_{50} was 311.38 µM.

The induction values of the test item did not exceed the 1.5-fold threshold at any tested concentrations compared to the respective negative control in either independent test. Thus, an $EC_{1.5}$ value could not be determined for any of the tests.
Moreover, according to the OECD Test Guideline 497 prediction model, there was only one induction value of the test item which exceeded the lower limit of the borderline threshold (1.35-fold) compared to the respective negative control at the interim concentration of 250.00 µg/mL in the first test.

Based on the obtained results and in accordance with the OECD 442D and OECD 497 prediction model criteria, the test item was concluded to be negative. The overall I_{max} value was determined as 1.25-fold. In addition, no dose-response-relationship was observed in any of the tests.

Table 1. Summary of the KeratinoSens™ results

#	Significant induction above 1.5-fold (yes/no)	Viability ≥ 70 % at lowest concentration with ≥ 1.5-fold (yes/no)	$EC_{1.5}$ < 1000 µM or 200 µg/ml (yes/no)	Showing clear dose response (yes/no)	KeratinoSens™ result obtained based on OECD 442D (positive/negative/ in-conclusive)	KeratinoSens™ result obtained based on OECD 497 (positive/negative/ borderline/ in-conclusive)
1	no	no	-	no	**negative**	**borderline***
2	no	no	-	no	**negative**	**negative**
3	no	no	-	no	**negative**	**negative**
OVERALL CONCLUSION					**negative**	

*At the concentration of 250.00 µg/mL the induction value is higher than 1.35 with cell viability > 70 %.

Overall, based on the obtained results and according to the OECD 442D and OECD 497 prediction model criteria, the test item "Tafluprost ethyl amide" is concluded negative for skin sensitisation potential under the experimental conditions of KeratinoSens™ method (ARE-Nrf2 Luciferase Test Method), when tested up to clear cytotoxic concentrations.

2.0 Study Objective and Introduction

The purpose of the ARE-Nrf2 luciferase test method (KeratinoSens™) is to contribute to the identification of skin sensitisers and non-sensitisers by addressing the second key event of the skin sensitisation Adverse Outcome Pathway [1].

The ARE-Nrf2 luciferase test method makes use of an immortalised adherent cell line derived from HaCaT human keratinocytes stably transfected with a selectable plasmid. The cell line contains the luciferase gene under the transcriptional control of a constitutive promoter fused with an ARE element from a gene that is known to be up-regulated by skin sensitisers. The luciferase signal reflects the activation by sensitisers of endogenous Nrf2 dependent genes. The dependence of the luciferase signal in the recombinant cell line allows quantitative measurement (by luminescence detection) of luciferase gene induction, using well established light producing luciferase substrates, as an indicator of the activity of the Nrf2 transcription factor in cells following exposure to the test items. The KeratinoSens™ method not only supports the discrimination between skin sensitisers from non-sensitisers but, it may also potentially contribute to the assessment of sensitising potency when used in integrated approaches such as the Integrated Approaches to Testing and Assessment (IATA).

3.0 Regulatory Guidelines and Test Methods

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD/OCDE KEY EVENT BASED TEST GUIDELINE NO. 442D – Appendix IA: *In vitro* Skin Sensitisation: The ARE-Nrf2 Luciferase KeratinoSens™ Test Method (30 June 2022) [2]
- EURL ECVAM DB-ALM (INVITTOX) Protocol n°155: KeratinoSens™ (2018) [3]
- OECD Guideline No. 497: Defined Approaches for Skin Sensitisation - Annex I. Prediction model for the individual *in chemico/in vitro* tests with multiple runs for use in 2o3 DA (14 June 2021) [8]
- SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21 [9]

4.0 Archiving

The study documents and samples as listed below will be archived according to the OECD GLP and to the Toxi-Coop Zrt. SOPs in the archives of Toxi-Coop Zrt. (H-8230 Balatonfüred, Galamb u. 12/A, Hungary):

- Study Plan (15 years)
- All raw data (15 years)
- Retained sample of the test item (5 years)
- Retained sample of the reference item (5 years)
- Correspondence (15 years)
- Study report and any amendments (15 years)

For the first 5 years archiving is included, thereafter archiving occurs at additional costs of the Sponsor. After this period, the Sponsor will be notified to decide on further archiving to comply with current legal requirements. After the retention time all the archived materials listed above will be returned to the Sponsor or retained for a further period if agreed by a contract or destroyed on their behalf.
None of the above cited documents or material will be discarded without the explicit written consent of the Sponsor. At the end of the studies, any remaining test item will be returned to the Sponsor, unless otherwise instructed by the Sponsor.

5.0 Materials and Methods

5.1 Test Item

Name:	Tafluprost ethyl amide
Batch No.:	0652603-2
Expiry date:	24 June 2023
CAS number:	1185851-52-8
Molecular weight:	437.5 g/mol
Purity (HPLC):	98.5 %
Appearance:	colourless to pale yellow, oily consistency
Storage:	refrigerator (2-8 °C)
Safety precautions:	According to the SDS

Test item information is based on written information given by the Sponsor.

5.1.1 Identification, Receipt

The test item of a suitable chemical purity was supplied by the Sponsor. All precautions required in the handling and disposal of the test item are outlined by the Sponsor.
Identification of the test item was made by TOXI-COOP ZRT. on the basis of the information included in the test item documentation supplied by the Sponsor.

5.2 Formulation of the Test Item

Suitable solvents for the test item were dimethyl sulfoxide (DMSO) and sterile ultrapure water or exposure medium. An appropriate solvent dissolves the test item completely (the solution must not be cloudy or have noticeable precipitate) or at least forms a stable dispersion without apparent phase separation. Solubility of test item was evaluated and confirmed visually.

Test item was first tried to be dissolved in DMSO to the final concentration of 200 mM. The DMSO solutions can be considered self-sterilising, so that no sterile filtration is needed. The test item could be properly dissolved in DMSO and a clear and homogenous solution was gained after 5 minutes of vortexing.

Since DMSO is the preferred vehicle according to the test guideline, and the solution complied with all requirements, it was chosen as the solvent.

The pre-experiments on solubility of the test item were not performed in compliance with the GLP-Regulations and will be excluded from the Statement of Compliance in the Final Report, but the raw data of these tests will be archived under the study code of present study.

5.3 Controls

Positive, negative (vehicle) and blank controls were included in the tests. In the case more studies were run in parallel, the same controls were used.

5.3.1 Positive control

Test Substance:	**TRANS-CINNAMALDEHYDE**
Batch number:	STBJ1063
Expiry date:	23 September 2023
Storage:	2-8 °C
Purity:	99.1 %
CAS number:	14371-10-9
Formula weight:	132.16 g/mol
Formula:	$C_6H_5CH=CHCHO$
Manufacturer/Supplier:	SIGMA-ALDRICH

Trans-Cinnamaldehyde was dissolved in Dimethyl sulfoxide (DMSO).

5.3.2 Negative Controls (Vehicles)

Name:	**Dimethyl sulfoxide** (DMSO)
Manufacturer/Supplier:	SIGMA-ALDRICH
Batch Number:	STBK4632
Expiry date:	30 May 2023
Storage:	Room temperature
CAS number:	67-68-5

The final concentration of DMSO was 1 % in exposure medium on the plates treated with the test item, the negative (solvent) control and the positive control. This concentration in DMSO is known not to affect cell viability.

5.4 Additional Chemicals Used in the Experiment

Table 2. Additional Chemicals Used in the Experiment

Name	Supplier / Manufacturer	Expiry date/ Retest date	Batch/Lot number
Dulbecco's Modified Eagle's Medium (DMEM)	SIGMA-ALDRICH	FEBRUARY 2023	RNBK8725
Dulbecco's Phosphate Buffered Saline (DPBS)	SIGMA-ALDRICH	MARCH 2024	RNBK9604
Fetal Bovine Serum (FBS)	SIGMA-ALDRICH	JULY 2026	0001655439
G418 Solution	Roche	MARCH 2023	55674300
Ethylenediaminetetraacetic acid trisodium salt hydrate (EDTA)	SIGMA-ALDRICH	JANUARY 2023	SLCF3494
1 M Solution hydrochloric acid	lach:ner	17 FEBRUARY 2023	PP/2022/00535
Thiazolyl Blue Tetrazolium Bromide (MTT)	SIGMA-ALDRICH	JANUARY 2027	MKCR0748
Trypsin-EDTA solution – 1x, sterile	SIGMA-ALDRICH	FEBRUARY 2023	SLCH7201
2-Propanol	SIGMA-ALDRICH	22 APRIL 2023	STBK4737
Passive Lysis Buffer, 5x	Promega	12 AUGUST 2023	0000438458
Luciferase Assay System 10-Pack	Promega	16 NOVEMBER 2024	0000504956
Millipore (ultrapure) water*	-	-	-

* Purified water was always freshly prepared by Millipore Direct Q5 water purification system (Serial number: F0DA13956K) in the laboratory of TOXI-COOP ZRT. The expiry date of the purified water itself was one week from the date of preparation and a new batch number was generated every day when purified water was prepared (the used batch of ultrapure water: 20221019, 20221027, 20221103, 20221104, 20221110).

5.5 Preparation of Solutions

EDTA solution 10 %, pH 8:
Ultrapure water supplemented with 10 (w/v) % EDTA, brought to pH 8 with hydrogen chloride (HCl)

DPBS-EDTA:
Dulbecco's Phosphate Buffered Saline (DPBS) containing 0.05 % EDTA solution

MTT stock solution:
MTT was dissolved in DPBS at the concentration of 5 mg/mL

MTT working solution:
8.4-fold dilution of MTT stock solution in exposure medium

1× Lysis Buffer:
5-fold dilution of 5× Lysis Buffer in ultrapure water

Luciferase Reagent:
1 vial Luciferase Assay Substrate dissolved in 10 mL (content of 1 vial) Luciferase Assay Buffer

5.6 Cell or Test System (KeratinoSens™ cell line)

The KeratinoSens™ cell line is a transgenic cell line with a stable Luciferase construct insertion.

5.6.1 KeratinoSens™ cell line

Name:	KeratinoSens™ cell line
Description:	immortalised adherent cell line derived from human keratinocytes (HaCaT) transfected with selectable plasmids
Supplier:	Givaudan Schweiz AG
Lot Number:	20160415
Date of production:	April 15, 2016 [7]
Storage:	Vapor phase of liquid nitrogen

The original cells (supplied by Givaudan upon establishing a license agreement) were propagated and subcultured into prepared cell line stocks (master cultures - MCs) in our laboratory. Cells from this original stock could be propagated up to maximum 25 passages and were employed for routine testing using the maintenance/growth medium.

5.6.2 KeratinoSens™ Master culture

Subcultured MC3 master culture was used for the study.

ID of the cell line:	KeratinoSens™ MC3 p4 20180302-20221017
Date of preparation:	March 02, 2018
Date of thawing:	October 17, 2022
Passage number at start:	p6
Passage number at the end:	p10

5.6.3 Preparation of Media for KeratinoSens™ cells

Maintenance (culture) medium:
DMEM supplemented with 9.0 (v/v) % fetal bovine serum (FBS) and ~ 500 µg/mL G418.

Thawing medium:
DMEM containing 9.1 (v/v) % FBS without G418

Exposure medium:
DMEM containing 1 (v/v) % FBS without G418

5.7 Apparatus

5.7.1 Spectrophotometer

Name:	Varioskan™ LUX Type 3020
Serial number:	3020-078

Absorbance measurement

Light source:	Xenon flash lamp (100Hz)
Detector:	Photodiode
Wavelength range:	200 – 1000 nm

Luminescence measurement - LAT module

Detector:	Photomultiplier tube
Wavelength selection:	8 optional filter position
Wavelength range:	360 - 670 nm

5.7.2 Evaluation software

SkanIt® Software for Microplate Readers were used for measurements and MS Excel for further calculations.

6.0 Description of the Test

6.1 Procedure of the KeratinoSens™ method

0. Solubility assessment of the test item
   Preincubation of cells
1. Seeding of cells for testing - 24 h incubation
2. Preparation of the test item stock solution
3. Preparation of master plates
4. Exposure – 48 h incubation
5. Luciferase activation measurement
6. Cytotoxicity assessment

6.2 Principle of the KeratinoSens™ method

The KeratinoSens™ method is an *in vitro* assay that quantifies the extent of luciferase gene induction following 48 hours incubation time of the KeratinoSens™ reporter cells with the test items. Luciferase gene induction is measured in the cell lysates by luminescence detection using a light producing luciferase substrate (Luciferase Reagent). Cytotoxicity and the relative luminescence intensity of luciferase substrate in the lysates are measured and luciferase induction compared to solvent/vehicle control is calculated.

KeratinoSens™ cells were derived from HaCaT human keratinocytes and transfected with selectable plasmids containing luciferase gene under the transcriptional control of the AKR1C2 ARE gene sequence, upstream of the SV40 promoter. AKR1C2 is known to be one of the genes up-regulated upon contacting skin sensitisers in dendritic cells. Therefore, this method is able to mimic the activation of the Keap1-Nrf2-ARE regulatory pathway, and is relevant for the assessment of the skin sensitisation potential of test items. A prediction model is used, to support the discrimination between sensitisers and non-sensitisers.

6.3 Preparation of the master plate

6.3.1 Test item Master Solutions

Based on the test item stock solutions made of DMSO, 2-fold serial dilution in the first test, 1.3-fold serial dilution in the second test and 1.2-fold serial dilution in the third test was made using the solvent to obtain twelve 100 × master concentrations of the test item creating a 100 × master plate.
The 100 × master concentrations were further diluted 25-fold into exposure medium to obtain the 4 × master plate, by adding 10 µL of the 100 × master concentrations to 240 µL exposure medium.

6.3.2 Positive control

The positive control used was Trans-Cinnamaldehyde for which a series of five 100 × master concentrations ranging from 0.4 to 6.4 mM were prepared in DMSO (from a 200 mM stock solution) and diluted as described for the 4 × master solutions. The final concentration of the positive control on the treated plates ranged from 4 to 64 µM.

6.3.3 Negative control

The negative (solvent) control used was DMSO, for which six wells per plate were prepared. It underwent the same dilution as described for the master and working solution concentrations in 6.3.1, so that the final negative (solvent) control concentration was 1 % DMSO in exposure medium on the treated plates.

This DMSO concentration is known not to affect cell viability and corresponds to the same concentration of DMSO used in the tested chemical and in the positive control.

6.4 Preparation of cells

Cells were subcultured upon reaching 80 - 90 % confluence and care was taken to ensure that cells were never grown to full confluence. One day prior to testing cells were harvested in thawing medium and distributed into 96-well plates (10 000 cells/well) homogenously. For each individual test in the study, three replicates were used for the luciferase activity measurements, and one parallel replicate for the cell viability assay. One well per plate was left empty to assess background values. Cells were grown for 24 ± 0.5 hours in 96-wells microplates at 37 ± 1 °C in the presence of 5 % CO_2.

6.5 Exposure

After the 24-hour incubation time, thawing medium was replaced with fresh exposure medium. The 4 × master solutions of the test item and control substances were added to each well in a way that an additional 4-fold dilution was achieved on the plate for the final concentrations to be established (50 µL of 4 × master solution to 150 µL of exposure medium). The treated plates were then incubated for about 48 ± 1 hours at 37 ± 1 °C in the presence of 5 % CO_2. Care was taken to avoid cross-contamination between wells by covering the plates with a foil prior to the incubation with the test item.

6.6 Luciferase activity measurements

After the 48-hour exposure time with the test item and control substances, cells were washed with DPBS (270 µL), and 1 × lysis buffer (20 µL) for luminescence readings was added to each well for 20 minutes at room temperature (on all three plates). Plates with the cell lysate were then placed in the luminometer for reading. First the luciferase substrate (50 µL) was added to each well and after one second, the luciferase activity was integrated for 2 seconds.

6.7 Cytotoxicity

For the cell viability assay, medium was replaced after the 48-hour exposure time with MTT working solution (200 µL) and cells were incubated for 4 hours at 37 ± 1 °C in the presence of 5 % CO_2. The MTT working solution was then removed and formazan (reduced MTT) was solubilized by the addition of isopropanol (50 µL). After shaking for 30 minutes the absorption was measured at 570 nm with a spectrophotometer.

7.0 Acceptance Criteria

For both, the test item and the positive control substance at least two independent tests, each containing three replicates that are needed for the luminescence measurements and one replicate for the viability measurement, in order to derive a prediction. In case of discordant results between the two independent tests, a third test should be performed.
Each independent test is performed on a different day or on the same day provided that for each run independent fresh stock solutions and master solutions of the test chemical are prepared and independently harvested cells are used. However, cells may come from the same passage. KeratinoSens™ prediction should be considered in the framework of an IATA and in accordance with the limitations stated in the OECD test guideline.

The luciferase activity induction obtained with the positive control substance (Trans-Cinnamaldehyde) should be statistically significantly above the threshold of 1.5 in at least one of the tested concentrations. The $EC_{1.5}$ value of the positive control should be within two standard deviations of the historical mean of the testing facility or between 7 µM and 30 µM (based on the validation dataset). In addition, the average induction in the parallel plates for Trans-Cinnamaldehyde at 64 µM should be between 2 and 8. If the latter criterion is not fulfilled, the dose-response of Trans-Cinnamaldehyde should be carefully checked, and tests may be accepted only if there is a clear dose-response with increasing luciferase activity induction at increasing concentrations for the positive control.

Finally, the average coefficient of variation (CV) of the luminescence reading for the negative (solvent) control DMSO should be below 20 % in each test which consists of 6 wells tested in triplicate. If the variability is higher, results should be discarded.

8.0 Data evaluation

The following parameters (endpoint values) are calculated in the KeratinoSens™ test method:

- the maximal average fold induction of luciferase activity (I_{max}) value observed at any concentration of the tested chemical and positive control;
- the $EC_{1.5}$ value representing the concentration for which induction of luciferase activity is above the 1.5-fold threshold (i.e. 50 % enhanced luciferase activity) was obtained;
- the IC_{50} and IC_{30} concentration values for 50 % and 30 % reduction of cellular viability.

8.1 Fold induction and maximal average fold induction

Fold luciferase activity induction is calculated by the equation below. Maximal fold induction is determined in each individual test, while the overall maximal fold induction (Imax) is calculated as the average of the individual tests.

$$fold\ induction = (L_{sample} - L_{blank}) / (L_{solvent} - L_{blank})$$

where: L_{sample} is the luminescence reading in the wells containing cells and test item or positive control

L_{blank} is the luminescence reading in the blank well containing no cells and no treatment

$L_{solvent}$ is the average luminescence reading in the wells containing cells and solvent (negative) control

8.2 Determination of $EC_{1.5}$

The concentrations of the test item needed for a 1.5-fold luciferase induction are calculated by linear interpolation according to the equation below, and the overall $EC_{1.5}$ is calculated as the geometric mean of the individual tests.

$$EC_{1.5} = (Cb - Ca) \times [(1.5 - Ia) / (Ib - Ia)] + Ca$$

where: Ca is the lowest concentration in µM with > 1.5-fold induction

Cb is the highest concentration in µM with < 1.5-fold induction

Ia is the fold induction measured at the lowest concentration with > 1.5-fold induction (mean of three replicate wells)

Ib is the fold induction at the highest concentration with < 1.5-fold induction (mean of three replicate wells)

8.3 Cytotoxicity (determination of IC_{50} and IC_{30})

Viability is calculated by the equation below:

$$viability = \left[(V_{sample} - V_{blank}) / (V_{solvent} - V_{blank}) \right] \times 100$$

where: V_{sample} is the MTT-absorbance reading in the wells containing cells and test item or positive control

V_{blank} is the MTT-absorbance reading in the blank well containing no cells and no treatment

$V_{solvent}$ is the average MTT-absorbance reading in the wells containing cells and solvent (negative) control

IC_{50} and IC_{30} are calculated by linear interpolation according to the equation below, and the overall IC_{50} and IC_{30} are calculated as the geometric mean of the individual tests.

$$ICx = (Cb - Ca) \times \left[(100 - x) - Va) / (Vb - Va) \right] + Ca$$

where: x is the % reduction at the concentration to be calculated (50 and 30)

Ca is the lowest concentration in µM with > x % reduction in viability

Cb is the highest concentration in µM with < x % reduction in viability

Va is the % viability at the lowest concentration with > x % reduction in viability

Vb is the % viability at the highest concentration with < x % reduction in viability

For each concentration showing a luciferase activity induction equal or higher than 1.5-fold, statistical significance is determined (e.g. using a two-tailed Student's t-test) by comparing the luminescence values of the three replicate samples with the luminescence values in the solvent/vehicle control wells to assess whether the luciferase activity induction is statistically significant ($p < 0.05$). Furthermore, it should be checked that no significant cytotoxic effects occur at the lowest concentration leading to ≥1.5-fold luciferase induction and that this concentration is below the IC_{30} value, indicating that there is less than or equal to 30 % reduction in cellular viability. In addition, at least two consecutive concentrations should have ≥ 70 % viability, otherwise the concentration range should be adjusted.

If no clear dose-response curve is observed, or if the dose-response curve obtained is biphasic, the experiment should be repeated to verify whether this is specific to the test item or due to an experimental artefact. In case the biphasic response is reproducible in an independent experiment, the lower $EC_{1.5}$ value (the concentration when the threshold of 1.5 is crossed the first time) is reported.

In the KeratinoSens™ test method, in the rare cases where a statistically non-significant luciferase induction equal or above 1.5-fold is observed followed by a higher concentration with a statistically significant induction, results from this repetition are only considered as valid and positive if the statistically significant induction equal or above the threshold of 1.5 was obtained for a non-cytotoxic concentration.

For test items generating a 1.5-fold or higher induction already at the lowest test concentration (i.e. 0.98 µM), the $EC_{1.5}$value of < 0.98 is set based on visual inspection of the dose-response curve.

9.0 Prediction model

A KeratinoSens™ prediction is considered positive if the following 4 conditions are all met in 2 of 2 or in the same 2 of 3 tests, otherwise the KeratinoSens™ prediction is considered negative:

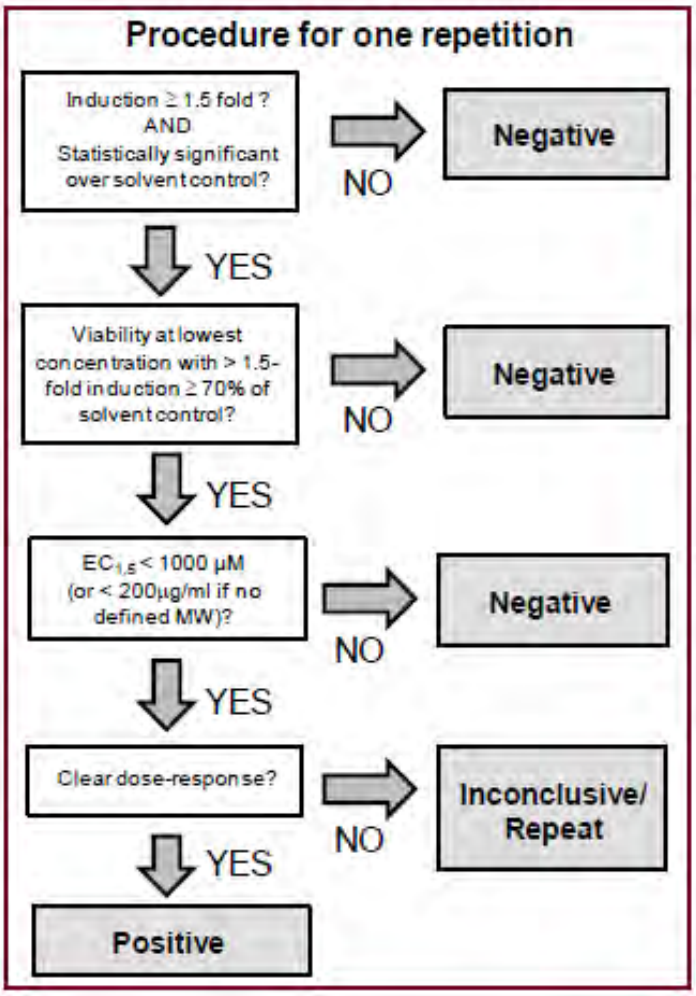
- the Imax is equal or higher than 1.5-fold and statistically significantly different as compared to the negative/solvent control (as determined by a two-tailed, unpaired Student's T-test);
- the cellular viability is equal or higher than 70 % at the lowest concentration with induction of luciferase activity ≥ 1.5-fold;
- the $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/mL for test items with no defined molecular weight);
- there is an apparent overall dose-response for luciferase induction (or a biphasic response).

If in a given test, all of the three first conditions are met but a clear dose-response for the luciferase induction cannot be observed, then the result of that test should be considered inconclusive and further testing may be required. In addition, a negative result obtained with concentrations < 1000 µM (or < 200 µg/mL for test items with no defined molecular weight) and which do not reach cytotoxicity (< 70 % viability) at the maximal tested concentration should also be considered as inconclusive.

In cases, if the test item induces the luciferase activity very close to the cytotoxic levels, it might be positive in some tests at non-cytotoxic levels and in other tests only at cytotoxic levels. Such test items shall be retested with a narrower dose-response analysis, using a lower dilution factor between wells, to determine if induction has occurred at cytotoxic levels or not.

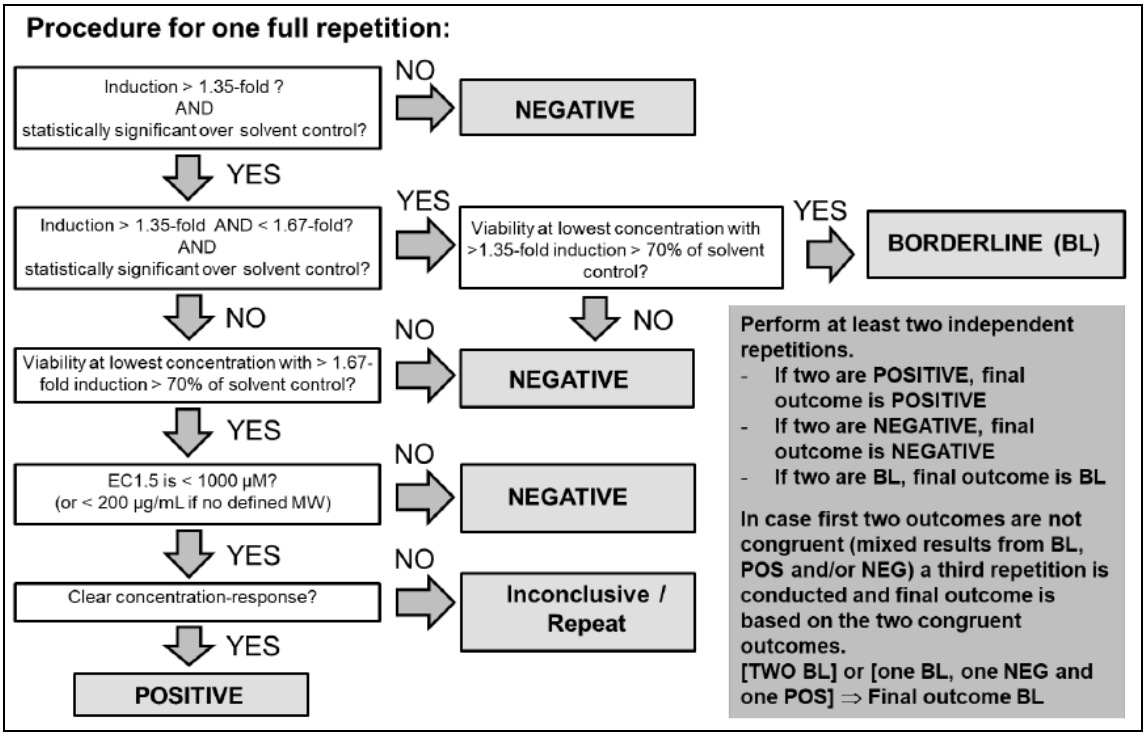
With respect to chemicals tested at lower concentrations than the default ones, results fulfilling the criteria for positivity could still be used to support the identification of the test item as a skin sensitiser. In cases where a negative result is obtained in a test with a maximal concentration lower than 1000 µM and no cytotoxicity is reached, the result should be considered as inconclusive. If cytotoxicity (< 70 % viability) is reached at a maximal soluble test concentration lower than 1000 µM, criteria for negativity can still be applied.

Figure 1. Prediction model used in the KeratinoSens™



The prediction model of the KeratinoSens™ assay requires multiple runs. For the assessment of whether the outcome of repeated runs yields a positive, negative or borderline final outcome in KeratinoSens™ the prediction model in Figure 2 shall be applied (adapted from the prediction model described in TG 442D to be used within the 2 out of three 3 defined approach (2o3 DA) to conclude on borderline cases). This prediction model introduces a third outcome (borderline) to be used within the 2o3 DA, based on the same decision cut-offs of the prediction model described in TG 442D. Thus, a negative in the original prediction model can only become negative or borderline, while a positive from the original prediction model can only become positive or borderline. The original threshold for a positive classification is 1.5-fold induction, and the statistically derived borderline range around this threshold is 1.35 – 1.67-fold. [8]

Figure 2. Flow-chart of the KeratinoSens™ prediction model taking into account borderline ranges and multiple runs to conclude on borderline results within the 2o3 DA



10.0 Demonstration of Proficiency

Prior to routine use of the test method described in the OECD Test Guideline 442D, our laboratory demonstrated technical proficiency (Study Number: 392-442-4012), using the 10 Proficiency Substances listed in APPENDIX IA - ANNEX 1 of TG 442D. Moreover, a historical database of data generated with the positive control is maintained over time to confirm the reproducibility of the test method in the laboratory.

11.0 Deviation from the Study Plan

No Deviation from the Study Plan was issued.

12.0 Amendment to the Study Plan

No Amendment to the Study Plan was issued.

13.0 Results and evaluation

For each individual test, four parallel plates were used: three replicates were used for the luciferase activity induction measurements and one was needed for the MTT cell viability assay to measure the cytotoxicity induced by the test item.

In order to derive a prediction for the test item the results of three independent tests were used. Since the results of the two tests were not concordant, a third was needed in order to derive a conclusion.

Dates for the individual tests conducted:

First test: from October 24, 2022 to October 27, 2022
Second test: from November 01, 2022 to November 04, 2022
Third test: from November 07, 2022 to November 10, 2022

Each independent test was performed on a different day provided that for each run independent fresh stock solutions and master solutions of the test chemical were prepared and independently harvested cells were used. KeratinoSens™ prediction was considered in the framework of an IATA and in accordance with the limitations stated in the OECD test guideline.

13.1 Test item

For the test item, twelve doses ranging from 2000.00 µM to 0.98 µM and 2-fold dilution factor were used in the first test. In order to be able to determine IC_{30} and IC_{50} values more precisely (since strong cytotoxicity was observed at the higher tested concentrations) and to investigate the possible positive effect of the test item, lower top concentration and narrower dilution factor was used in the second and third tests. Thus, twelve doses ranging from 1000.00 µM to 55.80 µM and 1.3-fold dilution factor were used in the second test, while twelve doses ranging from 500.00 µM to 67.29 µM and 1.2-fold dilution factor were used in the third test.

Average fold induction, significance and viability (%) values for the test item concentrations in the independent experiments are presented in Appendix IV. The summary of the results obtained in the three independent tests are described below.

First test:

The test item induced strong cytoxicity (viability below 70 %) at higher tested concentrations (2000.00 – 500.00 µM) compared to the solvent/vehicle control in KeratinoSens™ cells. Thus, IC_{30} as 313.83 µM and IC_{50} as 367.02 µM were determined for the first test.

The luciferase activity induction did not exceed the 1.5-fold threshold at any tested concentrations compared to the respective negative control. Thus, $EC_{1.5}$ value could not be determined for the first test (Table 3).
Moreover, according to the OECD Test Guideline 497 prediction model, only one induction value of the test item at the interim concentration of 250.00 µM was slightly above the lower limit of the borderline threshold range (1.35-1.67-fold) compared to the respective negative control.

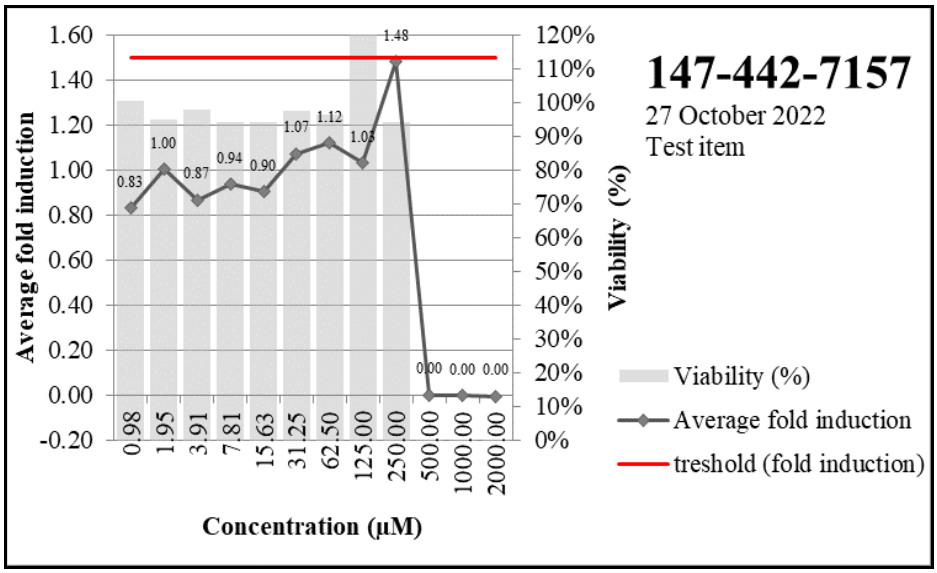
The maximal fold induction (I_{max}) was 1.48-fold in the first test.
Moreover, no dose response (Figure 3) and no precipitation were observed at any concentration during the first test.

Table 3. Summary of the KeratinoSens™ results for the test item in the first test

	Criterion for a positive response	
Test item	Statistically significant induction over 1.5-fold	no
	Viability ≥ 70 % at lowest concentration with ≥1.5-fold	no
	$EC_{1.5}$ (µg/mL)	-
	Clear dose response	no
	positive / negative (based on OECD 442D)	**negative**
	positive / borderline / negative (based on OECD 497)	**borderline***

*At the concentration of 250.00 µg/mL the induction value is higher than 1.35 with cell viability > 70 %.

Figure 3. Graphical presentation of the average fold luciferase activity inductions of the test item in the first test



Based on the prediction models and the above described results, the test item was concluded borderline in the first test (Table 3).

Second test:

The test item induced also strong cytoxicity (viability below 70 %) at higher tested concentrations (1000.00 – 350.13 µM) compared to the solvent/vehicle control in KeratinoSens™ cells. Thus, IC_{30} as 274.79 µM and IC_{50} as 296.63 µM were determined for the second test.

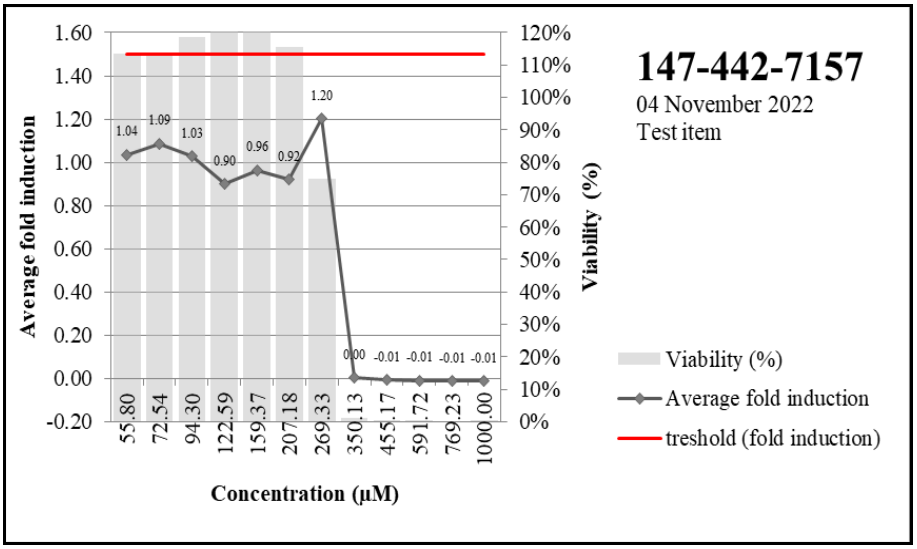
The luciferase activity induction did not exceed the 1.5-fold threshold at any tested concentrations compared to the respective negative control. Moreover, according to the OECD Test Guideline 497 prediction model, induction values of the test item were below the borderline threshold range (1.35-1.67-fold) at all tested concentrations compared to the respective negative control.
The maximal fold induction (I_{max}) was 1.20-fold. Therefore, $EC_{1.5}$ value could not be determined (Table 4).

Moreover, no dose response (Figure 4) and no precipitation were observed at any concentration during the second test.

Table 4. Summary of the KeratinoSens™ results for the test item in the second test

	Criterion for a positive response	
Test item	Statistically significant induction over 1.5-fold	no
	Viability ≥ 70 % at lowest concentration with ≥1.5-fold	no
	$EC_{1.5}$ (µg/mL)	-
	Clear dose response	no
	positive / negative (based on OECD 442D)	**negative**
	positive / borderline / negative (based on OECD 497)	**negative**

Figure 4. Graphical presentation of the average fold luciferase activity inductions of the test item in the second test



Based on the prediction models and the above described results, the test item was concluded negative in the second test, since the conditions for a positive result were not met (Table 4).

Third test:

The test item again induced cytoxicity (viability below 70 %) at higher tested concentrations (500.00 – 289.35 µM) compared to the solvent/vehicle control in KeratinoSens™ cells. Thus, IC_{30} as 250.51 µM and IC_{50} as 277.30 µM were determined for the third test.

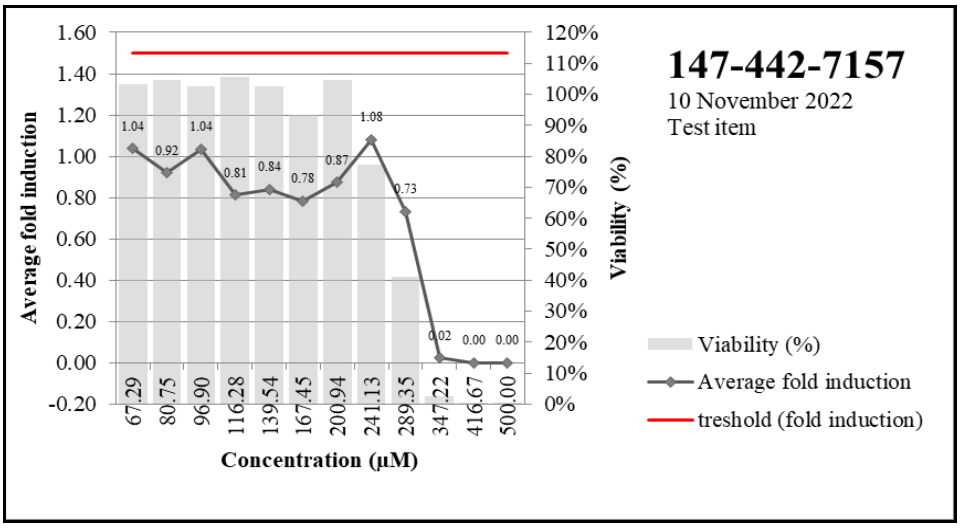
The luciferase activity induction did not exceed the 1.5-fold threshold at any tested concentrations compared to the respective negative control. Moreover, according to the OECD Test Guideline 497 prediction model, induction values of the test item were below the borderline threshold range (1.35-1.67-fold) at all tested concentrations compared to the respective negative control.
The maximal fold induction (I_{max}) was 1.08-fold. Therefore, $EC_{1.5}$ value could not be determined (Table 5).

Moreover, no dose response (Figure 5) and no precipitation were observed at any concentration during the third test.

Table 5. Summary of the KeratinoSens™ results for the test item in the third test

	Criterion for a positive response	
Test item	Statistically significant induction over 1.5-fold	no
	Viability ≥ 70 % at lowest concentration with ≥1.5-fold	no
	$EC_{1.5}$ (µg/mL)	-
	Clear dose response	no
	positive / negative (based on OECD 442D)	**negative**
	positive / borderline / negative (based on OECD 497)	**negative**

Figure 5. Graphical presentation of the average fold luciferase activity inductions of the test item in the third test



Based on the prediction models and the above described results, the test item was concluded negative in the third test, since the conditions for a positive result were not met (Table 5).

Overall result of the test item:

Based on the prediction models and the above described results, the test item was concluded to be negative (Table 3, Table 4 and Table 5). The overall I_{max} value was determined as 1.25-fold. Thus, an $EC_{1.5}$ value could not be determined for any of the tests.
The test item induced cytotoxicity according to the acceptance criteria in the form of viability below 70 % in KeratinoSens™ cells compared to the solvent/vehicle control in all three tests at the higher tested concentrations. Thus, IC_{30} and IC_{50} values could be determined for each independent test. The overall IC_{30} was determined as 278.51 µM, while the overall IC_{50} was 311.38 µM.

13.2 Negative and positive control

The coefficient of variation (CV %) of the luminescence reading for the negative control DMSO was below 20 % in all tests (7.8 %, 13.2 % and 8.7 % respectively).

The luciferase activity induction obtained with the positive control, Trans-Cinnamaldehyde was statistically significant above the threshold of 1.5 at several concentrations in all three tests. The $EC_{1.5}$ values of the positive control were 12 µM, 8 µM and 16 µM in the first, second and third tests respectively (Figure 6-7-8). These values were well within the historical control data range (1 µM - 40 µM) and fell between the OECD 442D Test Guideline example range, 7 µM - 30 µM.

The average inductions in the parallel plates for Trans-Cinnamaldehyde at 64 µM were 6.41-fold, 25.12-fold and 12.22-fold in the first, second and third tests, respectively.
In the first test the luciferase activity induction was within the 2 – 8-fold induction range and there was a clear dose response relationship in the luciferase activity induction for the positive control.
Although the luciferase activity induction in the second and third test was outside of the 2 – 8-fold induction range, there was a clear dose response relationship in the luciferase activity induction for the positive control for both tests. Consequently, both tests were accepted as valid.

In any of the tests, there was no cytotoxicity (or cell viability lower than 70 %) induced by the positive control at any of the tested concentrations.

Average fold induction values are presented in Table 6-7-8.

Figure 6. Graphical presentation of the average fold inductions of the positive control in the first test

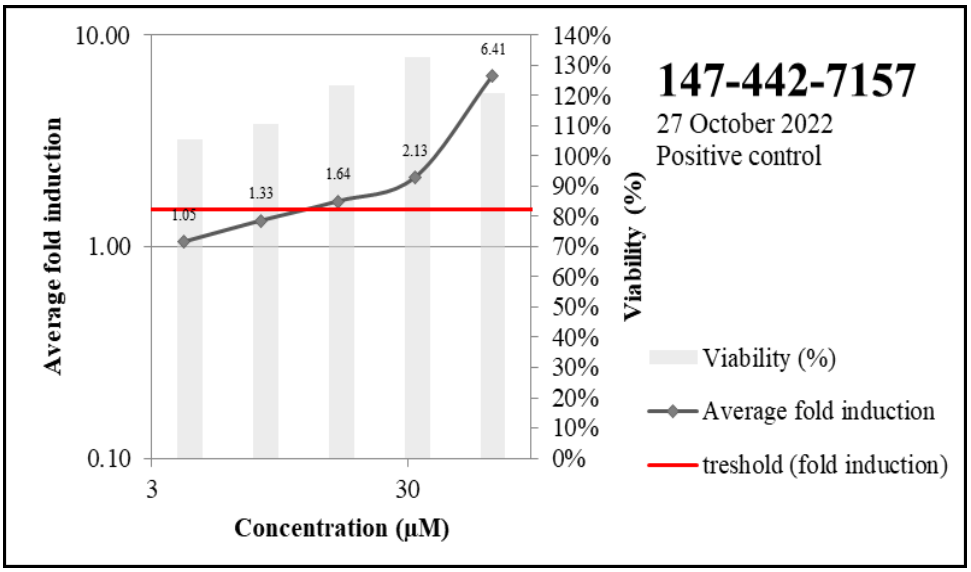


Figure 7. Graphical presentation of the average fold inductions of the positive control in the second test

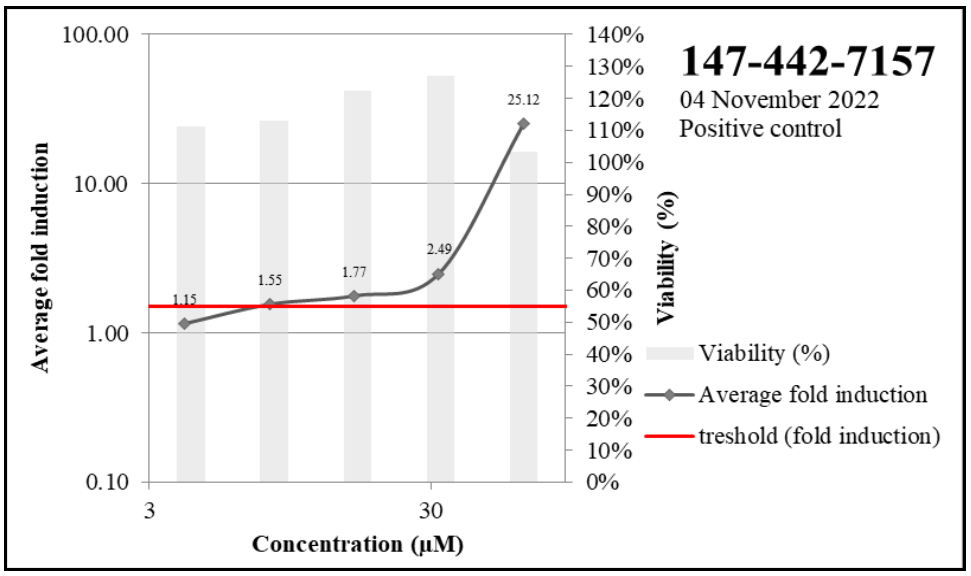


Figure 8. Graphical presentation of the average fold inductions of the positive control in the third test

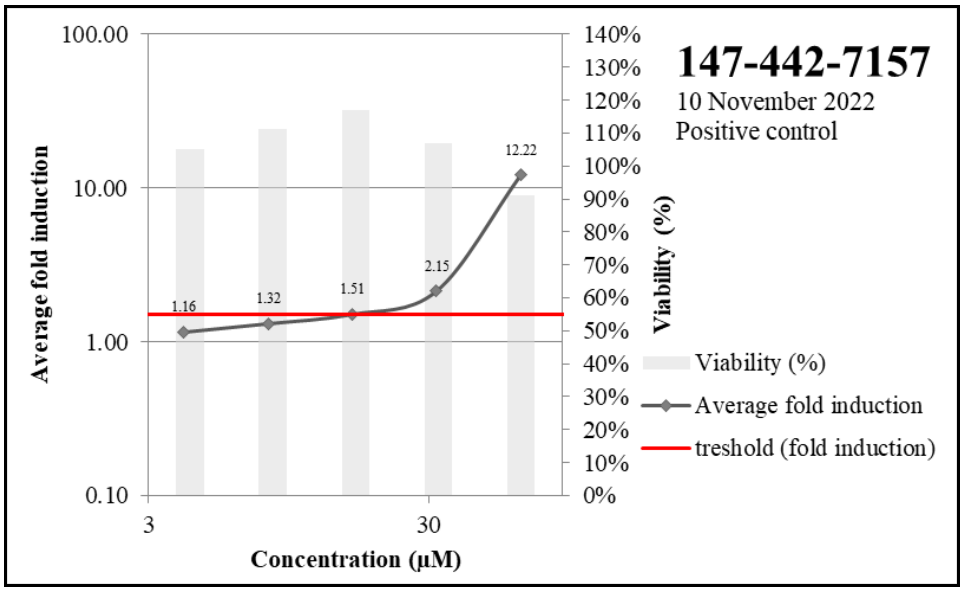


Table 6. Average fold induction, significance and viability (%) values for the positive control in the first test

	Trans-Cinnamaldehyde				
Concentration (µM)	**4**	**8**	**16**	**32**	**64**
average induction	**1.05**	**1.33**	**1.64**	**2.13**	**6.41**
significance	0.563	0.001	0.014	0.000	0.000
viability	106%	110%	123%	133%	121%

Table 7. Average fold induction, significance and viability (%) values for the positive control in the second test

	Trans-Cinnamaldehyde				
Concentration (µM)	**4**	**8**	**16**	**32**	**64**
average induction	**1.15**	**1.55**	**1.77**	**2.49**	**25.12**
significance	0.321	0.006	0.022	0.002	0.001
viability	111%	113%	122%	127%	103%

Table 8. Average fold induction, significance and viability (%) values for the positive control in the third test

	Trans-Cinnamaldehyde				
Concentration (µM)	**4**	**8**	**16**	**32**	**64**
average induction	**1.16**	**1.32**	**1.51**	**2.15**	**12.22**
significance	0.011	0.016	0.003	0.001	0.001
viability	105%	111%	117%	107%	91%

Each individual test met the acceptance criteria for the negative and positive controls, therefore considered valid under the conditions described.

14.0 Conclusion

In the course of this study the skin sensitisation potential of the test item "**Tafluprost ethyl amide**" was studied using the KeratinoSens™ method (ARE-Nrf2 Luciferase Test Method).

For the test item and positive control substance, in order to derive a prediction three valid independent tests were conducted, in which the concluded results were concordant.

The test item induced cytotoxicity in KeratinoSens™ cells compared to the solvent/vehicle control in all three tests at the higher tested concentrations. Thus, IC_{30} and IC_{50} values could be determined for each independent test. The overall IC_{30} was determined as 278.51 µM, while the overall IC_{50} was 311.38 µM.

The induction values of the test item did not exceed the 1.5-fold threshold at any tested concentrations compared to the respective negative control in either independent test. Thus, $EC_{1.5}$ value could not be determined for any of the tests.
Moreover, according to the OECD Test Guideline 497 prediction model, there was only one induction value of the test item which exceeded the lower limit of the borderline threshold (1.35-fold) compared to the respective negative control at the interim concentration of 250.00 µg/mL in the first test.

Based on the obtained results and in accordance with the OECD 442D and OECD 497 prediction model criteria, the test item was concluded to be negative. The overall I_{max} value was determined as 1.25-fold. In addition, no dose-response-relationship was observed in any of the tests.

Overall, based on the obtained results and according to the OECD 442D and OECD 497 prediction model criteria, the test item "Tafluprost ethyl amide" is concluded negative for skin sensitisation potential under the experimental conditions of KeratinoSens™ method (ARE-Nrf2 Luciferase Test Method), when tested up to clear cytotoxic concentrations.

15.0 References

[1] OECD (2012), The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No.168.

[2] OECD (2022), *Test No. 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. (30 June 2022)

[3] DB-ALM (INVITTOX) (2018), Protocol 155: keratinoSens™ 17pp.

[4] Hungarian Good Laboratory Practice Regulation: 42/2014 (VIII. 19.) EMMI decree of the Minister of Human Capacities which corresponds to the OECD GLP, NV/MC/CHEM(98)17

[5] OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998

[6] EURL-ECVAM (2014). Recommendation on the KeratinoSens™ assay for skin sensitisation testing, 42 pp.

[7] *KeratinoSens™* test report on seven chemicals of the ring-study re-tested on the KeratinoSens cell batch for transfer to test labs: P X+ 6, 15.04.2016, Final version 30.5.2016

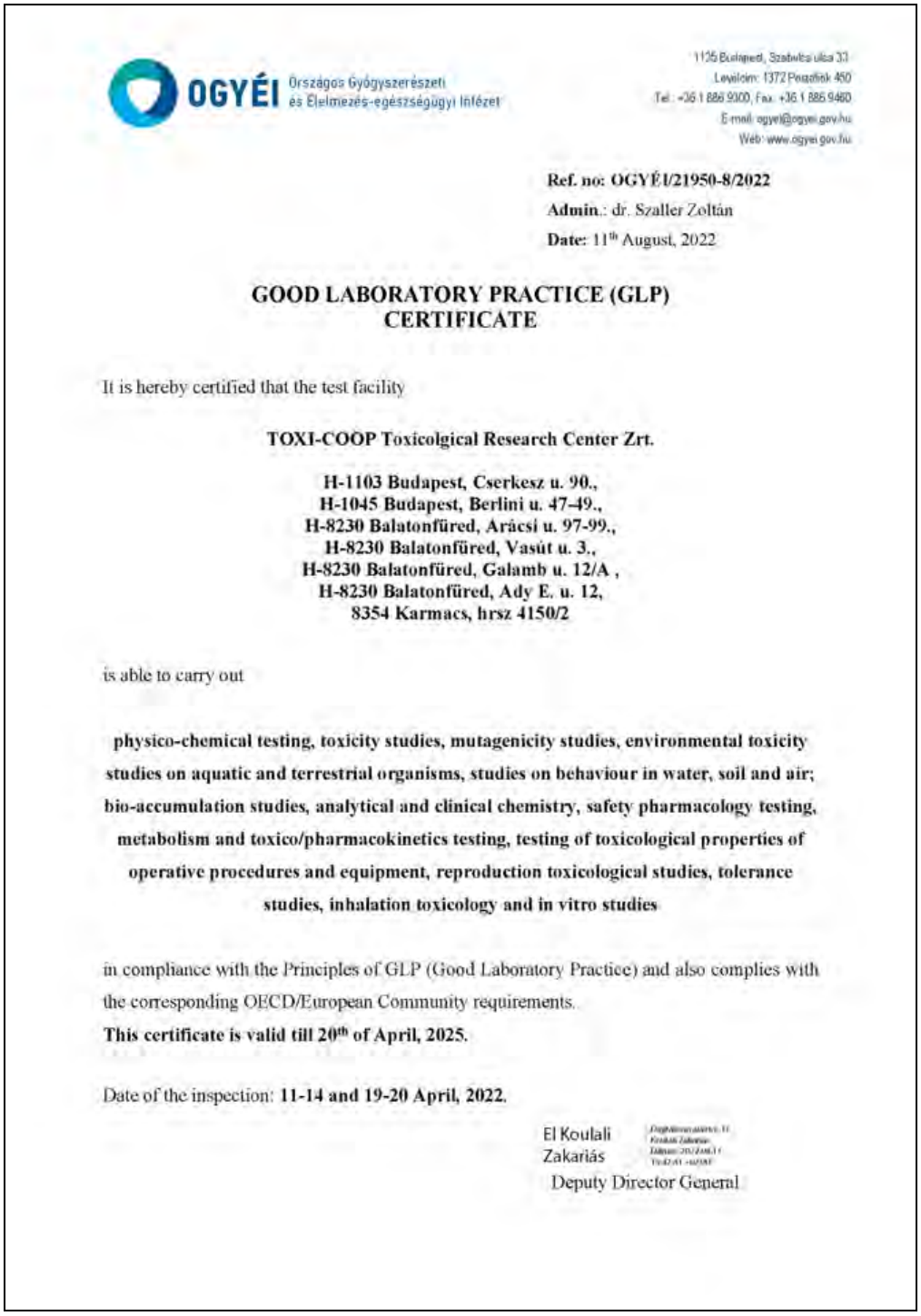
[8] OECD (2021), *Guideline No. 497: Defined Approaches for Skin Sensitisation*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. (14 June 2021)

[9] SCCS (2021), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21

APPENDICES

APPENDIX I

COPY OF THE GLP CERTIFICATE OF TOXI-COOP ZRT.



OGYÉI Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet

1135 Budapest, Szabolcs utca 33
Levelcím: 1372 Pf.: 450
Tel.: +36 1 886 9300, Fax: +36 1 886 9460
E-mail: ogyei@ogyei.gov.hu
Web: www.ogyei.gov.hu

Ref. no: OGYÉI/21950-8/2022
Admin.: dr. Szaller Zoltán
Date: 11th August, 2022

GOOD LABORATORY PRACTICE (GLP) CERTIFICATE

It is hereby certified that the test facility

TOXI-COOP Toxicolgical Research Center Zrt.

H-1103 Budapest, Cserkesz u. 90.,
H-1045 Budapest, Berlini u. 47-49.,
H-8230 Balatonfüred, Arácsi u. 97-99.,
H-8230 Balatonfüred, Vasút u. 3.,
H-8230 Balatonfüred, Galamb u. 12/A ,
H-8230 Balatonfüred, Ady E. u. 12,
8354 Karmacs, hrsz 4150/2

is able to carry out

physico-chemical testing, toxicity studies, mutagenicity studies, environmental toxicity studies on aquatic and terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation studies, analytical and clinical chemistry, safety pharmacology testing, metabolism and toxico/pharmacokinetics testing, testing of toxicological properties of operative procedures and equipment, reproduction toxicological studies, tolerance studies, inhalation toxicology and in vitro studies

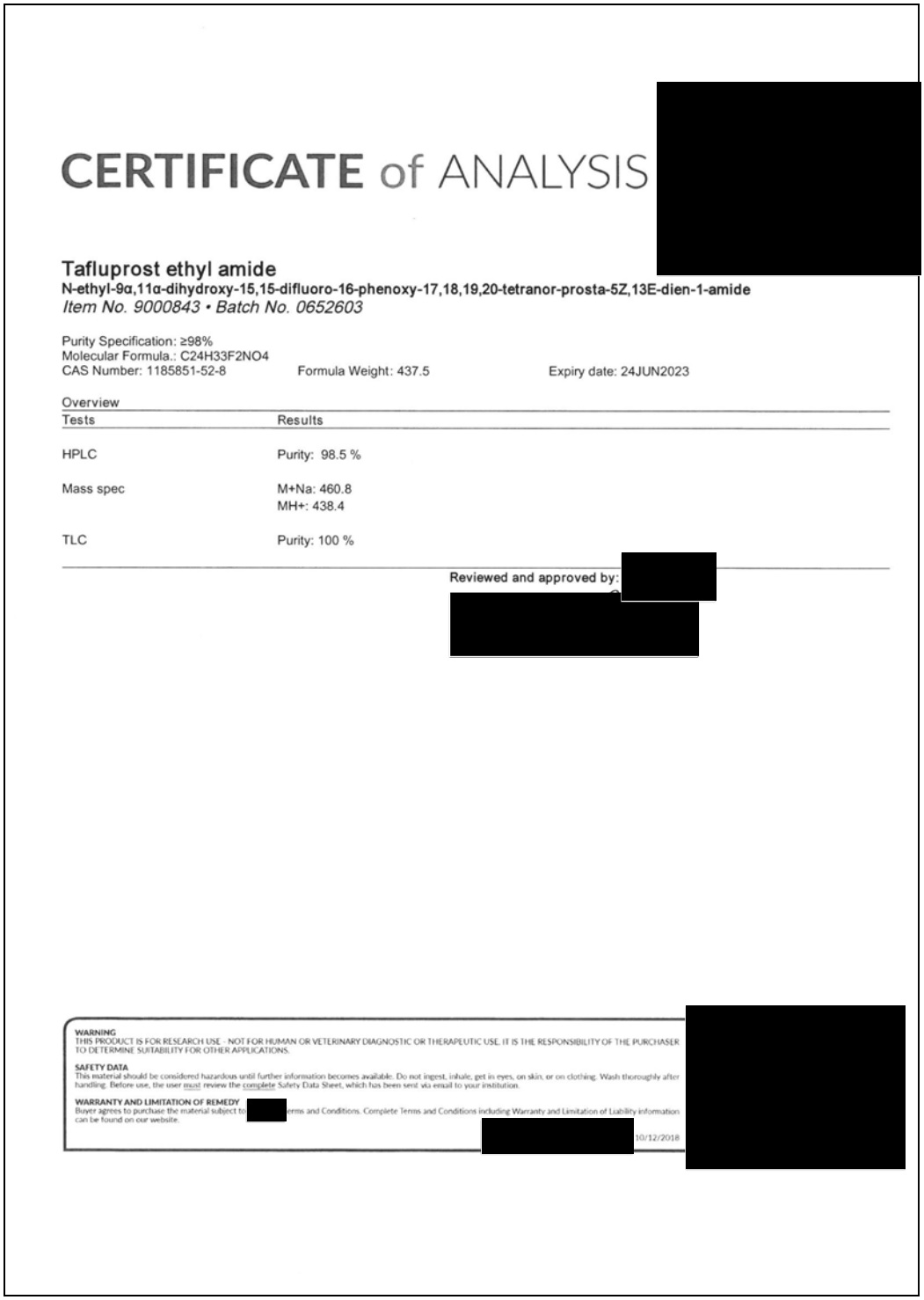
in compliance with the Principles of GLP (Good Laboratory Practice) and also complies with the corresponding OECD/European Community requirements.
This certificate is valid till 20th of April, 2025.

Date of the inspection: **11-14 and 19-20 April, 2022.**

El Koulali Zakariás
Deputy Director General

APPENDIX II

COPY OF THE CERTIFICATE OF ANALYSIS OF THE TEST ITEM



CERTIFICATE of ANALYSIS [redacted]

Tafluprost ethyl amide
N-ethyl-9α,11α-dihydroxy-15,15-difluoro-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide
Item No. 9000843 • Batch No. 0652603

Purity Specification: ≥98%
Molecular Formula.: C24H33F2NO4
CAS Number: 1185851-52-8 Formula Weight: 437.5 Expiry date: 24JUN2023

Overview

Tests	Results
HPLC	Purity: 98.5 %
Mass spec	M+Na: 460.8 MH+: 438.4
TLC	Purity: 100 %

Reviewed and approved by: [redacted]

WARNING
THIS PRODUCT IS FOR RESEARCH USE - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE. IT IS THE RESPONSIBILITY OF THE PURCHASER TO DETERMINE SUITABILITY FOR OTHER APPLICATIONS.

SAFETY DATA
This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent via email to your institution.

WARRANTY AND LIMITATION OF REMEDY
Buyer agrees to purchase the material subject to [redacted] erms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

[redacted] 10/12/2018

APPENDIX III

THE LABORATORY'S HISTORICAL CONTROL DATA RANGE

Historical control data of Trans-Cinnamaldehyde (PC)			
	$EC_{1.5}$ tartomány / $EC_{1.5}$ value range (µM)		
2018	1	-	40
2019	5	-	24
2020	7	-	17
2021	12	-	21
2022/I. (half-year)	16	-	25
Átlag/Average	8	-	25
Szórás/SD	6	-	9
Elfogadási tartomány / Acceptance range	1	-	40

APPENDIX IV

MEASURED VALUES FOR THE TEST ITEM AND CONTROLS

First test: 2022. 10. 24 – 2022. 10. 27.

Measured luminescence values for the test item 1st run: 2022.10.27

Concentration (µM)		Test item											
		0.98	**1.95**	**3.91**	**7.81**	**15.63**	**31.25**	**62.50**	**125.00**	**250.00**	**500.00**	**1000.00**	**2000.00**
Plate ID	20221024-0927-2	1093000	1035000	1103000	1090000	1248000	1136000	1465000	1491000	1788000	4038	334.2	79.07
	20221024-0927-3	999600	1405000	1043000	1199000	1147000	1493000	1283000	1178000	1470000	4231	348.3	91.48
	20221024-0927-4	903400	1171000	973200	1083000	854300	1234000	1277000	1034000	2072000	4664	309.7	93.73

Measured luminescence values for the controls

Concentration (µM)		DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	Trans-Cinnamaldehyde					blank
								4	**8**	**16**	**32**	**64**	
Plate ID	20221024-0927-2	1231000	1183000	1191000	1103000	1283000	1116000	1448000	1671000	2290000	2485000	8217000	4017
	20221024-0927-3	1230000	1183000	1146000	1287000	1176000	1148000	1129000	1533000	1659000	2418000	6903000	3408
	20221024-0927-4	1048000	1380000	1269000	1028000	1302000	1303000	1207000	1566000	1937000	2750000	7897000	3966

Measured absorbance values

Concentration (µM)		Test item											
		0.98	**1.95**	**3.91**	**7.81**	**15.63**	**31.25**	**62.50**	**125.00**	**250.00**	**500.00**	**1000.00**	**2000.00**
Plate ID	20221024-0927-1	0.9114	0.8641	0.8875	0.8571	0.8568	0.8857	0.8752	1.0823	0.8559	0.0404	0.0400	0.0418

Concentration (µM)		DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	Trans-Cinnamaldehyde					blank
								4	**8**	**16**	**32**	**64**	
Plate ID	20221024-0927-1	0.9577	0.9068	0.8991	0.9254	0.8698	0.8861	0.9565	0.9977	1.1113	1.1912	1.0891	0.0396

Individual and average fold induction, significance and viability 1st run: 2022.10.27

		Test item											
Concentration (µM)		**0.98**	**1.95**	**3.91**	**7.81**	**15.63**	**31.25**	**62.50**	**125.00**	**250.00**	**500.00**	**1000.00**	**2000.00**
Plate ID	20221024-0927-2	0.92	0.87	0.93	0.92	1.05	0.96	1.24	1.26	1.51	0.00	0.00	0.00
	20221024-0927-3	0.84	1.18	0.87	1.00	0.96	1.25	1.07	0.99	1.23	0.00	0.00	0.00
	20221024-0927-4	0.74	0.96	0.80	0.89	0.70	1.01	1.05	0.85	1.70	0.00	0.00	0.00
average induction		**0.83**	**1.00**	**0.87**	**0.94**	**0.90**	**1.07**	**1.12**	**1.03**	**1.48**	**0.00**	**0.00**	**0.00**
significance		0.023	0.977	0.015	0.123	0.379	0.461	0.087	0.814	0.030	0.000	0.000	0.000
viability		100%	95%	98%	94%	94%	97%	96%	120%	94%	0%	0%	0%

		Trans-Cinnamaldehyde				
Concentration (µM)		**4**	**8**	**16**	**32**	**64**
Plate ID	20221024-0927-2	1.22	1.41	1.94	2.10	6.96
	20221024-0927-3	0.94	1.28	1.39	2.03	5.79
	20221024-0927-4	0.99	1.28	1.59	2.26	6.48
average induction		**1.05**	**1.33**	**1.64**	**2.13**	**6.41**
significance		0.563	0.001	0.014	0.000	0.000
viability		106%	110%	123%	133%	121%

Average DMSO luminescence values for each plate	
20221024-0927-2	1184500
20221024-0927-3	1195000
20221024-0927-4	1221667

Average DMSO absorbance value	0.907

Significant induction above the threshold of 1.5-fold

Test item	Concentration (µM)	0.98	1.95	3.91	7.81	15.63	31.25	62.50	125.00	250.00	500.00	1000.00	2000.00
	Induction	-	-	-	-	-	-	-	-	-	-	-	-

Positive control	Concentration (µM)	**4**	**8**	**16**	**32**	**64**
	Induction	-	-	1.64	2.13	6.41

CV % for DMSO	7.8%

Second test: 2022. 11. 01. – 2022. 11. 04.

Measured luminescence values for the test item 2nd run: 2022.11.04

		Test item											
Concentration (µM)		**55.80**	**72.54**	**94.30**	**122.59**	**159.37**	**207.18**	**269.33**	**350.13**	**455.17**	**591.72**	**769.23**	**1000.00**
	20221101-0914-2	1051000	1180000	1093000	965400	1070000	927500	1325000	10000	4601	1250	171.1	48.18
Plate ID	20221101-0914-3	1286000	1166000	1203000	1030000	978900	1099000	1191000	22410	6051	1537	219.2	82.68
	20221101-0914-4	1102000	1247000	1106000	991500	1133000	1042000	1452000	12930	7309	2253	260.0	76.00

Measured luminescence values for the controls

								Trans-Cinnamaldehyde					
Concentration (µM)		**DMSO**	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**4**	**8**	**16**	**32**	**64**	**blank**
	20221101-0914-2	910500	1076000	885500	931000	953800	888700	1047000	1572000	1539000	2535000	29030000	13420
Plate ID	20221101-0914-3	1158000	1255000	1151000	1165000	1077000	1257000	1422000	1779000	2160000	2527000	30920000	13650
	20221101-0914-4	1161000	1149000	1272000	1358000	1194000	1261000	1390000	1792000	2227000	3178000	21400000	10330

Measured absorbance values

		Test item											
Concentration (µM)		**55.80**	**72.54**	**94.30**	**122.59**	**159.37**	**207.18**	**269.33**	**350.13**	**455.17**	**591.72**	**769.23**	**1000.00**
Plate ID	20221101-0914-1	0.9097	0.9054	0.9491	1.0324	0.9941	0.9258	0.6141	0.0470	0.0411	0.0407	0.0395	0.0398

								Trans-Cinnamaldehyde					
Concentration (µM)		**DMSO**	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**4**	**8**	**16**	**32**	**64**	**blank**
Plate ID	20221101-0914-1	0.8813	0.7817	0.7986	0.7912	0.7735	0.8072	0.8919	0.9055	0.9764	1.0135	0.8317	0.0395

Individual and average fold induction, significance and viability 2nd run: 2022.11.04

Concentration (µM)		**Test item**											
		55.80	**72.54**	**94.30**	**122.59**	**159.37**	**207.18**	**269.33**	**350.13**	**455.17**	**591.72**	**769.23**	**1000.00**
Plate ID	20221101-0914-2	1.12	1.26	1.16	1.03	1.14	0.99	1.41	0.00	-0.01	-0.01	-0.01	-0.01
	20221101-0914-3	1.09	0.99	1.02	0.87	0.83	0.93	1.01	0.01	-0.01	-0.01	-0.01	-0.01
	20221101-0914-4	0.89	1.01	0.90	0.80	0.92	0.84	1.18	0.00	0.00	-0.01	-0.01	-0.01
average induction		**1.04**	**1.09**	**1.03**	**0.90**	**0.96**	**0.92**	**1.20**	**0.00**	**-0.01**	**-0.01**	**-0.01**	**-0.01**
significance		0.809	0.433	0.867	0.255	0.604	0.411	0.153	0.000	0.000	0.000	0.000	0.000
viability		114%	113%	119%	130%	125%	116%	75%	1%	0%	0%	0%	0%

Concentration (µM)		**Trans-Cinnamaldehyde**				
		4	**8**	**16**	**32**	**64**
Plate ID	20221101-0914-2	1.11	1.68	1.64	2.72	31.28
	20221101-0914-3	1.21	1.52	1.84	2.16	26.56
	20221101-0914-4	1.13	1.46	1.81	2.59	17.50
average induction		**1.15**	**1.55**	**1.77**	**2.49**	**25.12**
significance		0.321	0.006	0.022	0.002	0.001
viability		111%	113%	122%	127%	103%

Average DMSO luminescence values for each plate	
20221101-0914-2	940917
20221101-0914-3	1177167
20221101-0914-4	1232500

Average DMSO absorbance value	0.806

Significant induction above the threshold of 1.5-fold

Test item	Concentration (µM)	55.80	72.54	94.30	122.59	159.37	207.18	269.33	350.13	455.17	591.72	769.23	1000.00
	Induction	-	-	-	-	-	-	-	-	-	-	-	-

Positive control	Concentration (µM)	**4**	**8**	**16**	**32**	**64**
	Induction	-	1.55	1.77	2.49	25.12

CV % for DMSO	13.2%

Third test: 2022. 11. 07. – 2022. 11. 10.

Measured luminescence values for the test item 3rd run: 2022.11.10

		Test item											
Concentration (µM)		**67.29**	**80.75**	**96.90**	**116.28**	**139.54**	**167.45**	**200.94**	**241.13**	**289.35**	**347.22**	**416.67**	**500.00**
	20221107-0925-2	637000	719600	787600	596300	627700	522400	689200	754500	505500	15560	115.1	49.71
Plate ID	20221107-0925-3	766100	719400	765800	603000	598600	717100	628100	724200	600400	29090	942.4	59.50
	20221107-0925-4	975500	659000	808900	651900	689600	552200	672600	996600	568200	12220	333.1	43.88

Measured luminescence values for the controls

		DMSO	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**Trans-Cinnamaldehyde**					**blank**
Concentration (µM)								**4**	**8**	**16**	**32**	**64**	
	20221107-0925-2	688900	774200	682400	721600	726900	730000	890200	1108000	1037000	1479000	10270000	55.14
Plate ID	20221107-0925-3	771900	935000	733100	717100	743700	755600	849000	961100	1235000	1601000	7500000	51.20
	20221107-0925-4	722600	864200	747100	742800	879600	764300	908000	927800	1190000	1835000	10020000	50.64

Measured absorbance values

		Test item											
Concentration (µM)		**67.29**	**80.75**	**96.90**	**116.28**	**139.54**	**167.45**	**200.94**	**241.13**	**289.35**	**347.22**	**416.67**	**500.00**
Plate ID	20221107-0925-1	0.8069	0.8155	0.8008	0.8246	0.8020	0.7335	0.8167	0.6121	0.3456	0.0576	0.0438	0.0410

		DMSO	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**Trans-Cinnamaldehyde**					**blank**
Concentration (µM)								**4**	**8**	**16**	**32**	**64**	
Plate ID	20221107-0925-1	0.8184	0.8107	0.7425	0.7486	0.7508	0.8188	0.8199	0.8644	0.9071	0.8334	0.7165	0.0397

Individual and average fold induction, significance and viability 3rd run: 2022.11.10

		Test item											
Concentration (µM)		**67.29**	**80.75**	**96.90**	**116.28**	**139.54**	**167.45**	**200.94**	**241.13**	**289.35**	**347.22**	**416.67**	**500.00**
Plate ID	20221107-0925-2	0.88	1.00	1.09	0.83	0.87	0.72	0.96	1.05	0.70	0.02	0.00	0.00
	20221107-0925-3	0.99	0.93	0.99	0.78	0.77	0.92	0.81	0.93	0.77	0.04	0.00	0.00
	20221107-0925-4	1.24	0.84	1.03	0.83	0.88	0.70	0.85	1.27	0.72	0.02	0.00	0.00
average induction		**1.04**	**0.92**	**1.04**	**0.81**	**0.84**	**0.78**	**0.87**	**1.08**	**0.73**	**0.02**	**0.00**	**0.00**
significance		0.769	0.098	0.335	0.006	0.022	0.062	0.023	0.510	0.004	0.000	0.000	0.000
viability		103%	105%	103%	106%	103%	94%	105%	77%	41%	2%	1%	0%

		Trans-Cinnamaldehyde				
Concentration (µM)		**4**	**8**	**16**	**32**	**64**
Plate ID	20221107-0925-2	1.24	1.54	1.44	2.05	14.25
	20221107-0925-3	1.09	1.24	1.59	2.06	9.66
	20221107-0925-4	1.15	1.18	1.51	2.33	12.74
average induction		**1.16**	**1.32**	**1.51**	**2.15**	**12.22**
significance		0.011	0.016	0.003	0.001	0.001
viability		105%	111%	117%	107%	91%

Average DMSO luminescence values for each plate	
20221107-0925-2	720667
20221107-0925-3	776067
20221107-0925-4	786767

Average DMSO absorbance value	0.782

Significant induction above the threshold of 1.5-fold

Test item	Concentration (µM)	67.29	80.75	96.90	116.28	139.54	167.45	200.94	241.13	289.35	347.22	416.67	500.00
	Induction	-	-	-	-	-	-	-	-	-	-	-	-

Positive control	Concentration (µM)	**4**	**8**	**16**	**32**	**64**
	Induction	-	-	1.51	2.15	12.22

CV % for DMSO	8.7%

BioScreen® Testing Services, Inc.
3892 Del Amo Boulevard • Torrance, California 90503
(310) 214-0043 • Fax(310) 370-3642
Web Site: www.bioscreen.com • E-Mail: info@bioscreen.com

50 SUBJECT HUMAN REPEAT INSULT PATCH TEST FOR SKIN IRRITATION AND SKIN SENSITIZATION EVALUATION

Date: November 20, 2009

BCS Study No.: 09-110A

Sponsor: [redacted]

1.0 Objective: To determine the irritation and sensitization (contact allergy) potential of a test material after repeated application to the skin of human subjects.

2.0 Test Material:

2.1 Test Material Description:

Date Received: 9/23/2009

Received From: [redacted]

Number Of Test Samples Received: 3

Label On Test Samples: Eye Lash Conditioner TEA .025%
Enhanced Peptide, Formula #31
MFG: 9/9/09, Lot #: 090309-2

Accession No.: 635537

2.2 Handling:

Upon arrival at BioScreen Clinical Services (BCS) the test material was assigned a unique laboratory code number and entered into a daily log identifying the lot number, sample description, sponsor, date received and tests requested.

Samples will be retained for a period of thirty (30) days beyond submission of final report unless otherwise specified by the sponsor or, if sample is known to be in support of governmental applications, in which case representative retained samples are kept two (2) years beyond final report submission.

Sample disposition will be conducted in compliance with appropriate federal, state and local ordinances.

2.3 Test Material Evaluation Prerequisite:

Prior to induction of a human test panel, animal toxicology, microbiology and other in-vivo or in-vitro performance data were required to assess the feasibility of commencement.

3.0 Panel Selection:

3.1 Standards for Inclusion in a Study:

- Individuals who were not currently under a doctor's care.
- Individuals who were free of any dermatological or systemic disorder that would interfere with the results, at the discretion of the Investigator.
- Individuals who were free of any acute or chronic disease that would interfere with or increase the risk of study participation.
- Individuals who completed a preliminary medical history form mandated by BCS and were in general good health.
- Individuals who read, understood and signed an informed consent document relating to the specific type of study.
- Individuals who were able to cooperate with the Investigator and research staff, and were willing to have test materials applied according to the protocol, and complete the full course of the study.

3.2 Standards for Exclusion from a Study:

- Individuals who were under 18 years of age.
- Individuals who were currently under a doctor's care.
- Individuals who were currently taking any medication (topical or systemic) that might mask or interfere with the test results.
- Individuals who had a history of any acute or chronic disease that might interfere with or increase the risk associated with study participation.

- Individuals who were diagnosed with chronic skin allergies.
- Female volunteers who indicated that they were pregnant or nursing.

3.3 Recruitment:

Panel selection was accomplished by advertisements in local periodicals, community bulletin boards, phone solicitation, electronic media or any combination thereof.

3.4 Informed Consent and Medical History Forms:

An informed consent was obtained from each volunteer prior to initiating the study describing reasons for the study, possible adverse effects, associated risks and potential benefits of the treatment and their limits of liability. Panelists signed and dated the informed consent document to indicate their authorization to proceed and acknowledge their understanding of the contents. Each subject was assigned a permanent identification number and completed an extensive medical history form. These forms along with the signed consent forms are available for inspection on the premises of BCS only. [Reference 21 CFR Ch. 1 Part 50, Subpart B]

The parties agree to comply with applicable state and federal privacy laws for the use and disclosure of a subject's personal health information by taking reasonable steps to protect the confidentiality of this information. This obligation shall survive the termination or expiration of this Agreement.

4.0 Population Demographics:

Number of subjects enrolled	52
Number of subjects completing study	51
Age Range	18-59
Sex	
Male	3
Female	48
Fitzpatrick Skin Type*	
1 – always burn, does not tan	0
2 – burn easily, tan slightly	5

3 – burn moderately, tan progressively	39
4 – burn a little, always tan	7
5 – rarely burn, tan intensely	0
6 – never burn, tan very intensely	0

*[Agache P., Hubert P.. Measuring the skin. (p. 473, table 48.1) Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)]

5.0 Equipment:

Test materials to be tested under occlusive conditions were placed on an 8-millimeter aluminum chamber (Finn Chamber, Epitest Ltd Oy, Tuussula, Finland) supported on a sheet of Scanpore® (occlusive) tape (Norgesplaster A/S, Kristiansand, Norway), or an equivalent.

Test materials to be tested under semi-occlusive conditions were placed on Curad™ sensitive skin bandages or on a 7.5mm filter paper disc affixed to a strip of hypoallergenic tape (Johnson & Johnson 1 inch First Aid Cloth Tape).

Test materials to be tested in an open patch were applied and rubbed directly onto the back of the subject.

Approximately 0.02-0.05 mL (in case of liquids) and/or 0.02-0.05 gm (in case of solids) of the test material was used for the study. Liquid test material was dispensed on a 7.5mm paper disk, which fit in the Finn Chamber.

6.0 Procedure:

- Subjects were requested to bathe or wash as usual before arrival at the facility.
- Patches containing the test material were then affixed directly to the skin of the intrascapular regions of the back, to the right or left of the midline and subjects were dismissed with instructions not to wet or expose the test area to direct sunlight.
- Subjects were instructed to remove the patches approximately 48 hours after the first application and 24 hours thereafter for the remainder of the study.
- This procedure was repeated until a series of nine (9) consecutive, 24-hour exposures had been made three (3) times a week for three (3) consecutive weeks.
- Prior to each reapplication, the test sites were evaluated by trained laboratory personnel.

- Following a 10-14 day rest period a retest/challenge dose was applied once to a previously unexposed test site. Test sites were evaluated by trained laboratory personnel 48 and 96 hours after application.
- In the event of an adverse reaction, the area of erythema and edema were measured. Edema is estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin.
- Subjects were instructed to report any delayed reactions that might occur after the final reading.
- Clients will be notified immediately in the case of an adverse reaction and a determination is made as to treatment program if necessary.

7.0 Scoring:

Scoring scale and definition of symbols shown below are based on the scoring scheme according to the International Contact Dermatitis Research Group scoring scale [Rietschel, R.L., Fowler, J.F., Ed., Fisher's Contact Dermatitis (fourth ed.). Baltimore, Williams & Wilkins, 1995] listed below:

0 no reaction (negative)
1 erythema throughout at least ¾ of patch area
2 erythema and induration throughout at least ¾ of patch area
3 erythema, induration and vesicles
4 erythema, induration and bullae

D Site discontinued
Dc Subject discontinued

NOTE: Clinical evaluations are performed by a BCS investigator or designee trained in the clinical evaluation of the skin. Whenever feasible, the same individual will do the scoring of all the subjects throughout the study and will be blinded to the treatment assignments and any previous scores.

8.0 Results:

Accession No.: 635537
Test Material Description: Eye Lash Conditioner TEA .025% Enhanced Peptide, Formula #31 MFG: 9/9/09, Lot #: 090309-2
Patch Description: Occlusive

Subject Information					Induction									Challenge	
No.	ID	Sex	Age	Skin Type	1	2	3	4	5	6	7	8	9	1	2
1	881883	M	28	3	0	0	0	0	0	0	0	0	0	0	0
2	1182022	F	26	3	1	0	0	0	0	0	0	0	0	0	0
3	1378015	F	59	3	0	0	0	0	0	0	0	0	0	0	0
4	1448967	F	53	3	0	0	0	0	0	0	0	0	0	0	0
5	1451172	F	53	2	0	0	0	0	0	0	0	0	0	0	0
6	1467351	F	52	3	0	0	0	0	0	0	0	0	0	0	0
7	1482918	F	51	3	0	0	0	0	0	0	0	0	0	0	0
8	1503292	F	50	3	0	0	0	0	0	0	0	0	0	0	0
9	1506150	F	51	3	0	0	0	0	0	0	0	0	0	0	0
10	1558008	F	47	4	0	0	0	0	0	0	0	0	0	0	0
11	1587917	F	47	3	0	0	0	0	1	0	0	0	0	0	0
12	1624080	F	44	3	0	0	0	0	0	0	0	0	0	0	0
13	1629774	F	45	2	0	0	0	0	0	0	0	0	0	0	0
14	1661539	F	44	2	0	0	0	0	0	0	0	0	0	0	0
15	1683726	F	43	3	0	0	0	0	0	0	0	0	0	0	0
16	1702024	F	42	3	0	0	0	0	0	0	0	0	0	0	0
17	1724958	F	41	3	0	0	0	0	0	0	0	0	0	0	0
18	1757522	F	39	3	0	0	0	0	0	0	0	0	0	0	0
19	1793066	F	38	3	0	0	0	0	0	0	0	0	0	0	0
20	1793508	F	38	2	0	0	0	0	0	0	0	0	0	0	0
21	1801529	F	38	4	0	0	0	0	0	0	0	0	0	0	0
22	1834973	F	36	2	0	0	0	0	0	0	0	0	0	0	0
23	1874042	F	36	4	0	0	0	0	0	0	0	0	0	0	0
24	1970523	F	32	3	0	0	0	0	0	0	0	0	0	0	0
25	1972053	F	32	3	0	0	0	0	0	0	0	0	0	0	0
26	2538365	F	30	3	0	0	0	0	0	0	0	0	0	0	0
27	2678946	F	18	3	0	0	0	0	0	0	0	0	0	0	0
28	4144848	F	44	3	0	0	0	0	0	0	0	0	0	0	0
29	6149765	F	49	3	0	0	0	0	0	0	0	0	0	0	0
30	7091205	M	41	3	0	0	0	0	0	0	0	0	0	0	0
31	11029524	F	30	3	0	0	0	0	0	0	0	0	0	0	0
32	11033715	F	30	3	0	0	0	0	0	0	0	0	0	0	0
33	11090185	F	28	3	0	0	0	0	0	0	0	0	0	0	0

Subject Information					Induction									Challenge	
No.	ID	Sex	Age	Skin Type	1	2	3	4	5	6	7	8	9	1	2
34	11095433	M	28	3	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc
35	11111345	F	28	3	0	0	0	0	0	0	0	0	0	0	0
36	11134872	F	27	3	0	0	0	0	0	0	0	0	0	0	0
37	11358160	F	29	4	0	0	0	0	0	0	0	0	0	0	0
38	11377591	F	20	3	0	0	0	0	0	0	0	0	0	0	0
39	11400240	M	19	3	0	0	0	0	0	0	0	0	0	0	0
40	11482852	F	18	3	0	0	0	0	0	0	0	0	0	0	0
41	11655152	F	44	4	0	0	0	0	0	0	0	0	0	0	0
42	11770926	F	39	3	0	0	0	0	0	0	0	0	0	0	0
43	12310527	F	24	3	0	0	0	0	0	0	0	0	0	0	0
44	13770953	F	20	3	0	0	0	0	0	0	0	0	0	0	0
45	109710080	F	32	3	0	0	0	0	0	0	0	0	0	0	0
46	110660492	F	29	4	0	0	0	0	0	0	0	0	0	0	0
47	112420975	F	24	4	0	0	0	0	0	0	0	1	0	0	0
48	113060306	F	22	3	0	0	0	0	0	0	0	0	0	0	0
49	113070844	F	22	3	0	0	0	0	0	0	0	0	0	0	0
50	113100107	F	22	3	0	0	0	0	0	0	0	0	0	0	0
51	800800039	F	33	3	0	0	0	0	0	0	0	0	0	0	0
52	800890684	F	54	3	0	0	0	0	0	0	0	0	0	0	0

9.0 Evaluation Period:

The study was conducted from October 12, 2009 to November 13, 2009.

10.0 Observations:

No adverse reactions of any kind were reported during the course of this study.

11.0 Study Archives:

All original samples, raw data sheets, technician's notebooks, correspondence files and copies of final reports and remaining specimens will be maintained on premises of BCS in limited access storage files marked "Archive".

12.0 Conclusions:

Under conditions of the study, there were no identifiable signs or symptoms of sensitization (contact allergy) noted for Eye Lash Conditioner TEA .025% Enhanced Peptide, Formula #31 MFG: 9/9/09, Lot #: 090309-2; Accession No. 635537.

[redacted] M.D.
Consulting Dermatologist

Hemali B. Gunt, Ph.D.
Clinical Manager

Mallyc Murray
Quality Assurance Supervisor

ALS
3904 Del Amo Boulevard #801
Torrance, CA 90503
(310) 214-0043
www.alsglobal.com www.bioscreen.com
info.bioscreen@alsglobal.com

50 SUBJECT HUMAN REPEAT INSULT PATCH TEST FOR SKIN IRRITATION AND SKIN SENSITIZATION EVALUATION

Date: December 1, 2022

Study No.: 22-525A

Sponsor: [redacted]

1.0 Objective: To determine the irritation and sensitization (contact allergy) potential of a test material after repeated application to the skin of human subjects.

2.0 Test Material:

2.1 Test Material Description:

Date Received: September 30, 2022

Received From: [redacted]

Number Of Test Samples Received: 3

Label On Test Samples: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide; Lot/Batch Number: TAF-F-0522-01

Accession No.: 1200670

2.2 Handling:

Upon arrival at ALS Pharmaceutical, Beauty, and Personal Care the test material was assigned a unique laboratory code number and entered into a daily log identifying the lot number, sample description, sponsor, date received and tests requested.

Samples will be retained for a period of thirty (30) days beyond submission of final report unless otherwise specified

by the sponsor or, if sample is known to be in support of governmental applications, in which case representative retained samples are kept two (2) years beyond final report submission.

Sample disposition will be conducted in compliance with appropriate federal, state and local ordinances.

3.0 Panel Selection:

3.1 Standards for Inclusion in a Study:

- Individuals who were not currently under a doctor's care.
- Individuals who were free of any dermatological or systemic disorder that would interfere with the results, at the discretion of the Investigator.
- Individuals who were free of any acute or chronic disease that would interfere with or increase the risk of study participation.
- Individuals who completed a preliminary medical history form mandated by ALS and were in general good health.
- Individuals who read, understood and signed an informed consent document relating to the specific type of study.
- Individuals who were able to cooperate with the Investigator and research staff, and were willing to have test materials applied according to the protocol, and complete the full course of the study.

3.2 Standards for Exclusion from a Study:

- Individuals who were under 18 years of age.
- Individuals who were currently under a doctor's care.
- Individuals who were currently taking any medication (topical or systemic) that might mask or interfere with the test results.
- Individuals who had a history of any acute or chronic disease that might interfere with or increase the risk associated with study participation.
- Individuals who were diagnosed with chronic skin allergies.
- Female volunteers who indicated that they were pregnant or nursing.

3.3 Recruitment:

Panel selection was accomplished by advertisements in local periodicals, community bulletin boards, phone solicitation, electronic media or any combination thereof

3.4 Informed Consent and Medical History Forms:

An informed consent was obtained from each volunteer prior to initiating the study describing reasons for the study, possible adverse effects, associated risks and potential benefits of the treatment and their limits of liability. Panelists signed and dated the informed consent document to indicate their authorization to proceed and acknowledge their understanding of the contents. Each subject was assigned a permanent identification number and completed an extensive medical history form. These forms along with the signed consent forms are available for inspection on the premises of ALS only. [Reference 21 CFR Ch. 1 Part 50, Subpart B]

The parties agree to comply with applicable state and federal privacy laws for the use and disclosure of a subject's personal health information by taking reasonable steps to protect the confidentiality of this information. This obligation shall survive the termination or expiration of this Agreement.

4.0 Population Demographics:

Number of subjects enrolled	55
Number of subjects completing study	54
Age Range	18-64
Sex	
Male	12
Female	42
Fitzpatrick Skin Type*	
1 – always burn, does not tan	0
2 – burn easily, tan slightly	10
3 – burn moderately, tan progressively	23
4 – burn a little, always tan	17
5 – rarely burn, tan intensely	4
6 – never burn, tan very intensely	0

*[Agache P., Hubert P.. Measuring the skin. (p. 473, table 48.1) Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)]

5.0 Equipment:

Test materials to be tested under occlusive conditions were placed on an adhesive tape with paper filter discs with 1.0 cm2 (Adhesive tapes from 3M Company – Durapore (Code 1538) and Blenderm (Code 1525) or placed on an 8-millimeter aluminum Finn Chamber® (Epitest Ltd. Oy, Tuusula, Finland) supported on Scanpor® Tape (Norgesplaster A/S, Kristiansand, Norway) or an 8-millimeter filter paper coated aluminum Finn Chamber® AQUA supported on a thin flexible transparent polyurethane rectangular film coated on one side with a medical grade acrylic adhesive, consistent with adhesive used in state-of-the-art hypoallergenic surgical tapes or a 7mm IQ-ULTRA® closed cell system which is made of additive-free polyethylene plastic foam with a filter paper incorporated (It is supplied in units of 10 chambers on a hypoallergenic non-woven adhesive tape; the width of the tape is 52mm and the length is 118mm) or other equivalents.

Test materials to be tested under semi-occlusive conditions were placed on an adhesive tape with paper filter discs with 1.0 cm2 (Adhesive tapes from 3M Company – Durapore (Code 1538) or placed on a test strip with a Rayon/Polypropylene pad or on a 7.5mm filter paper disc affixed to a strip of hypoallergenic tape (Johnson & Johnson 1 inch First Aid Cloth Tape).

Test materials to be tested in an open patch were applied and rubbed directly onto the back of the subject.

Approximately 0.02-0.05 mL (in case of liquids) and/or 0.02-0.05 gm (in case of solids) of the test material was used for the study. Liquid test material was dispensed on a paper disk, which fit in the patch chamber.

6.0 Procedure:

- Subjects were requested to bathe or wash as usual before arrival at the facility.
- Patches containing the test material were then affixed directly to the skin of the intrascapular regions of the back, to the right or left of the midline and subjects were dismissed with instructions not to wet or expose the test area to direct sunlight.
- Approximately 1-3% sodium lauryl sulfate in distilled water will be applied as a positive control and approximately 0.02-0.05 mL of distilled water will be applied as a negative control. These will be applied on an occlusive patch the first induction only.
- Patches remained in place for 48 hours after the first application. Subjects were instructed not to remove the patches prior to their 48 hour scheduled visit. Thereafter, subjects were instructed to remove patches 24 hours after application for the remainder of the study.
- This procedure was repeated until a series of eight (8) to nine (9) consecutive, 24-hour exposures had been made three (3) times a week for three (3) consecutive weeks.
- Prior to each reapplication, the test sites evaluated by trained laboratory personnel.
- Following a 10-14 day rest period a retest/challenge dose was applied once to a previously unexposed test site. Test sites were evaluated by trained laboratory personnel 48 and 96 hours after application.
- In the event of a reaction, the area of erythema and edema were measured. Edema is estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin.
- Subjects were instructed to report any delayed reactions that might occur after the final reading.
- Clients will be notified immediately in the case of an adverse reaction and a determination is made as to treatment program if necessary.

7.0 Scoring:

Scoring scale and definition of symbols shown below are based on the scoring scheme according to the International Contact Dermatitis Research Group scoring scale [Rietschel, R.L., Fowler, J.F., Ed., Fisher's Contact Dermatitis (fourth ed.). Baltimore, Williams & Wilkins, 1995] listed below:

0 no reaction (negative)
1 erythema throughout at least ¾ of patch area
2 erythema and induration throughout at least ¾ of patch area
3 erythema, induration and vesicles
4 erythema, induration and bullae

D Site discontinued
Dc Subject discontinued voluntarily
DcI Subject discontinued per Investigator

NOTE: Clinical evaluations are performed by an ALS investigator or designee trained in the clinical evaluation of the skin. Whenever feasible, the same individual will do the scoring of all the subjects throughout the study and will be blinded to the treatment assignments and any previous scores.

8.0 Results:

Accession No.: 1200670
Test Material Description: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide; Lot/Batch Number: TAF-F-0522-01
Patch Description: Semi-Occlusive (Diluted to 0.267% in DI Water)

Subject Information					Induction									Challenge	
No.	Subject ID	Sex	Age	Skin Type	1	2	3	4	5	6	7	8	9	1	2
1	3000202	M	20	3	0	0	0	0	0	0	0	0	0	0	0
2	3000209	F	26	3	0	0	0	0	0	0	0	0	0	0	0
3	3000250	F	20	3	0	0	0	0	0	0	0	0	0	0	0
4	3000336	F	38	2	0	0	0	0	0	0	0	0	0	0	0
5	3000377	F	40	4	0	0	0	0	0	0	0	0	0	0	0
6	3000539	F	38	3	0	0	0	0	0	0	0	0	0	0	0
7	3002078	F	44	3	0	0	0	0	0	0	0	0	0	0	0
8	3002105	F	28	3	0	0	0	0	0	0	0	0	0	0	0
9	3005246	F	24	2	0	0	0	0	0	0	0	0	0	0	0
10	3007313	F	44	3	0	0	0	0	0	0	0	0	0	0	0
11	3008310	F	29	4	0	0	0	0	0	0	0	0	0	0	0
12	3009361	F	56	3	0	0	0	0	0	0	0	0	0	0	0
13	3009455	F	62	3	0	0	0	0	0	0	0	0	0	0	0
14	3010616	F	38	4	0	0	0	0	0	0	0	0	0	0	0
15	3011821	F	35	4	0	0	0	0	0	0	0	0	0	0	0
16	3011850	M	49	5	0	0	0	0	0	0	0	0	0	0	0
17	3020110	F	33	3	0	0	0	0	0	0	0	0	0	0	0
18	3021209	F	52	2	0	0	0	0	0	0	0	0	0	0	0
19	3021213	F	48	3	0	0	0	0	0	0	0	0	0	0	0
20	3022428	F	55	3	0	0	0	0	0	0	0	0	0	0	0
21	3022453	M	26	2	0	0	0	0	0	0	0	0	0	0	0
22	3022615	F	27	2	0	0	0	0	0	0	0	0	Dc	Dc	Dc
23	3022776	F	54	4	0	0	0	0	0	0	0	0	0	0	0
24	3022781	M	61	3	0	0	0	0	0	0	0	0	0	0	0
25	3022782	F	34	4	0	0	0	0	0	0	0	0	0	0	0
26	3023168	F	34	3	0	0	0	0	0	0	0	0	0	0	0
27	3023462	F	23	4	0	0	0	0	0	0	0	0	0	0	0
28	3023496	M	64	4	0	0	0	0	0	0	0	0	0	0	0
29	3023541	F	40	3	0	0	0	0	0	0	0	0	0	0	0
30	3023588	M	19	4	0	0	0	0	0	0	0	0	0	0	0
31	3023796	F	29	3	0	0	0	0	0	0	0	0	0	0	0
32	3023797	F	44	2	0	0	0	0	0	0	0	0	0	0	0
33	3023865	F	32	5	0	0	0	0	0	0	0	0	0	0	0

34	3023866	F	43	4	0	0	0	0	0	0	0	0	0	0	0
35	3023877	M	51	4	0	0	0	0	0	0	0	0	0	0	0
36	3023945	F	39	4	0	0	0	0	0	0	0	0	0	0	0
37	3023984	F	18	2	0	0	0	0	0	0	0	0	0	0	0
38	3023987	F	32	4	0	0	0	0	0	0	0	0	0	0	0
39	3023998	F	48	2	0	0	0	0	0	0	0	0	0	0	0
40	3024143	F	48	3	0	0	0	0	0	0	0	0	0	0	0
41	3024144	F	49	3	0	0	0	0	0	0	0	0	0	0	0
42	3024145	F	54	3	0	0	0	0	0	0	0	0	0	0	0
43	3024146	M	30	2	0	0	0	0	0	0	0	0	0	0	0
44	3024147	F	41	3	0	0	0	0	0	0	0	0	0	0	0
45	3024148	M	26	4	0	0	0	0	0	0	0	0	0	0	0
46	3024149	F	21	2	0	0	0	0	0	0	0	0	0	0	0
47	3024150	F	45	4	0	0	0	0	0	0	0	0	0	0	0
48	3024151	M	52	4	0	0	0	0	0	0	0	0	0	0	0
49	3024152	F	56	3	0	0	0	0	0	0	0	0	0	0	0
50	3024153	M	47	5	0	0	0	0	0	0	0	0	0	0	0
51	3024154	F	37	3	0	0	0	0	0	0	0	0	0	0	0
52	3024155	M	57	5	0	0	0	0	0	0	0	0	0	0	0
53	3024156	F	32	4	0	0	0	0	0	0	0	0	0	0	0
54	3024157	F	35	3	0	0	0	0	0	0	0	0	0	0	0
55	3024158	F	18	2	0	0	0	0	0	0	0	0	0	0	0

9.0 Evaluation Period:

The study was conducted from October 17, 2022 to November 25, 2022.

10.0 Observations:

No adverse reactions of any kind were reported during the course of this study.

There were one (1) subject with a Grade 2 reaction and eighteen (18) subjects with a Grade 1 reaction to the positive control (1.0% Sodium Lauryl Sulfate Solution).

No subjects showed any signs of reaction to the negative control (DI Water).

11.0 Study Archives:

All original samples, raw data sheets, technician's notebooks, correspondence files and copies of final reports and remaining specimens will be maintained on premises of ALS in limited access storage files marked "Archive".

12.0 Conclusions:

The test product was dermatologist tested and under the conditions of the study, there was no indication of a potential to elicit dermal irritation or sensitization (contact allergy) noted for Dechloro Dihydroxy Difluoro Ethylcloprostenolamide; Lot/Batch Number: TAF-F-0522-01. Accession No. 1200670.

[redacted]
[redacted], MD
Consulting Dermatologist

Ashley Glavis
Clinical Supervisor

Steve Park
Clinical Quality Assurance Lead

Final Report

Original 1 of 1

Determination of Eye Hazard Potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) using the EpiOcular™ Reconstructed human Cornea-like Epithelium (RhCE) Test Method following OECD 492

Study No.: 22120103G891

Sponsor:

[redacted]

Monitor:

ToxMinds BVBA
Dr. Thomas Petry
Avenue de Broqueville, 116
1200 Brussels
Belgium

Test Facility:

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler
Germany

Study Director:

Diana Brandt

1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following documents:

- OECD Principles of Good Laboratory Practice and Compliance Monitoring, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998 and subsequent advisory/consensus OECD GLP documents (where appropriate).
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemicals Act of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 from 28. Aug. 2013, published in Federal Law Gazette, Germany (BGBl) No. 55/2013 as of 06. Sep. 2013, and further revisions.

Responsibility for the accuracy of the information concerning the test item as well as for its authenticity rests with the sponsor.

I herewith accept responsibility for the data presented within this report.
There were no circumstances that may have affected the quality or integrity of the study.

This report contains the following parameter which was not performed under GLP conditions: Determination of the proficiency chemicals and the pre-tests.

Diana Brandt
Study Director

19 JUN 2023
Date

Information on Study Organisation:

Study Director	Diana Brandt
Deputy Study Director	Caroline Przewalla
Study Plan dated	14. Feb. 2023
Experimental Starting Date	08. Mar. 2023
Experimental Completion Date	09. Mar. 2023

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice.

All phases of the study (Study plan, performance of the study and Final report) were checked by the quality assurance. Dates of inspections are given below. Findings are reported to the Study Director and Test Facility Management.

The inspection of the performance of short-term studies (duration less than four weeks) may be carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the Final report was written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study plan (if any) were acknowledged and assessed by the Study Director and included in the Final report.

The reported results reflect the raw data of the study.

Phases of Study	Inspected on	Findings reported on	Audit report no.
Study plan	06. Feb. 2023	06. Feb. 2023	230206-14
Study plan Amendment No. 1	06. Apr. 2023	06. Apr. 2023	230406-03
Study plan Amendment No. 2	14. Jun. 2023	14. Jun. 2023	230614-03
Performance of study (audit of process to studies 22111101G891, 22112110G891 and 22121502G891)	18. Jan. 2023 19. Jan. 2023 02. Mar. 2023	18. Jan. 2023 19. Jan. 2023 02. Mar. 2023	230118-03 230119-10 230302-06
Final report	05. Jun. 2023	05. Jun. 2023	230605-20

Schedler

Dr. Anette Schedler
Quality Assurance

19 JUN 2023

Date

Table of Contents

3 SUMMARY

Title of Study: Determination of Eye Hazard Potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) using the EpiOcular™ Reconstructed human Cornea-like Epithelium (RhCE) test method following OECD 492

Findings and Results:

This study was conducted to determine the eye irritation potential of the test item, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil), using the *in vitro* EpiOcular™ reconstructed human cornea-like epithelium (RhCE) assay according to OECD Guideline 492, in compliance with GLP. One valid experiment was performed.

The test item was applied to a three-dimensional human cornea tissue model in duplicate for an exposure time of 28 minutes. Sterile demineralised water was used as negative control and methyl acetate was used as positive control.

The respective substances were rinsed from the tissue after treatment. Cell viability of the tissues was then evaluated by addition of MTT, which can be reduced to formazan. The formazan production was evaluated by measuring the optical density (OD) of the resulting solution.

After treatment with the negative control, the absorbance values were within the required acceptability criterion of mean OD > 0.8 and < 2.8, OD was 1.927. The positive control showed clear eye irritating effects and the mean value of the relative tissue viability was 22.6 % (required: < 50 %).

The difference within tissue replicates of the controls and the test item was acceptable (< 20 %).

After treatment with the test item, the mean value of relative tissue viability was 98.8 %.

This value is above the threshold for eye irritation potential (≤ 60 %). Test items that induce values above the threshold are considered non-eye irritant.

Under the conditions of the test, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) is considered non-eye irritant (GHS No Category) in the *in vitro* EpiOcular™ Eye Irritation Test.

4 PURPOSE AND PRINCIPLE OF THE STUDY

This *in vitro* study was performed in order to evaluate the potential of Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) to evoke eye irritation or serious eye damage in a Reconstructed human Cornea-like Epithelium (RhCE) model (e. g. EpiOcular™ Eye Irritation Test).

The EpiOcular™ Eye Irritation Test (EIT) predicts the acute eye hazard potential of chemicals by measurement of tissue damage caused by cytotoxic effects in the reconstructed human cornea-like tissue model. Within a testing strategy, the EpiOcular™ EIT can be used as a replacement of the *in vivo* Draize Eye Irritation Test.

It is utilized for the classification and labelling of chemicals concerning their eye hazard potential. The EpiOcular™ EIT can be used to identify chemicals that do not require classification for eye irritation or serious eye damage according to the UN GHS classification system. A limitation of the guideline is that it neither allows discrimination between eye irritation/reversible effects on the eye (Category 2) and serious eye damage/irreversible effects on the eye (Category 1), nor between eye irritants (optional Category 2A) and mild eye irritants (optional Category 2B). For these purposes, further testing with other suitable test methods is required.

The liquid test item was applied topically to a three-dimensional RhCE tissue construct in duplicate for an exposure time of 30 ± 2 minutes.

Eye hazard materials are identified by their ability to produce a decrease in cell viability, measured by dehydrogenase conversion of MTT (3-(4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazolium bromide), present in cell mitochondria, into a blue formazan salt. The formazan is quantitatively measured after extraction from tissues. The percentage reduction of cell viability in comparison with untreated negative controls is used to predict the eye hazard potential.

5 LITERATURE

The study was conducted in compliance with the following guideline:

- OECD Guideline for the Testing of Chemicals, Part 492, adopted 18. Jun. 2019, “Reconstructed Human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye Irritation or serious eye damage”

Corresponding SOP of LAUS GmbH:

- SOP 118 00 891, edition 5 valid from 22. Aug. 2022, “Bestimmung der Augenreizung mit dem EpiOcular™ Eye Irritation Test (EIT)“

Additional literature:

- MatTek Protocol: EpiOcular™ Eye Irritation Test (OCL-200-EIT) for the prediction of acute ocular irritation of chemicals, for use with MatTek Corporation’s Reconstructed Human EpiOcular™ Model, 04. Aug. 2022
- Stern M., Klausner M., Alvarado R., Renskers K., Dickens M., 1998. “Evaluation of the EpiOcular Tissue Model as an Alternative to the Draize Eye Irritation Test”. Toxicology *in Vitro* 12, 455-461

6 MATERIALS AND METHODS

6.1 Test Item

Designation in Test Facility:	22120103G
Date of Receipt:	01. Dec. 2022
Condition at Receipt:	cooled, in proper conditions

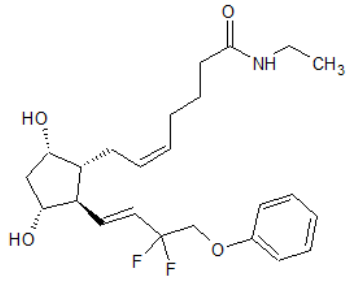
6.1.1 Specification

The following information concerning identity and composition of the test item was provided by the sponsor.

Name	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Batch no.	TAF-10-1122-01
CAS no.	1185851-52-8
Composition	Dechloro Dihydroxy Difluoro Ethylcloprostenolamide
Storage	fridge (2 - 8 °C); keep under inert gas
Expiry date	23. Nov. 2026
Stability	stable under storage conditions
Appearance	clear, colorless to light yellow liquid
Purity	99.78 %
Homogeneity	homogeneous
Production date	18. Nov. 2022
EC no.	867-521-0
Molecular formula	$C_{24}H_{33}F_2NO_4$
Molecular weight	437.52 g/mol
Vapour pressure	unknown
Solubility in solvents	water: not stated; ethanol: >1g/L; acetone: not stated; acetonitril: not stated; DMSO: >1g/L; methanol: >1g/L; DMF: 0.1-1g/L
Stability in solvents	water: not stated; ethanol: not stated; acetone: not stated; acetonitrile: not stated; DMSO: not stated; methanol: not stated; DMF: not stated

A certificate of analysis was provided by the sponsor and is attached (in copy) in annex 5, chapter 17.

6.1.2 Structural Formula



O[C@@H]2C[C@H](O)[C@H](C/C=C\CCCC(=O)NCC)[C@H]2/C=C/C(F)(F)COc1ccccc1

6.1.3 Storage in Test Facility

The test item was stored in a closed vessel in a fridge (2 – 8 °C), kept under inert gas.

6.2 Test System

6.2.1 Specification

The test system was a commercially available EpiOcular™ tissue kit (e. g. OCL-200-EIT / OCL-212-EIT), procured by MatTek In Vitro Life Science Laboratories.

The EpiOcular™ tissue consists of primary human-derived keratinocytes, which have been cultured to form a stratified squamous epithelium similar to that found in the human cornea. It consists of highly organized basal cells. These cells are not transformed or transfected with genes to induce an extended life span. The EpiOcular™ tissues are cultured in specially prepared cell culture inserts with a porous membrane through which nutrients can pass to the cells. The tissue surface is 0.6 cm^2.

6.2.2 Origin

EpiOcular™ tissues were procured from MatTek In Vitro Life Science Laboratories, Mlynské Nivy 73, 82105 Bratislava, Slovakia and used for this study.

Designation of the kit:	OCL-200-EACH
Day of delivery:	07. Mar. 2023
Batch no.:	38509

6.3 Controls

6.3.1 Negative Control

Sterile demineralised water, prepared by LAUS GmbH using an ion exchanger and membrane filtration through sterile filters, batch no.: T20230102.

6.3.2 Positive Control

Methyl acetate ($C_3H_6O_2$, CAS No. 79-20-9), portioned and delivered from MatTek In Vitro Life Science Laboratories, batch no.: 010323MSA.

6.4 Solutions and Media

6.4.1 MTT-Solution

Contained 1 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (=MTT), which can be reduced to a blue formazan salt/dye and was prepared by LAUS GmbH.

A MTT stock solution of 5 mg/mL in DPBS was prepared and stored in aliquots of 2 mL in the freezer (– 20 ± 5 °C). One aliquot of 2 mL of the stock solution was thawed and diluted with 8 mL assay medium (resulting in 1 mg/mL). This MTT-solution with the concentration of 1 mg/mL was used in the test (batch no.: T20230308).

6.4.2 DPBS-Buffer

“Dulbecco`s Phosphate Buffered Saline” (DPBS, without Ca^{2+} and Mg^{2+}) buffer was used for the rinsing the test item of the tissues and as solvent for the MTT concentrate. A subset was portioned and delivered from MatTek In Vitro Life Science Laboratories; the other subset was prepared by LAUS GmbH.

Composition of the subset prepared at LAUS GmbH (batch no.: T20230130):

KCl	0.4 g
KH_2PO_4	0.4 g
NaCl	16.01 g
$Na_2HPO_4 * 2H_2O$	2.90 g
sterile H_2O	ad 2 L

pH was adjusted to 6.997 using 1 M HCl.

Molar composition of the subset from MatTek In Vitro Life Science Laboratories (batch no.: 022123MSA) is the same, but different salts (crystal water) may have been used.

The buffer which was delivered from MatTek In Vitro Life Science Laboratories was used for rinsing the test item from the tissues. The buffer which was prepared by LAUS GmbH was only used for preparing the MTT concentrate.

6.4.3 Assay Medium

Serum-free DMEM (Dulbecco’s Modified Eagle’s Medium) was used as assay medium and procured from MatTek In Vitro Life Science Laboratories, batch no.: 030623ISA.

6.4.4 Isopropanol

CH_3-CH(OH)-CH_3, for synthesis., ≥ 99.5 %, batch no.: 190296551, used as extracting solvent for formazan.

6.5 Test Vessels

All vessels used are made of glass or plastic (sterilized). The glassware was sterilized before use by autoclaving.

The following vessels were used:

- 96-well-plates
- 24-well plates
- 12-well plates
- 6-well plates

6.6 Instruments and Devices

The instruments and devices that were used in the test are listed in the following table.

Table 6.6 Instruments and Devices

Device	Device name	Manufacturer
Autoclave	Autoklav 3870 ELV-B	Tuttnauer
Microtiter plate photometer	Photometer Anthos Reader 2010 Flexi	Anthos Microsysteme GmbH
Clean bench	Mars 1200	Scanlaf
Suction pump	VacuSip	Integra
Pipetting device	AccuJet pro	Brand
Auto Rep E	Auto Rep E	Rainin
Precision scales	ME5002T/M00	Mettler Toledo
Analytical scales	XS205 Dual Range	Mettler Toledo
Incubation chamber	Inkubator CB-150 (E3)	Binder
Glass thermometer	Glass thermometer 20210422-1	--
Glass thermometer	Glass thermometer 20020912-15	--
Thermohygrometer	Thermohygrometer Dewpoint Pro	DOSTMANN electronic GmbH
Table water bath	WBS-11	neolab
Pipette 20 – 200 µL	Pipet-lite XLS	Rainin
Pipette 200 – 2000 µL	Pipet-lite XLS	Rainin
Orbital shaker	Schüttelapparat 3005	GFL GmbH
Freezer	LGex 3410-23A-001	Liebherr
Refrigerator	LKv 3913 Index 21A/001	Liebherr
Stop watch	Stop watch	Roth
pH meter	3310	wtw

-- = various suppliers

Usage and, if applicable, calibration followed the corresponding SOP in the current edition. Standard laboratory material and equipment was also used.

6.7 Demonstration of Proficiency

The validity of the EpiOcular™ Eye Irritation study at LAUS GmbH was demonstrated in a non-GLP proficiency study. For this purpose, 15 proficiency chemicals (indicated by the OECD 492 guideline) were tested.

All of the 15 proficiency chemicals were correctly categorized. Therefore, the proficiency of the EpiOcular™ Eye Irritation study was demonstrated (see chapter 15).

7 PERFORMANCE OF THE STUDY

The test item was heated up to 37 ± 1 °C for 15 minutes before usage. Afterwards, the test item was handled as liquid substance.

7.1 Pre-Tests

7.1.1 Non-GLP Pre-Test: Assessment of Colored or Staining Test Items

The test item is colorless (which was visually determined). To assess, whether the test item will become colored after contact with isopropanol, 50 µL test item were added to 2 mL isopropanol, incubated in 6-well plates on an orbital shaker for 2 hours at room temperature. Then, two 200 µL aliquots of the resulting solution and two 200 µL aliquots of neat isopropanol were transferred into a 96-well plate and measured with a plate reader at 570 nm.

After subtraction of the mean OD for isopropanol, the mean OD of the test item solution was 0.0005 (≤ 0.08). Therefore, no additional test was performed.

To assess, whether the test item will become colored after contact with demineralized water, 50 µL of the test item were added to 1 mL of sterile demineralized water in a 6-well plate and incubated in the dark for 1 hour at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity. Then, two 200 µL aliquots of the resulting solution and two 200 µL aliquots of sterile demineralized water were transferred into a 96-well plate and measured with a plate reader at 570 nm.

After subtraction of the mean OD for demineralized water, the mean OD of the test item solution was -0.0005 (≤ 0.08). Therefore, no additional test was performed.

7.1.2 Non-GLP Pre-Test: Assessment of Direct Reduction of MTT by the Test Item

The test item was tested for the ability of direct MTT reduction. To test for this ability, 50 µL of the test item were added to 1 mL of MTT solution in a 6-well plate and the mixture was incubated in the dark for 3 hours at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity. 1 mL of MTT solution plus 50 µL of sterile demineralized water were used as negative control. After incubation a potential color formation was assessed.

The color of the MTT solution was not changed to blue/purple, therefore, the test item is not presumed to have reduced the MTT and no additional test on freeze killed tissues was performed.

7.2 Main Test

7.2.1 Pre-Incubation of Tissues

The assay medium was pre-warmed to 37 ± 1 °C.

6-well-plates were labelled with “negative control”, “test item number” and “positive control”. The plates were filled with 1 mL assay medium in the appropriate wells.

After arrival, all inserts were inspected for viability and the presence of air bubbles between agarose gel and insert. Cultures with air bubbles under the insert covering more than 50 % of the insert area were discarded. Viable tissues were transferred (2 tissues per negative control, test item and positive control) in the prepared wells of the 6-well-plates and incubated for 1 hour at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

After the pre-incubation, the medium was replaced and the wells were filled with 1 mL fresh assay medium. All 6-well-plates were incubated for 17 hours 40 minutes at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity. The rest of the assay medium was stored in the refrigerator.

7.2.2 Exposure and Post-Treatment

Two tissues were used for the negative control (sterile demineralised water), two for the positive control (methyl acetate) and two for the test item.

After overnight incubation, the tissues were pre-wetted with 20 µL DPBS buffer and then incubated for 30 minutes at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

At the beginning of each experiment (application of negative control), a stop watch was started. Afterwards, 50 µL of the controls and the test item were applied in duplicate in one-minute-intervals. This was done in such a fashion that the upper surface of the tissue was covered. After dosing the last tissue of each plate, each plate was transferred into the incubator for 28 minutes at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

At the end of the exposure time, the inserts were removed from the plates in one-minute-intervals using sterile forceps and rinsed immediately. The inserts were thoroughly rinsed with DPBS. Then, the tissues were immediately transferred to and immersed in 5 mL of pre-warmed assay medium in a pre-labelled 12-well plate for 12 minutes post soak at room temperature.

After that, each insert was removed from the medium, the medium was decanted off the tissue and the insert was blotted on absorbent material and transferred into the respective well of a pre-labelled 6-well plate containing 1 mL assay medium. For post-treatment incubation, the tissues were incubated for 120 minutes at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95% relative humidity.

After the post-treatment incubation, the MTT assay was performed.

7.2.3 MTT Assay and Extraction

On the day of the start of the MTT assay, one aliquot of the MTT stock solution was thawed. The MTT stock solution was diluted with the MTT solvent directly before usage.

After the post-incubation, a 24-well-plate was prepared with 300 µL freshly prepared MTT solution in each well. The tissue inserts were blotted on absorbent material and then transferred into the MTT solution. Then, the 24-well-plate was incubated for 180 minutes at 37 ± 1 °C, 5 ± 1 % CO_2 and ≥ 95 % relative humidity.

At last, each insert of the negative and positive control and the test item was thoroughly blotted on absorbent material and set into an empty, pre-labelled 24-well-plate. Into each well, 2 mL isopropanol were pipetted, taking care to reach the upper rim of the insert.

The plates were sealed, placed in an airtight box and stored in the refrigerator overnight. On the next day, the plates were shaken for 2 hours at room temperature.

7.2.4 Measurement

The tissues of the negative and positive control and the test item were pierced with an injection needle, taking care that all color was extracted. The inserts were then discarded and the content of each well was thoroughly mixed in order to achieve homogenization.

From each well, two replicates with 200 µL solution were pipetted into a 96-well-plate which was read in a plate spectrophotometer at 570 nm. In addition, eight wells of the 96-well-plate were filled with 200 µL isopropanol each, serving as blank.

8 EVALUATION

The values of the 96-plate-reader were transferred into a validated spreadsheet (Microsoft Excel®).

Note: All calculations are performed with unrounded values. Therefore, re-calculation with rounded values may lead to slightly different results.

8.1 Calculation

Calculation was performed as follows:

- Calculation of mean OD of the blank control wells (Isopropanol) (OD_{Blk})
- Subtraction of OD_{Blk} from each OD value (blank corrected values)
- Calculation of mean value of the two replicates for each tissue (= e. g. OD corrected test item)
- Calculation of mean value of the two relating tissues for each control and the test item (= e. g. OD corrected mean negative control)

Note: Corrected mean OD value of the negative control corresponds to 100 % viability. For the mean of the two replicates of test item and positive control, tissue viability was calculated as % photometric absorbance compared to the negative control.

To calculate the relative tissue viability of each test item and positive control replicate, the following equation was used:

$$\%\ \text{Viability} = \left[\frac{\text{OD corrected test item or positive control}}{\text{OD corrected mean negative control}}\right] \cdot 100\ \%$$

9 FINDINGS AND RESULTS

9.1 Measured Values

As blank, the optical density of isopropanol was measured in eight wells of the 96-well-plate. The measured values and their mean are given in the following table:

Table 9.1-a Absorbance Values Blank Isopropanol (OD at 570 nm)

Replicate	1	2	3	4	5	6	7	8	Mean
Absorbance	0.042	0.044	0.042	0.044	0.042	0.045	0.042	0.044	**0.043**

The absorbance values of negative control, test item and positive control are given in the following table:

Table 9.1-b Absorbance Values Negative Control, Positive Control and Test Item (OD at 570 nm)

Designation	Measure-ment	Negative Control	Positive Control	Test Item
Tissue 1	1	1.974	0.541	1.878
	2	2.061	0.555	1.999
Tissue 2	1	1.882	0.414	1.959
	2	1.962	0.407	1.954

From the measured absorbances, the mean of each tissue was calculated, subtracting the mean absorbance of isopropanol as given in table 9.1-a (= corrected values).

Table 9.1-c Mean Absorbance Negative Control, Positive Control and Test Item

Designation	Negative Control	Positive Control	Test Item
Mean – blank (Tissue 1)	1.975	0.505	1.896
Mean – blank (Tissue 2)	1.879	0.368	1.914

9.2 Comparison of Tissue Viability

For the test item and the positive control, the following percentage values of tissue viability were calculated in comparison to the negative control:

Table 9.2 % Viability Positive Control and Test Item

Designation	Positive Control	Test Item
% Viability (Tissue 1)	26.2 %	98.4 %
% Viability (Tissue 2)	19.1 %	99.3 %
% Viability Mean	**22.6 %**	**98.8 %**

9.3 Assessment

Eye hazard potential is assessed using the criteria given in the following table:

Table 9.3 Assessment of Eye Hazard Potential

% Viability	Assessment	UN GHS classification
> 60 %	Non eye irritant	No Category
≤ 60 %	At least eye irritant	No Prediction Can Be Made (category 1 or 2)

9.4 Validity

Validity criteria and results are stated in the following table:

Table 9.4 Validity

Criterion	Demanded	Found
Mean OD of negative control	> 0.8 and < 2.8	1.927
% mean relative viability of positive control	< 50 % of negative control	22.6 %
Difference within replicates	< 20 %	5.0 % (negative control) 7.1 % (positive control) 0.9 % (test item)

The values for negative control and for positive control were within the range of historical data of the test facility (see annex 2, page 22).

10 DISCUSSION

In this study, one valid experiment was performed.

The test item Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) was applied to a three-dimensional human cornea tissue model in duplicate for an exposure time of 28 minutes. 50 µL of the liquid test item was applied to two tissue replicates.

After treatment with the test item, the mean value of relative tissue viability was 98.8 %. This value is above the threshold for eye irritation potential (≤ 60 %).

All validity criteria were met. The criterion for optical density of the negative control was fulfilled. The OD value was 1.927 (> 0.8 and < 2.8).

The positive control induced a decrease in tissue viability as compared to the negative control to 22.6 %.

The difference within the replicates of the controls and the test item was acceptable (< 20 %).

For these reasons, the result of the study is considered valid.

Under the conditions of the test, Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil) is considered non-eye irritant in the EpiOcular™ Eye Irritation Test.

11 DEVIATIONS

11.1 Deviations from the Study Plan

No deviations were ascertained.

11.2 Deviations from the Guidelines

No deviations were ascertained.

12 RECORDING AND ARCHIVING

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP-Document-Archive of the test facility for 15 years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP-Substance Archive for 15 years and then discarded.

Number of originals of the final report to be sent to the sponsor: 0, PDF-file only

13 ANNEX 1: COPY OF GLP-CERTIFICATE

RheinlandPfalz
LANDESAMT FÜR UMWELT

GUTE LABORPRAXIS – GOOD LABORATORY PRACTICE

GLP-BESCHEINIGUNG
STATEMENT OF GLP COMPLIANCE

gemäß/according to § 19b Abs. 1 Chemikaliengesetz

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

Prüfeinrichtung / Test facility

LAUS GmbH
Auf der Schafweide 20
67489 Kirrweiler

Prüfung nach Kategorien / Areas of Expertise
(gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6, 8, 9 (toxikologische in Vitro Prüfungen an Säugerzellen und Bakterien / toxicological in vitro studies on mammalian cells and bacteria)

Datum der Inspektion / Date of Inspection
(Tag.Monat.Jahr / day.month.year)
28. und 29.04.2021

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist spätestens drei Jahre nach der letzten Inspektion zu beantragen. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Principles of the GLP has to be applied for not later than three years after the last inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Landesamt für Umwelt Rheinland-Pfalz

Unterschrift, Datum / Signature, Date Mainz, 21.06.21

Sabine Riewenherm - Präsidentin -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt
Kaiser-Friedrich-Straße 7, 55116 Mainz
(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

MESSEN BEWERTEN BERATEN

14 ANNEX 2: COMPARISON WITH HISTORICAL DATA

In the following table, the means of negative control and positive control of all performed experiments up to 01. Mar. 2023 are stated and compared with the values which were found in this study.

Table 14 Historical Data

Parameter	**Optical Density Negative Control**	**Relative Tissue Viability Positive Control**
	Sterile Demineralised Water	Methyl Acetate
Exposure time	30 minutes	
Mean	1.891	31.9 %
Standard deviation	0.236	7.1 %
Range Min-Max	1.167 - 2.437	12.4 - 57.2 %
Range mean ± 2SD	1.437 - 2.367	18.0 - 46.3 %
Study 22120103G891	**1.927**	**22.6 %**

SD = Standard Deviation

15 ANNEX 3: LIST OF PROFICIENCY CHEMICALS

In the following table the outcome of the proficiency chemical testing is stated.

All 15 proficiency chemicals were correctly classified.

The demonstration of proficiency was performed under non-GLP conditions but within the GLP-environment at LAUS GmbH.

Table 15 Results of Proficiency Chemicals

Chemical Name	CAS No.	Physical State	Prediction OECD 492 UN GHS Category	Findings LAUS GmbH
Methylthioglycolate	2365-48-2	liquid	no prediction can be made	no prediction can be made
Hydroxyethyl acrylate	818-61-1	liquid	no prediction can be made	no prediction can be made
2,5-Dimethyl-2,5-hexanediol	110-03-2	solid	no prediction can be made	no prediction can be made
Sodium oxalate	62-76-0	solid	no prediction can be made	no prediction can be made
2,4,11,13-Tetraazatetradecanediimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-Dgluconate(20%, aqueous)	18472-51-0	liquid	no prediction can be made	no prediction can be made
Sodium benzoate	532-32-1	solid	no prediction can be made	no prediction can be made
Diethyl toluamide	134-62-3	liquid	no prediction can be made	no prediction can be made
2,2-Dimethyl-3-methylenebicyclo[2.2.1] heptane	79-92-5	solid	no prediction can be made	no prediction can be made
1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	liquid	No category	No category
Dicaprylyl ether	629-82-3	liquid	No category	No category
Piperonyl butoxide	51-03-6	liquid	No category	No category
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	viscous liquid	No category	No category
1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	solid	No category	No category
2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	solid	No category	No category
Potassium tetrafluoroborate	14075-53-7	solid	No category	No category

16 ANNEX 4: QUALITY CONTROL DATA OF THE TEST SYSTEM

Provided by MatTek In Vitro Life Science Laboratories (supplier)

16.1 Certificate of Analysis

MATTEK
A BICO COMPANY

Certificate of Analysis

Product: EpiOcular™ Tissue

Lot Number: 38509

Part#: OCL-200, OCL-212, OCL-200-EIT, OCL-212-EIT

Description: Reconstructed ocular tissue containing normal human keratinocytes. *This product is for research use only. Not for use in animals, humans or diagnostic purposes.*

I. Cell source

All cells used to produce EpiOcular™ are purchased or derived from tissue obtained by MatTek Corporation from accredited institutions. In all cases, consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the cells or derivatives of the tissue for research purposes.

Keratinocyte Strain: 4F1188

II. Analysis for potential biological contaminants

The cells used to produce EpiOcular™ tissue are screened for potential biological contaminants. Tests performed for each of the potential biological contaminant listed in the analysis that follows, where performed according to the test method given. The product resulted in "no detection" for the following potential biological contaminants determined by the stated test method:

Keratinocytes:

HIV-1 virus – Oligonucleotide-directed amplification	Not detected
Hepatitis B virus – Oligonucleotide- directed amplification	Not detected
Hepatitis C virus – Oligonucleotide- directed amplification	Not detected
Bacteria, yeast, and other fungi – long term antibiotic, antimycotic free culture	Not detected

III. Analysis for tissue functionality

Test	Specification	Acceptance criteria	Result and QA Statement	
Tissue viability	MTT QC assay, 1 hour, n=3	OD (540-570 nm) [1.1-3.0]	1.505 ± 0.046	**Pass**
Barrier function	ET-50 assay, 100 µl 0.3% Triton X-100, 3 time-points, n=2, MTT assay	ET-50 [12.2-37.5 min]	20.12 min	**Pass**
Sterility	Long term antibiotic and antimycotic free culture	No contamination	Sterile	**Pass**

Tissue viability and the barrier function tests are within the acceptable ranges and indicate appropriate formation of the mucosal barrier and a viable basal cell layer.

Initials: IS
Date: 3/7/23

March 7, 2023
Date

Nelson Rivas
Quality Assurance Department
Document Control Manager

CAUTION: Whereas all information above is believed to be accurate and correct, no absolute guarantee that human derived material is non-infectious can be made or is implied by this certificate of analysis. All tissues should be treated as potential pathogens. The use of protective clothing and eyeware and appropriate disposal procedures is strongly recommended.

MatTek Corporation	**www.mattek.com**	**support@mattek.com**
MatTek Headquarters	200 Homer Avenue, Ashland, MA - USA	+1-508-881-6771
MatTek Europe	Mlynské Nivy 73, Bratislava - Slovakia	+421-2-3260-7401

QC-10-012-0011 Rev. B

Page 1 of 1

16.2 Functionality Test

MatTek Corporation

EpiOcular QC (OCL-200)

LOT 38509 TESTING DATE 07.03.2023
TESTED Post Refrigerated Storage
COMMENTS No

Dosed with: 0.3% Triton X-100 (100uL)

Exposure Time (min)	Well	OD	MTT (OD)	Std Dev (OD)	Viability %	Std Dev (%)
5	1	1.2309	1.283	0.046	84.0	3.0
	1	1.2958				
20	1	0.7042	0.755	0.072	50.2	4.8
	1	0.8057				
60	1	0.2081	0.261	0.075	17.4	5.0
	1	0.3147				
H20	1	1.4612	1.505	0.046	100.0	3.0
	1	1.5004				
	1	1.5525				

Avg. cv (%): 11.3 6.3 Exp. Cv (%): 8.8

ET-50 (min): 20.12

EPIOCULAR (OCL-200) Acceptance Criteria
Based on 1996 QC Database

	TRI (MIN)	*H2O MTT (OD)*
greater than	12.2	1.10
less than	37.5	n.a.
1996 avg.	24.9	
std. dev.	6.3	

QC Evaluation: PASS

Initials: IS
Date: 3/7/2023

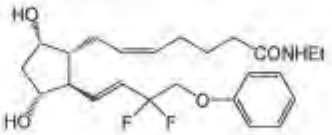
17 ANNEX 5: COPY OF THE CERTIFICATE OF ANALYSIS OF THE TEST ITEM

[redacted]

CERTIFICATE OF ANALYSIS

COA No.: TAFEA-10-022-007

Product: Dechloro Dihydroxy Difluoro Ethylcloprostenolamide (Neat Oil)
Chemical Structure:



CAS: 1185851-52-8
MF: $C_{24}H_{31}F_2NO_4$
MW: 437.52

Manufacturing Date: NOV 2022
Release Date: 23-NOV-2022
Re-Test Date: 23-NOV-2026

Batch/Lot: TAF-10-1122-01
Storage: 2-8°C, well-closed containers

Test for	*Specification*	*Result*
Description	Colorless to pale yellow oil	Conforms
Identity 01	NMR, IR, or HPLC	Conforms
Identity 02	LCMS M+1 = 438.3	Conforms
Purity: (including isomers NMT 2.5%)	NLT 99.00%	99.78%
Other Impurities	NMT 1.00%	0.22%

Result: Product Conforms to Specifications.

Quality Control: [redacted] *Date:* 23NOV2022

Approved: [redacted] *Date:* 23 Nov 2022

Quality Control Laboratory
[redacted]

Consumer Product Testing Co.

EST 1975

FINAL REPORT

CLIENT: [redacted]

ATTENTION: [redacted]

TEST: The Hen's Egg Test - Utilizing the Chorioallantoic Membrane (HET-CAM)

TEST ARTICLE: Eyelash Conditioner TEA Enhanced .025% Lot 090309-2

EXPERIMENT REFERENCE NO.: V09-4466-2

10/21/09

Steven Nitka
Vice President
Laboratory Director

70 New Dutch Lane • Fairfield, New Jersey 07004-2514 • (973) 808-7111 • Fax (973) 808-7234

Consumer Product Testing Co.
EST. 1975

QUALITY ASSURANCE UNIT STATEMENT

Study No.: V09-4466-2

The objective of the Quality Assurance Unit (QAU) is to monitor the conduct and reporting of nonclinical laboratory studies. This study has been performed under Good Laboratory Practice principles (including government regulations to the extent applicable) and in accordance with standard operating procedures and applicable standard protocols. The QAU maintains copies of study protocols and standard operating procedures and has inspected this study on the date listed below. The findings of this inspection may have been reported to management and the Study Director.

Date of data inspection: 10/19/09

Quality Assurance:

Christine Hendricks 10/21/09
Signature/Date

70 New Dutch Lane • Fairfield, New Jersey 07004-2514 • (973) 808-7111 • Fax (973) 808-7234
Clinical • Toxicology • Analytical Chemistry • Microbiology

Objective:

To evaluate the test article for irritancy potential utilizing the HET-CAM test. The test is a modification of that described by Kemper and Luepke.[1]

Introduction:

The chick embryo has been used extensively in toxicology. "The chorioallantoic membrane (CAM) of the chick embryo is a complete tissue with organoid elements from all germ cell layers. The chorionic epithelium is ectodermal and the allantoic epithelium is endodermal. The mesoderm located between these epithelia is a complete connective tissue including arteries, capillaries, veins and lymphatic vessels. The CAM responds to injury with a complete inflammatory reaction, comparable to that induced in the rabbit eye test. It is technically easy to study, and is without nerves to sense pain."[2]

Test Article: Eyelash Conditioner TEA Enhanced .025% Lot 090309-2

Reference Articles: Almay One Coat Mascara
Maybelline Waterproof Ultra Eyeliner

Date of Assay: October 8, 2009

[1]Kemper, F.H. & Luepke, N.P., (1986). The HET-CAM Test: An Alternative to the Draize Test. *FD Chem. Toxic.* 24, p. 495 - 496.

[2]Leighton, J., Tchao, R., Verdone, J. & Nassauer, J. Macroscopic Assay of Focal Injury in the Chorioallantoic Membrane. In: *Alternative Methods in Toxicology*, Vol. 3, *In Vitro* Toxicology E2, pp. 357 - 369, Alan M. Goldberg, (ed.), Mary Ann Liebert Publishers, Inc., New York, 1985.

Method:

Fresh, fertile, White Leghorn eggs were obtained from Moyer's Chicks, Inc., in Quakertown, Pennsylvania. They were stored at this facility for up to seven (7) days, at approximately 13° C (± 3° C), before being incubated. For incubation the eggs were placed, on their sides, in a Kuhl, humidified incubator. The incubator is such that the eggs are automatically rotated once every hour. The temperature was controlled at 37° C (± 2° C) for the ten (10) days of incubation. On day eight (8) the eggs were turned so that the acutely angled end faced down.

On day ten (10) each egg was removed from the incubator and placed in a Plexiglas work enclosure. This enclosure had been preheated and humidified so that its environment approached that of the incubator. A cut was made in the larger end of each egg, where the air sack is located. A Dremel® Moto-Flex Tool (model 232-5) equipped with a Dremel® Cut-Off Wheel (No. 409) was used to make each cut. Forceps were then used to remove the shell down to the shell-membrane junction. The inner egg membrane was then hydrated with a warm, physiological saline solution. The saline was removed after a two (2) to five (5) minute exposure. Utilizing pointed forceps, the inner egg membrane was then carefully removed to reveal the CAM.

The test or reference article, at a dosage of three-tenths of one milliliter (0.3 ml) of a liquid or three-tenths of one gram (0.3 g) of a solid, was then administered to each of four (4) CAM's. Twenty seconds later, the test or reference article was rinsed from each CAM with five (5) milliliters of physiological saline. All CAM's were observed immediately prior to test article administration and at 30 seconds, two (2) and five (5) minutes after exposure to the test article. The reactions of the CAM, the blood vessels, including the capillaries, and the albumin were examined and scored for irritant effects as detailed below:

		Score		
Effect	**Time (min.)**	**0.5**	**2**	**5**
Hyperemia		5	3	1
Minimal Hemorrhage ("Feathering")		7	5	3
Hemorrhage (Obvious Leakage)		9	7	5
Coagulation and/or Thrombosis		11	9	7

The numerical, time dependent scores were totaled for each CAM. Each reaction type can be recorded only once for each CAM, therefore the maximum score per CAM is 32. The mean score was determined for all CAM's similarly tested.

Results:

Test Article (%)	CAM #	Scores @ 0.5 min.	2 min.	5 min.	Total
Eyelash Conditioner TEA Enhanced .025% Lot 090309-2 (50%)	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
				Average:	**0.00**

Reference Article (%)	CAM #	Scores @ 0.5 min.	2 min.	5 min.	Total
Almay One Coat Mascara (50%)	1	0	0	1	1
	2	0	0	1	1
	3	0	0	0	0
	4	0	0	0	0
				Average:	**0.50**

Reference Article (%)	CAM #	Scores @ 0.5 min.	2 min.	5 min.	Total
Maybelline Waterproof Ultra Eyeliner (50%)	1	0	0	1	1
	2	0	0	1	1
	3	0	0	1	1
	4	0	0	0	0
				Average:	**0.75**

Each article was then classified as indicated in the following:

Mean Score	Irritation Potential
0.0 - 4.9	Practically none
5.0 - 9.9	Slight
10.0 - 14.9	Moderate
15.0 - 32.0	Severe

Discussion:

Previous studies have shown that the CAM of the hen's egg is more sensitive to liquid irritants than is the rabbit eye. Therefore, dilutions of the liquid test and reference articles were used.

Historical *In Vivo* Results:

The reference products have historically been categorized as being practically non-irritating, eliciting scores approaching 0, at 24 hours, when dosed at 100% and tested using the Draize ocular irritation methodologies (Draize Scale: 0 – 110).

Conclusion:

Under the conditions of this test, the results indicate that the sponsor-submitted product, Eyelash Conditioner TEA Enhanced .025% Lot 090309-2, at 100%, would have practically no ocular irritation potential *in vivo*.

Professional personnel involved:

Steven Nitka, B.S.	-	Vice President Laboratory Director (Study Director)
Lillian Vazquez, B.S.	-	Laboratory Supervisor
Christine Hendricks	-	Quality Assurance Group Leader

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: October 27, 2023

SUBJECT: Isopropyl Cloprostenate

Anonymous. 2023. Additional Data Supporting the Safe Use of Isopropyl Cloprostenate in Cosmetics.

Additional Data Supporting the Safe Use of Isopropyl Cloprostenate in Cosmetics

Submitted on Behalf of:

Company 1
(markets cosmetic lash serum containing 0.0044% isopropyl cloprostenate)

&

Company 2
(markets cosmetic lash serum containing 0.005% isopropyl cloprostenate)

Submitted To:
Bart Heldreth, PhD
Executive Director
Cosmetic Ingredient Review
Washington, DC
heldrethb@cir-safety.org

October 27, 2023

Table of Contents

1. Introduction

This report provides additional data supporting the safe use of Isopropyl Cloprostenate ("IC") in the concentration contained in Company 1's cosmetic lash serum (0.0044% IC) and Company 2's cosmetic lash serum (0.005% IC).

In response to the Expert Panel's Scientific Literature Notice to Proceed, Personal Care Product Council ("PCPC"), on behalf of Company 1 and Company 2, previously submitted **6** Het-Cams, **4** HRIPTs, **3** Ocular Irritation Studies, **1** In Use Eye Assessment, **1** Ocular Irritation Assay, **2** IC Assays and **2** Average Weight Usage Tests.

Company 1 and Company 2 now present additional data in response to the Panel's Insufficient Data Announcement ("IDA").

2. 8-Month / 120 Subject Ophthalmological In-Use Safety Evaluation

Company 1 engaged Consumer Product Testing Company ("CPTC") to perform an 8-month Ophthalmological In-Use Safety Evaluation on 120 female subjects to evaluate the safety of its cosmetic eyelash serum (0.0044% IC) (the "8-Month Clinical Study"). The 8-Month Clinical Study evaluated three distinct endpoints (i) general safety and ocular irritation potential, (ii) potential for change in ocular pigmentation, and (iii) potential for change in periorbital volume. We expect the final report to be issued imminently and will send under separate cover once available as Annex 1.

The 8-Month Clinical Study is, to the knowledge of Company 1, the longest and highest-powered study conducted on any cosmetic eyelash serum containing prostaglandin analogues. A board-certified ophthalmologist performed gross and/or slit lamp examination of each subject's eyelids, conjunctivae, corneas, anterior chambers, and pupillary reactions, in addition to measuring visual acuity, dryness, erythema, and edema at baseline, 1-month, 2-month, 4-month and 8-month intervals. At the same intervals, a bioinstrumentation technician captured VISIA-CR® Digital Imaging and Aeva® 3D HE Imaging images to measure the potential for change in ocular pigmentation and periorbital volume.

The 8-Month Clinical Study concludes:

> **General Safety and Ocular Irritation Potential**: The product tested was determined to be safe for use by both contact lens and non-contact lens wearers. The lead investigator did note the product had a slight potential for transient ophthalmological irritation.
>
> **Potential for Change in Ocular Pigmentation**: Study participants exhibited no statistically significant differences in visible eye color of the iris on the RGB scale (e.g., R/RGB (red color) G/RGB (green color), B/RGB (blue color) or L* (luminosity/brightness) from baseline after 8 months of use. In depth photography did indicate a statistically significant increase in overall color change of the iris over the length of the study (delta E) that can be attributed to

changes in a*(redness /irritation) and b* (yellowness / sharpness), but these changes were determined to not be clinically relevant to the issue of ocular pigmentation.

Potential for Change in Periorbital Volume: There was no change in periorbital fat volume from baseline after 8 months of use.

3. Intraocular Pressure Assay

Company 2 engaged CPTC to perform a 28-Day clinical trial on 24 female participants to evaluate its cosmetic eyelash serum's (0.005% IC) potential to impact intraocular pressure (the "Intraocular Pressure Assay"). The final report is attached hereto as Annex 2.

A board-certified ophthalmologist measured the intraocular pressure measurements of both right and left eyes at baseline and day 28 using a Reichert Tonopen. The Intraocular Pressure Assay concludes:

> "After 28 days of Lash Enhancing Serum usage, subjects exhibited no statistically significant differences in intraocular eye pressure in either the left or right eyes."

4. Toxicological Safety Assessment of IC in Cosmetic Lash Serums

Company 1 and Company 2 engaged Tox Services LLC to evaluate the likelihood of potential systemic and localized toxicity potential of IC in the companies' cosmetic lash serums. The report entitled "Additional Data for Consideration by CIR Pertaining to the Use of Isopropyl Cloprostenate in Cosmetic Products" (the "Toxicological Safety Assessment") is attached hereto as Annex 3.

a. Low Risk of Potential Systemic Toxicity Due to De Minimis Exposure

The Toxicological Safety Assessment concludes:

> "A comprehensive evaluation of published data for identified surrogates and finished product safety testing substantiate the safety of lash serums containing up to 0.005% IPC for its intended cosmetic use. A large margin of safety ("MOS") for IPC present in lash serums containing up to 0.005% IPC confirms the overall safety of IPC in terms of systematic toxicity; intended use of cosmetic lash serums result in *de minimis* exposure to IPC."
>
> "As shown in Table E-1, the MOS for cosmetic lash serum use of IPC at up to 0.05% is 1,029, indicating no significant risk of adverse systemic effects."

b. Low Risk of Eye Irritation and Skin Irritation or Sensitization

The Toxicological Safety Assessment, analyzing safety tests previously provided to CIR by PCPC on behalf of Company 1 and Company 2, concludes:

> "…the results of four *in vitro* ocular irritation assays and two clinical studies indicate that IPC as used in cosmetic lash serums is not expected to be irritating to the eyes. Additionally, three human repeat insult patch tests (HRIPTs) demonstrate that cosmetic lash serums containing up to 0.005% IPC are neither irritating nor sensitizing to the skin."

5. Concentration of Use Data

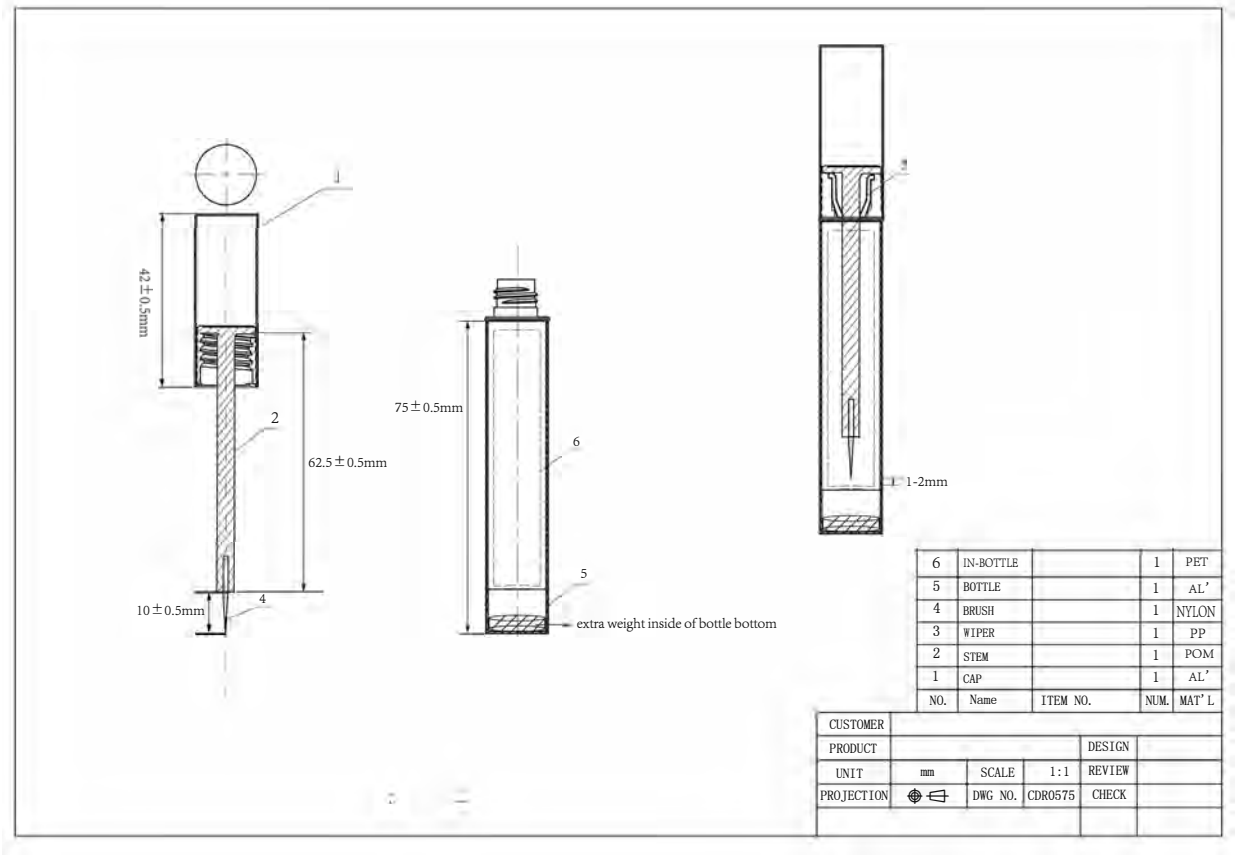
Company 1's marketed cosmetic lash serum contains 0.0044% IC. A previous formulation of the product sold until 2019 contained 0.005% IC. Company 2's marketed cosmetic lash serum contains 0.005% IC. Company 1 and Company 2's cosmetic lash serums are formulated to prevent dripping into the eye.

Application of Company 1 and Company 2's lash serums in accordance with product use instructions results in exceedingly low exposure to IC. PCPC, on behalf of Company 1 and Company 2, previously submitted testing demonstrating that, on average, 0.0000084mg of IC is applied to the upper lash line of both eyes with each application of Company 1's product, while 0.000013mg of IC is applied to the upper lash line of both eyes with each application of Company 2's product. As described in Section 6 below, the applicator, wiper and instructions for use are specifically designed to prevent the serum from dripping from the upper lash line into the eye. Further, consumers are instructed to immediately rinse with cool water if serum does contact the eye.

6. Packaging and Directions for Consumer Use

Company 1 and Company 2's lash serums are intended to be applied once each day as a thin line on the eyelid just above the upper lash line. The products include a multi-use applicator wand that is attached to the container's screw on cap. The tip of the applicator is similar to applicators used with eyeliners and consists of a very fine brush that is designed to optimize precise application of a small amount of serum, as directed by the Directions for Use.

A technical drawing is included below. The tube neck includes a wiper (#3) that is specifically designed to tightly hug the stem (#2) resulting in the removal of all excess serum from the stem (#2) and the brush (#4) when the applicator is removed from the bottle. The thin nylon brush is designed so that only a very small amount of serum remains on the brush.



NO.	Name	ITEM NO.	NUM.	MAT'L
6	IN-BOTTLE		1	PET
5	BOTTLE		1	AL'
4	BRUSH		1	NYLON
3	WIPER		1	PP
2	STEM		1	POM
1	CAP		1	AL'

CUSTOMER					
PRODUCT				DESIGN	
UNIT	mm	SCALE	1:1	REVIEW	
PROJECTION		DWG NO.	CDR0575	CHECK	

Company 1 and Company 2's Directions for Use and Caution Statement are below. They include instructions designed to prevent ocular exposure. For example, users are instructed to: (i) apply to a clean, dry lash line, (ii) use one dip into the bottle for both eyes, (iii) allow serum to completely dry, (iv) not to get serum in eye and (v) if serum contacts eye to immediately rinse with cool water. Therefore, even in the unlikely event that serum comes in contact with the eye, it would be immediately flushed with water.

Company 1	
Directions for Use	Caution Statement
Apply once daily on a clean, dry lash line using a single stroke on your eyelid just above your upper lash line (like a liquid eyeliner). Use one dip into the bottle for both eyes and allow 1-2 minutes for the serum to dry. Due to the length of the lash cycle, apply every day for a full 3 months. After desired improvement is achieved, apply every other day for maintenance. Product 1 requires continued use to maintain benefits.	Do not get into the eyes; in event of direct contact, rinse with cool water. Do not use if you are pregnant or nursing, under the age of 18, are prone to dry eyes or styes or undergoing chemotherapy. Keep out of reach of children. If redness or irritation occurs, stop using product. Consult physician if you are being treated for an eye-related condition.

Company 2	
Directions for Use	Caution Statement
Apply lash serum just above your upper eyelash line on clean, dry skin. One dip of the wand is enough serum	For external use only. Keep out of reach of children. Only apply with applicator. Do not share applicator.

for both eyes. Allow 1-2 minutes for the serum to dry before applying other products or touching your eyes. Use once daily in the evening, on upper lash line only. Once satisfied with results, use 2-3 times per week to maintain your look. We recommend evaluating your results after 12 weeks of use.	Secure lid tightly after each use. Avoid getting in eyes; if so, immediately rinse with cool water. Do not use if pregnant or nursing, undergoing treatment for glaucoma or cancer, or if you have ever experienced conjunctivitis, dry eyes, eye infections, styes, irritation from other cosmetics applied in the eye area, or any eye-related disorder or illness. Discontinue use immediately if irritation occurs. If symptoms persist, seek medical attention.

Annex 1
(8-Month Clinical Study)
To Be Submitted Once Final Report Available

Annex 2
(Intraocular Pressure Assay)

CONSUMER PRODUCT TESTING COMPANY
cptclabs.com

FINAL REPORT

CLIENT: [redacted]

ATTENTION: [redacted]

TEST: Safety Evaluation of an Eyelash Serum using changes in Intraocular Pressure
Protocol No.: LPQU01-003
Protocol Date: 07/25/23

TEST MATERIAL: Eyelash Conditioner containing IC

lash serum containing 0.005% Isopropyl Cloprostenate

STUDY NUMBER: [redacted]

Reviewed by: [redacted] M.D.
Board Certified Ophthalmologist

Approved by: [redacted] R.N.
Executive Vice President, Clinical Evaluations

CONSUMER PRODUCT TESTING COMPANY
cptclabs.com

FDA Registration# 1000151293
DEA Registration# RC0199744 Schedule I-V
US EPA/NJ DEP Registration# NJD982726648
ISO/IEC 17025 2017 Accreditation # 88071

QUALITY ASSURANCE UNIT STATEMENT

Study Number: [redacted]

The Consumer Product Testing Company, Incorporated (CPTC) Quality Assurance Unit (QAU) is responsible for auditing the conduct, content and reporting of all clinical trials that are conducted at CPTC.

This trial has been conducted in accordance with the Declaration of Helsinki, the ICH Guideline E6 for *Good Clinical Practice*, the requirements of 21 CFR Parts 50 and 56, other applicable laws and regulations, CPTC Standard Operating Procedures, and the approved protocol.

The CPTC QAU has reviewed all data, records, and documents relating to this trial and also this Final Report. The following QAU representative signature certifies that all data, records, and documents relating to this trial and also this Final Report have been reviewed and are deemed to be acceptable, and that the trial conforms to all of the requirements as indicated above.

All records and documents pertaining to the conduct of this trial shall be retained in the CPTC archives for a minimum of five (5) years. At any time prior to the completion of the fifth archival year, a Sponsor may submit a written request to the CPTC QAU to obtain custody of trial records once the CPTC archive period has been completed. This transfer shall be performed at the Sponsor's expense. In the absence of a written request, trial-related records shall be destroyed at the end of the CPTC archive period with no further notice in a manner that renders them useless.

[redacted]
Quality Assurance Representative

9/28/23
Date

[redacted]

Objective:

To evaluate the potential for change in intraocular pressure through Ophthalmologic evaluations of both eyes, over a 28-day period of once nightly usage of an eyelash serum.

Participants:

Twenty-four female subjects, aged 24 to 65 years, were recruited for this trial. Twenty-two subjects qualified for this trial. Subject #s 11 and 14 did not qualify. Subject #3 did not complete the trial for personal reasons unrelated to the test material. Twenty-one subjects completed the trial.

Inclusion Criteria:

1. Subjects who read, signed, and dated an Informed Consent Form that included a HIPAA statement;
2. Approximately 20 female subjects aged 18 to 65 years, inclusive;
3. Subjects who arrived at each Testing Facility visit with a clean face, wearing no eye or face make-up;
4. Subjects who were considered dependable and able to follow directions; and
5. Subjects who signed a Non-Disclosure/Confidentiality Agreement ("NDA") agreeing to keep their participation in the study confidential.

Exclusion Criteria:

1. Subjects who were in ill health, as determined by the PI;
2. Subjects who were taking medication, other than birth control, such as any systemic or topical corticosteroids, immunosuppressants, anti-inflammatories, antihistamines, antibiotics, or other medication that, in the opinion of Investigator, may have influenced the purpose, integrity, or outcome of the trial;
3. Subjects who were pregnant, planning to become pregnant, or lactating during the course of the trial;
4. Subjects who experienced any type of ophthalmological issues within the past 6 months;
5. Subjects who had any visible ocular infection or disease which might have been confused with a reaction to the test material;
6. Subjects who had previously undergone refractive surgery;
7. Subjects who currently had glaucoma, were undergoing glaucoma treatments, have had glaucoma surgery or incision surgery;
8. Subjects who were allergic to proparacaine, a local anesthetic to numb the eye;
9. Subjects who were regular contact lens wearers;
10. Subjects who had a history of adverse reactions to cosmetics, OTC drugs, or other personal care products;
11. Subjects who introduced the use of any new cosmetic, toiletry, or personal care products during the trial; or
12. Subjects who were prone to dry eyes, styes, or undergoing chemotherapy.

Test Material: Eyelash Conditioner containing IC

Trial Schedule:

Initiation Date	Completion Date
August 16, 2023	September 13, 2023

Trial Design: This was a single-center, baseline controlled, monadic clinical trial design of 28-days duration.

Descriptions of the trial timepoints are shown below:

Description	Qualification & Baseline (Day 0)	Test Phase (Day 28)
Distribution of Informed Consent, Subject N-DA Form and MHF	X	
Board-Certified Ophthalmologist Evaluations	X	X
Application of Proparacaine Drops & Intraocular Pressure Measurements of Right & Left Eyes	X	X
Distribution of Test Material, Daily Diaries, and Instruction for Use	X	
Return of unused Test Material and Completed Daily Diaries		X

Methodology: The informed consent process fully apprised each potential subject of the risks and benefits associated with the research clinical trial and of the confidentiality requirements relating to the subject's clinical trial records. If the potential subject agreed to participate in the research clinical trial, then the potential subject executed the Informed Consent Form (ICF) after which the potential subject entered the clinical trial as a subject. Staff who conducted the informed consent process also executed the form.

Each subject received a signed copy of the fully executed ICF. If at any time during the clinical trial the subject had questions, the ICF directed the subject to a Subject Rights Advocate, whose contact information was in the ICF.

Intraocular Pressure Measurement Procedure

Proparacaine

Proparacaine, a local anesthetic, works by blocking the pain signals at the nerve endings in the eye.

Methodology (continued):

Before Intraocular Pressure measurements were evaluated, each subject was given 1 drop of 0.5% of Proparacaine, in both the right and left eye, respectively, numbing the area before measurements were taken.

Reichert Tonopen

The Reichert Tonopen is a handheld electronic device that uses a small plunger to gauge the resistance of an anesthetized cornea when in contact. It has a known area of flattening and correlates well with Goldmann Applanation Tonometer (GAT) in "normal" IOP ranges.

Qualification & Baseline (Day 0)

Potential subjects reported to the Testing Facility at their scheduled appointment time, with a clean face wearing no eye or face make-up and executed an ICF to become subjects. Subjects completed an NDA Form and Medical History Form to determine initial qualification.

Subjects were screened by a Board-Certified Ophthalmologist to determine qualification. The Ophthalmologist studied and evaluated by gross and/or slit lamp examination each subject's eyelids, conjunctivae, corneas, anterior chambers, and pupillary reactions, in addition to measuring visual acuity. Observations included evaluations of dryness, erythema, and edema of the eye area, which were recorded on CRFs. Findings were noted on individual CRFs.

Evaluation Key:

0	None
0.5	Barely perceptible
1	Mild
2	Moderate
3	Marked
4	Severe

Subjects who presented a score of moderate (2) or greater for any evaluation were disqualified. All evaluation scores were recorded on CRFs.

Subjects then had Proparacaine eye drops administered to both the right and left eye by the Board-Certified Ophthalmologist. After approximately 10 seconds of the anesthetic drop application, intraocular pressure measurements of both the right and left eyes were taken, respectively, by the Board-Certified Ophthalmologist, as described.

Intraocular Pressure Measurements
Ranges

Normal Range:
10-20mm hg

Abnormal Range:
21-29 mm hg

Severe Range:
≥ 30

Subjects with measurements between abnormal (21 – 29 mm hg) to severe (≥ 30 mm hg), on the right and/or left eyes were disqualified.

The Study Coordinator referred these subjects to see their private Ophthalmologist, or to visit the Emergency room, respectively for treatment. All evaluation scores were recorded on CRFs.

Test Phase (Day 0)

Qualified subjects were provided with a 28-day supply of the test material and a daily diary to document use. Written and verbal instructions for use and restrictions, supplied by the Sponsor, were provided to each subject.

Instructions:

Arrive at each Testing Facility visit with a clean face, wearing no eye or face makeup.

Use only the test material for all eyelash enhancement purposes for the duration of the trial. Do not use other products designed to promote the growth of lashes.

You may wear your usual eye and facial makeup products during the trial.

Do not introduce any new cosmetic, toiletry, or personal care products during the trial.

Instructions for use: (Once Daily) (PM Use)

- **Use once daily in the evening, on upper lash line only.**
- **Apply the lash serum on your eyelid just above your upper eyelash line on clean, dry skin.**
- **Allow 1-2 minutes for the serum to dry.**
- **One dip of the wand is enough serum for both eyes.**

Methodology (continued):

WARNING: For external use only. Keep out of reach of children. Only apply with applicator. Do not share applicator. Secure lid tightly after each use. Avoid getting in eyes; if so, immediately rinse with cool water. Do not use if pregnant or nursing, undergoing treatment for glaucoma or cancer, or if you have ever experienced conjunctivitis, dry eyes, eye infections, styes, irritation from other cosmetics applied in the eye area, or any eye-related disorder or illness. Discontinue use immediately if irritation occurs. If symptoms persist, seek medical attention.

Do not let anyone else use the test material. Report any adverse reactions or problems immediately to the Testing Facility staff. After using the test material, place a "√" in the appropriate box under the APPLICATIONS column on the daily diary.

Subjects were encouraged to document comments pertaining to the test material in their daily diaries.

Subjects must have agreed to use only the test material for all eyelash enhancement purposes for the duration of the trial. Subjects may have worn their usual eye and facial makeup products during the trial. Subjects may not have introduced any new cosmetic, toiletry, or personal care products during the course of the trial.

Test Phase (Day 28)

After 28 days of test material use, subjects reported to the Testing Facility at their scheduled appointment time with a clean face, wearing no eye or facial make-up.

The Board-Certified Ophthalmologist conducted an evaluation of each subject, as previously described.

Daily diaries were reviewed for completeness and retained by the Testing Facility.

Statistical Methods (Per-Protocol Analysis):

Within groups statistical analysis was performed on the mean calculated values from the changes in Intraocular pressure. Prior to performing the analyses, diagnostic tests on the data were performed to determine whether normality and/or homogeneity of variances of the data were maintained. If the above conditions were maintained, a parametric Student's t-Test or Analysis of Variance was performed. If any of the above conditions were not maintained, a non-parametric equivalent to the above statistical tests was utilized.

Statistical Methods (Per-Protocol Analysis) (continued):

If statistical significance was observed and further statistical comparisons were required, multiple comparison testing was performed and appropriate adjustments to the p-values were made accordingly. For all of the above analyses, statistical significance was achieved at the 95% Confidence Level ($p < 0.050$).

For all of the above analyses, statistical significance was achieved at the 95% Confidence Level ($p < 0.050$).

Amendments:

ADMINISTRATIVE CHANGE 1

OLD VERSION:

Protocol:	LPQU01-003
Date:	07/25/23
Page:	1 of 19

NEW VERSION:

Protocol:	LPQU01-003
Date:	07/25/23
Page:	1 of 16

REASON FOR CHANGE: Updating protocol with correct total number of pages.

ADMINISTRATIVE CHANGE 2

OLD VERSION:

Test Material: Lash Enhancing Serum

NEW VERSION:

Test Material: Eyelash Conditioner containing IC

REASON FOR CHANGE: Updating protocol with test material identification.

Deviations:

Subject #21 missed applications on the following days as per her diary: 7, 17, 24, 25 and 27. The subject did not follow the instructions per the protocol. The Principal Investigator determined that there would be no impact to the trial.

Results:

Subject demographics are presented in Table 1.

Statistical analyses of Intraocular Pressure values are presented in Table 2.

A statistically significantly greater number of subjects rated a positive/favorable response for each question answered.

Positive

No. of Subjects	Comments
1	It's ok
1	I enjoyed using this product!

Negative

No. of Subjects	Comments
1	A little glammy and sometimes I wake up with my lids sealed

Summary:

Under the conditions of this trial, test material, Eyelash Conditioner containing IC, indicated no statistically significant differences in the intraocular eye pressure of subjects in either the left or right eyes after 28 days of use.

Table 1

Subject Demographics

Subject Number	ID#	Age
1	91792	60
2	92808	61
3	55559	36
4	68131	62
5	31484	58
6	60579	65
7	89232	63
8	10503	58
9	46410	54
10	72637	59
11	79677	53
12	9412	59
13	76861	61
14	90130	24
15	57454	52
16	29339	62
17	44968	65
18	92866	61
19	74926	49
20	25858	63
21	93986	52
22	3142	63
23	3128	65
24	7106	62

Did Not Qualify: Subject #s 11 and 14
Did Not Complete: Subject #3

Table 2

Intraocular Pressure Values (Right Eye)

Lash Enhancing Serum

Subject #	Baseline	Final	*Difference From Baseline At :* Final
1	16.0	13.0	-3.0
2	15.0	19.0	4.0
4	15.0	12.0	-3.0
5	14.0	15.0	1.0
6	12.0	13.0	1.0
7	14.0	10.0	-4.0
8	16.0	16.0	0.0
9	15.0	16.0	1.0
10	12.0	9.0	-3.0
12	9.0	14.0	5.0
13	11.0	12.0	1.0
15	12.0	14.0	2.0
16	15.0	9.0	-6.0
17	11.0	12.0	1.0
18	10.0	13.0	3.0
19	14.0	16.0	2.0
20	15.0	13.0	-2.0
21	8.0	13.0	5.0
22	15.0	15.0	0.0
23	16.0	11.0	-5.0
24	12.0	10.0	-2.0
Mean =	**13.2**	**13.1**	**-0.1**
Median =	**14.0**	**13.0**	**1.0**
Standard Deviation =	**2.4**	**2.5**	**3.2**
Maximum =	**16.0**	**19.0**	**5.0**
Minimum =	**8.0**	**9.0**	**-6.0**

Mean % Change From Baseline	
	Final
	-0.7%
Did not Complete: Subject # 3	Decrease
Did not Qualiify: Subjects # 11, 14	

Tallies & Percentages	
	Final
Total	21
Increased Values	11
Decreased Values	8
No Change	2
Increased Values	52.38%
Decreased Values	38.10%
No Change	9.52%

Table 2
(continued)

Intraocular Pressure Values (Left Eye)

Lash Enhancing Serum

Subject #	Baseline	Final	*Difference From Baseline At :* Final
1	13.0	11.0	-2.0
2	12.0	17.0	5.0
4	17.0	11.0	-6.0
5	13.0	19.0	6.0
6	11.0	13.0	2.0
7	18.0	11.0	-7.0
8	17.0	14.0	-3.0
9	13.0	14.0	1.0
10	11.0	10.0	-1.0
12	11.0	12.0	1.0
13	10.0	13.0	3.0
15	10.0	13.0	3.0
16	13.0	9.0	-4.0
17	12.0	12.0	0.0
18	8.0	13.0	5.0
19	13.0	16.0	3.0
20	15.0	12.0	-3.0
21	10.0	14.0	4.0
22	12.0	18.0	6.0
23	18.0	16.0	-2.0
24	16.0	10.0	-6.0
Mean =	**13.0**	**13.2**	**0.2**
Median =	**13.0**	**13.0**	**1.0**
Standard Deviation =	**2.8**	**2.7**	**4.1**
Maximum =	**18.0**	**19.0**	**6.0**
Minimum =	**8.0**	**9.0**	**-7.0**

Mean % Change From Baseline
Final
1.8%
Increase

Did not Complete: Subject # 3
Did not Qualify: Subjects # 11, 14

Tallies & Percentages	
	Final
Total	21
Increased Values	11
Decreased Values	9
No Change	1
Increased Values	52.38%
Decreased Values	42.86%
No Change	4.76%

Table 2
(continued)

Statistical Analysis of Intraocular Pressure Values

Within-Groups Analysis For :
Lash Enhancing Serum (Right eye)

Baseline VS.	Test Type	P-value	Significance	Direction
Final	Dependent (Paired) t-Test	0.892	No	Decrease

Within-Groups Analysis For :
Lash Enhancing Serum (Left eye)

Baseline VS.	Test Type	P-value	Significance	Direction
Final	Dependent (Paired) t-Test	0.792	No	Increase

Summary:

After 28 days of Lash Enhancing Serum usage, subjects exhibited no statistically significant differences in intraocular eye pressure in either the left or right eyes.

Annex 3
(Toxicological Safety Assessment)

TOXSERVICES
TOXICOLOGY RISK ASSESSMENT CONSULTING

DATA SUBMISSION

DOCUMENT STATUS: FINAL

REPORT TITLE

Additional Data for Consideration by CIR Pertaining to the Use of Isopropyl Cloprostenate (IPC) in Cosmetic Products

PREPARED BY

ToxServices LLC
1526 New Hampshire Ave., N.W., Suite 350
Washington, D.C. 20036

PREPARED FOR

REDACTED COMPANY 1
(markets cosmetic lash serum containing 0.0044% IPC)

&

REDACTED COMPANY 2
(markets cosmetic lash serum containing 0.005% IPC)

APPROVAL

AUTHOR: [redacted] Ph.D., M.H.S., D.A.B.T.
Principal Toxicologist
ToxServices LLC

SENIOR REVIEWER: [redacted] Ph.D., M.P.H., C.Biol., F.R.S.B., E.R.T., D.A.B.T.
Managing Director and Chief Toxicologist
ToxServices LLC

DATE: October 17, 2023

TOXSERVICES
TOXICOLOGY RISK ASSESSMENT CONSULTING

1526 New Hampshire Ave., N.W., Suite 350
Washington, D.C. 20036

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INTRODUCTION

In response to the Cosmetic Ingredient Review ("CIR") Expert Panel's Insufficient Data Announcement (CIR 2023a), this report provides additional data pertaining to the systemic and localized toxicity potential of isopropyl cloprostenate ("IPC") in cosmetic lash serums.

IPC EXPOSURE DATA

Application of REDACTED COMPANY1's and REDACTED COMPANY 2's lash serums in accordance with product use instructions results in exceedingly low exposure to IPC. PCPC, on behalf of Company 1 and Company 2, previously submitted testing demonstrating that, on average, 0.0000084 mg of IPC is applied to the upper lash line of both eyes with each application of REDACTED COMPANY 1's product, while 0.000013 mg of IPC is applied to the upper lash line of both eyes with each application of REDACTED COMPANY 2's product.

SAFETY ASSESSMENT OF IPC IN COSMETIC LASH SERUMS

An extensive search of the literature yielded no repeated-dose dermal toxicity data for IPC, and prohibitions on animal testing of cosmetic ingredients preclude *de novo* animal testing. Consequently, this report identifies appropriate surrogates for IPC in a read-across approach. Read-across is a widely used approach that can be used to support cosmetic ingredient safety assessments by determining whether a cosmetic ingredient has the inherent ability to cause specific human health effects without performing new animal tests.

A comprehensive evaluation of published data for identified surrogates and finished product safety testing substantiate the safety of lash serums containing up to 0.005% IPC for their intended cosmetic use. A large margin of safety ("MOS") for IPC present in lash serums containing up to 0.005% IPC confirms the overall safety of IPC in terms of systemic toxicity; intended use of cosmetic lash serums results in *de minimis* exposure to IPC. The safety of IPC for its intended cosmetic use is evaluated below.

Low Risk of Potential Systemic Toxicity Due to *De Minimis* Exposure

ToxServices relied upon read-across and conservative MOS calculations to assess the likelihood of adverse systemic effects potentially arising from the intended use of IPC in cosmetic lash serums. In this approach, appropriate surrogates are first identified and then a dose-response analysis is performed to select the point of departure for use in MOS calculations.

IPC is a synthetic analog of an endogenous biomolecule, prostaglandin F2α (PGF2α). PGF2α analogs are formulated as esters that undergo hydrolysis to the active acid form (SCCS 2022). Based on data for other PGF2α analogs, IPC is expected to be rapidly hydrolyzed to cloprostenol (CAS #54276-21-0), reducing the potential for systemic exposure to the intact IPC molecule. Data on the extent of IPC hydrolysis are not available, however. In the absence of repeated-dose systemic safety data for IPC itself, its hydrolysis product cloprostenol serves as a surrogate. Cloprostenol also served as a surrogate in the EU's Scientific Committee on Consumer Safety's (SCCS's) recent

evaluation of prostaglandin analogs including IPC (SCCS 2022). In contrast to IPC, cloprostenol has a robust toxicological dataset.

ToxServices also performed a detailed similarity analysis to ascertain whether other PGF2α analogs could serve as surrogates for IPC. A detailed description of procedures used to identify suitable surrogates is included in Appendix A, along with structural similarity comparisons generated from ChemMine and QSAR Toolbox in Appendices B through D. Based on the results of this analysis, ToxServices identified the PGF2α analog travoprost (CAS#157283-68-6) as an additional surrogate for IPC.

ToxServices characterized the likelihood of adverse systemic effects by calculating an MOS for IPC (SCCS 2023). The MOS is the ratio between the no (or lowest) observed adverse effect level (NOAEL or LOAEL, when no NOAEL is available) in a toxicity study and the estimate of consumer exposure to a chemical through use of a product and is a commonly used and protective approach to assess the safety of personal care product ingredients. A sufficiently large MOS demonstrates safety of the ingredient when used in a specific product application. For purposes of establishing safety, an MOS of at least 100 is required (SCCS 2023). The approach for the MOS calculations is described in detail in Appendix E. As shown in Table E-1, the MOS for cosmetic lash serum use of IPC at up to 0.005% is 1,029, indicating no significant risk of adverse systemic effects.

The SCCS's evaluation of prostaglandin analogs including IPC identified a different MOS that utilized a toxicological screening value (TSV) as the point of departure. According to the reference cited by SCCS,[1] the TSV can be used to assess risks of pharmacologically-active veterinary substances present in food-use animals. The specific TSV selected for the IPC MOS calculation appears, based on ToxServices' reverse calculations, to have been a value of 0.0042 µg/kg/day for non-genotoxic substances that can affect receptors in the reproductive system, divided by 2 as recommended by SCCS for oral-route data to obtain a PODsys of 0.0021 µg/kg/day. The TSV of 0.0042 µg/kg/day is the lowest available established acceptable daily intake (ADI) value across a group of drug substances affecting the nervous system, reproductive system, or that are corticoids/glucocorticoids. Specifically, the 0.0042 µg/kg/day TSV was the lowest ADI for agents affecting the nervous system; the lowest ADI for agents affecting the reproductive system is 0.01 µg/kg/day and, for comparison, the mean ADI for agents affecting the reproductive system is 0.20 µg/kg/day. However, ADIs are based on experimental NOAELs that have been adjusted by an uncertainty factor of 100 or greater.[2] In contrast, MOS calculations for cosmetic ingredients adjust the experimental N(L)OAEL by a systemic availability factor (2 for oral-route NOAELs) (SCCS 2023). Therefore, the MOS of 2.5 obtained using the TSV approach is overly conservative.

In summary, intended use of lash serums containing up to 0.005% IPC results in *de minimis* exposure to IPC that is unlikely to result in adverse systemic health effects, as demonstrated using a conservative MOS calculation.

[1] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5332
[2] https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2012.2537

[redacted]

Detailed Description of Literature

Since cloprostenol and IPC likely differ in their dermal absorption potential, but the active moiety is the same (i.e., the acid form), available parenteral-route toxicity data for cloprostenol are germane to the assessment of IPC. For completeness, oral-route toxicity data for cloprostenol are also considered, even though oral bioavailability information is not available. The use of cloprostenol as a surrogate is supported by two toxicity studies that evaluated both cloprostenol and IPC at comparable parenteral doses: these studies identified similar effects of IPC and cloprostenol on a sensitive target tissue, the testis (Indrei et al. 2001, Sava et al. 2015).

Cloprostenol has low acute oral toxicity potential, as evidenced by an LD_{50} >25,000 mg/kg in rats, as initially reported in a 2004 European Medicines Agency (EMA) report. Additionally, cloprostenol did not cause adverse effects when applied to rat skin at a dose of 1.25 mg/kg in a study published in 1995 (SCCS 2022). However, due to the expected differences in absorption for cloprostenol and IPC, these studies are of limited utility in predicting potential acute effects of IPC.

At a dose of 15 mg/kg given to Wistar rats by intraperitoneal (i.p.) injection daily for 7 days, IPC did not adversely affect red or white blood cell parameters, which were the only evaluated endpoints. Information on the authors' statistical evaluations of the data was not provided; however, which limits confidence in the data (Udeanu et al. 2008). These authors also evaluated the acute toxicity potential of some additional prostamide derivatives of cloprostenol but did not appear to include IPC in the acute toxicity test.

With respect to longer-term systemic toxicity potential, available evidence from parenteral and oral-route studies identifies the male and female reproductive systems as sensitive targets for cloprostenol. However, data are limited because the most relevant parenteral studies assessed only one or two endpoints each.

For example, daily subcutaneous (s.c.) injection of cloprostenol for one month resulted in vacuolization of ovarian luteal cells in female rats. The LOAEL for this effect was 12.5 µg/kg/day, which was the lowest dose tested. There was no NOAEL (SCCS 2022). In non-pregnant female rats, i.p. injection of a much higher dose of cloprostenol, 50 mg/kg/day, for 4 weeks resulted in considerable ovarian blood vessel dilation and follicular degeneration (atresia) beginning after one week of treatment. The number of secretory cells in the uterine tubes (i.e., oviducts) was significantly reduced beginning after one week of treatment (Indrei et al. 1999). Thus, 50 mg/kg/day is the LOAEL for this study. Similarly, oral administration of cloprostenol for 3 months produced ovarian vacuolization in female rats; the NOAEL was 50 µg/kg/day, while the LOAEL was 150 µg/kg/day (SCCS 2022).

Intraperitoneal injection of cloprostenol or IPC (designated as CIPG and CIPG IE, respectively, by the authors) daily for 28 days resulted in microscopic effects on the testes in adult male rats at a dose of 100 µg/kg/day. Specific effects included dilation of testicular blood vessels, interstitial macrophage accumulation, and tubular degeneration.

[redacted]

Such effects were also observed in adult rats and mice at a dose of 25 µg/kg/day. Both test substances produced similar effects (Indrei et al. 2001).

Sava et al. (2015) evaluated the potential effects of cloprostenol and a substance identified as "CIPG isopropyl ester" on mouse testes. Although Sava et al. (2015) did not define "CIPG," Indrei et al. (2001) previously identified "CIPG IE" as IPC. This evaluation therefore assumes that CIPG isopropyl ester is IPC. In their study, Sava et al. (2015) administered either cloprostenol or IPC to adult male mice at 25 µg/kg/day for 4 weeks by i.p. injection. Authors evaluated testes excised from treated mice after 7, 14, and 28 days of treatment using electron microscopy. Both treatments induced similar ultrastructural changes: dilation of capillaries accompanied by endothelial cell enlargement, interstitial macrophage and fibroblast accumulation, and germ cell apoptosis.

The similarity of effects reported for cloprostenol and IPC in the Indrei et al. (2001) and Sava et al. (2015) studies suggests that either IPC itself is an active species or that IPC is rapidly and extensively hydrolyzed *in vivo* to cloprostenol. The latter is more plausible, as the ester forms of other prostaglandin analogs are extensively and rapidly hydrolyzed to their respective active acid forms. Moreover, the ester forms of other prostaglandin analogs are not as effective at receptor activation compared to the acid forms (i.e., the hydrolysis products).

Effects measured in marmosets included unspecified myocardial changes and significantly increased testicular weights following oral exposure to cloprostenol for 3 months. This study also yielded a NOAEL of 50 µg/kg/day and a LOAEL of 150 µg/kg/day (SCCS 2022).

A 3-generation oral-route study in rats did not identify adverse effects of cloprostenol on reproductive performance, although premature parturition, resulting in slightly reduced neonatal viability, was reported. The NOAEL for the 3-generation study was 15 µg/kg/day and the LOAEL was 20 µg/kg/day. Cloprostenol does not, however, adversely affect fetal development, based on results of an oral-route study in rats and a subcutaneous-route study in rabbits that yielded NOAELs of 100 µg/kg/day and 0.25 µg/kg/day, respectively, which were the highest doses tested in each species (SCCS 2022). ToxServices has less confidence in the relevance of the oral-route studies with cloprostenol for the safety evaluation of IPC due to their anticipated differences in absorption.

Cloprostenol tested negative in multiple genotoxicity assays, including an Ames bacterial reverse mutation test, *in vitro* tests in mammalian cells for mutagenicity and chromosomal aberrations, and an *in vivo* mouse micronucleus assay reported in 2004 (SCCS 2022).
The results of multiple studies in rats and mice identify reproduction and embryonic development as sensitive endpoints for parenterally administered travoprost, although parental reproductive tissues do not appear to be directly affected. Twenty-eight-day toxicity studies in CD-1 mice and CD rats revealed no effects on reproductive tissue

histopathology at intravenous doses up to 1 mg/kg/day. Similarly, there were no microscopic findings on male or female reproductive tissues in CD rats following 13 weeks of intravenous exposure at up to 1 mg/kg/day. CD-1 mice exposed to travoprost at up to 1 mg/kg/day via intravenous and, subsequently, i.p. injection had no evidence of treatment-related microscopic lesions in their reproductive tissues (U.S. FDA 2000). There were no microscopic findings in male and female reproductive tissues in CD rats following 6 months' s.c. exposure to travoprost at up to 0.1 mg/kg/day. The critical effect in the 6-month study was altered bone histopathology (NOAEL = 0.01 mg/kg/day, LOAEL = 0.1 mg/kg/day) (U.S. FDA 2000).

Travoprost was evaluated for reproductive and developmental toxicity in rats. Pregnant Sprague-Dawley rats were dosed at 0, 1, 3, and 10 µg/kg/day by intravenous injection on gestation days (GD) 6-17 and were sacrificed on GD 20. Maternal body weight gain and gravid uterine weight were reduced. The incidence of total litter resorptions was increased, as were the numbers of early and late resorptions. Premature delivery was also observed. The numbers of corpora lutea and implantations were reduced below historical control values, but there was no net effect on pre-implantation loss. Fetal viability and fetal body weights were also reduced. Lastly, the incidences of fetal external, visceral, and skeletal malformations and variations were increased. Authors identified a NOAEL of 3 µg/kg/day for this study (U.S. FDA 2000).

In another study in Sprague-Dawley rats, animals were dosed by s.c. injection at 0, 1, 3, and 10 µg/kg/day travoprost for 4 weeks prior to and through GD 13 (females) or 2 weeks prior to mating through GD 7 (males). Corpora lutea, implantations, fetal viability, estrous cyclicity, and sperm parameters were not affected by treatment. Authors reported an increase in early fetal resorptions at 10 µg/kg/day. The NOAEL for this study was 3 µg/kg/day (U.S. FDA 2000).

One developmental study in mice was also identified. Female CD-1(ICR)BR mice were given s.c. injections of 0, 0.1, 0.3, and 1 µg/kg travoprost on GD 6-16 and were sacrificed on GD 18. The incidences of early deliveries, litter loss, and total litter resorption as well as the number of early resorptions were increased, while the number of viable fetuses was reduced. No teratogenic effects were observed in the fetuses. Authors identified a NOAEL of 0.3 µg/kg/day for this study (U.S. FDA 2000).

In a 3-generation study, Sprague-Dawley rats were dosed by s.c. injection at 0, 0.12, 0.36, and 0.72 µg/kg/day on GD 7 to postnatal day (PND) 21. Reported effects included a decrease in gestation length and an increase in litters with stillborn pups at all dose levels in the F0 generation and an increase in litter loss and a decrease in litters with viable pups and numbers of pups per litter were reported at 0.72 µg/kg/day. Effects reported in the F1 generation included decreases in pup survival on PND 1 to 4, physical development, and motor activity at all dose levels in the F1 generation. No treatment-related effects were reported in the F2 generation (U.S. FDA 2000). The LOAEL for this study was 0.12 µg/kg/day, which was the lowest dose tested. A similar developmental toxicity study was available in which one generation of Sprague-Dawley rats received slightly lower doses of 0, 0.01, 0.03, and 0.1 µg/kg travoprost on GD 7 to PND 21 by s.c.

[redacted]

injection. There were no effects on pup development. The NOAEL for this study was 0.1 µg/kg/day (U.S. FDA 2000).

Travoprost was not carcinogenic in 2-year studies in male and female CD-1 mice and CD rats dosed at 10, 30, and 100 µg/kg/day by s.c. injection. There was no evidence of carcinogenic potential associated with treatment, although the U.S. FDA reviewer noted that the doses administered to mice were too low and were below the maximum tolerated dose (MTD), a dose level that produces mild evidence of systemic toxicity, which serves as an indicator that negative results are not attributable to factors such as low bioavailability. At the 100 µg/kg/day dose level, male rats were only dosed for 82 weeks (U.S. FDA 2000), which is shorter than the duration of a typical lifetime carcinogenicity bioassay.

ToxServices identified a point of departure for MOS calculations by comparing available experimental NOAELs and LOAELs for identified surrogates, as shown below in Table 1. The point of departure (POD) is typically the highest NOAEL that is lower than the lowest LOAEL. The selected POD is shown in the shaded gray cell in Table 1, below.

Table 1: Dose-Response Evaluation of IPC Surrogates

Treatment	Study Design	Route of Exposure	NOAEL	LOAEL	Critical Effect	Reference
Travoprost	Developmental toxicity study in rats	s.c.	0.1 µg/kg/day	N/A	No evidence of adverse developmental effects.	U.S. FDA 2000
Travoprost	3-Generation rat study	s.c.	N/A	**0.12 µg/kg/day**	Reduced litter size	U.S. FDA 2000
Travoprost	Developmental toxicity study in mice	s.c.	0.3 µg/kg/day	1 µg/kg/day	Increased resorptions	U.S. FDA 2000
Travoprost	6-Month rat study	s.c.	10 µg/kg/day	100 µg/kg/day	Altered bone histopathology	U.S. FDA 2000
Travoprost	Reproductive toxicity study in rats	s.c.	3 µg/kg/day	10 µg/kg/day	Increased resorptions	U.S. FDA 2000
Travoprost	Developmental toxicity study in rats	i.v.	3 µg/kg/day	10 µg/kg/day	Increased resorptions	U.S. FDA 2000
Travoprost	28-Day toxicity studies in mice and rats	i.v.	1,000 µg/kg/day	N/A	No treatment-related effects on reproductive tissues.	U.S. FDA 2000
Travoprost	Carcinogenicity studies in rats and mice	s.c.	100 µg/kg/day	N/A	No evidence of carcinogenicity.	U.S. FDA 2000
Cloprostenol	1-Month rat study	s.c.	N/A	12.5 µg/kg/day	Ovarian vacuolization	SCCS 2022

[redacted]

Cloprostenol	3-month rat study	oral	50 µg/kg/day	150 µg/kg/day	Ovarian vacuolization	SCCS 2022
Cloprostenol	3-Month study in marmosets	oral	50 µg/kg/day	150 µg/kg/day	Myocardial changes; increased testes weights	SCCS 2022
Cloprostenol	28-Day study in rats and mice	i.p.	N/A	100 µg/kg/day	Altered testicular histopathology	SCCS 2022
Cloprostenol	3-Generation rat study	oral	15 µg/kg/day	20 µg/kg/day	Reduced neonatal viability	SCCS 2022
Cloprostenol	Developmental toxicity study in rats	oral	100 µg/kg/day	N/A	No evidence of adverse developmental effects.	SCCS 2022
Cloprostenol	Developmental toxicity study in rabbits	s.c.	0.25 µg/kg/day	N/A	No evidence of adverse developmental effects.	SCCS 2022
Cloprostenol	4-Week ovarian toxicity study in rats	i.p.	N/A	50 mg/kg/day	Ovarian follicular degeneration.	Indrei et al. 1999
Cloprostenol or IPC	28-Day testicular toxicity study in rats and mice	i.p.	N/A	25 µg/kg/day	Degeneration of seminiferous tubules.	Indrei et al. 2001
Cloprostenol or IPC	4-Week testicular toxicity study in mice	i.p.	N/A	25 µg/kg/day	Degeneration of seminiferous tubules.	Sava et al. 2015

Based on the weight of available evidence, ToxServices identified the subcutaneous LOAEL of 0.12 µg/kg/day (0.00012 mg/kg/day) from the 3-generation rat study with travoprost as the point of departure for margin of safety calculations (U.S. FDA 2000). This value is >100-fold lower than LOAELs for adverse effects on reproductive tissues following parenteral cloprostenol exposure. Even though travoprost is not a cosmetic ingredient, use of cloprostenol as the sole surrogate for IPC would result in a less conservative risk assessment because the identified N(L)OAELs for cloprostenol are higher than those for travoprost for the critical reproductive endpoints.

IRRITATION AND SKIN SENSITIZATION STUDIES

As described below, the results of four *in vitro* ocular irritation assays and two clinical studies indicate that IPC as used in cosmetic lash serums is not expected to be irritating to the eyes. Additionally, three human repeat insult patch tests (HRIPTs) demonstrate that cosmetic lash serums containing up to 0.005% IPC are neither irritating nor sensitizing to the skin.

Low Risk of Eye Irritation

Consumer Product Testing Company (CPTC) evaluated the eye irritancy potential of IPC (0.05%) in a GLP-compliant hen's egg test – utilizing the chorioallantoic membrane

[redacted]

(HET-CAM) assay. Investigators applied 0.3 mL IPC (0.05% in an unspecified vehicle) for 20 seconds to each of four chorioallantoic membranes (CAM) prepared from White Leghorn eggs, followed by rinsing. Each CAM was scored for irritation at three time points (0.5 min, 2 min, and 5 min) by evaluating hyperemia, degree of hemorrhage, coagulation, and thrombosis; the maximum possible score was 32 for each CAM. The mean score was 1.50 for IPC (0.05%), which indicates that 0.1% IPC would have practically no ocular irritation potential under the conditions of the test (CPTC 2022a).

A cosmetic lash serum containing 0.0044% IPC exhibited low ocular irritation potential in a GLP-compliant HET-CAM assay. Investigators applied 0.3 mL of a 50% dilution of the formulation for 20 seconds to each of four chorioallantoic membranes prepared from White Leghorn eggs, followed by rinsing. Each CAM was scored for irritation at three time points (0.5 min, 2 min, and 5 min) by evaluating hyperemia, degree of hemorrhage, coagulation, and thrombosis; the maximum possible score was 32 for each CAM. The average score of a 50% dilution of the product was 1.25, indicating that the undiluted formulation would have practically no eye irritation potential (CPTC 2022b).

A cosmetic lash serum containing 0.005% IPC exhibited similarly low ocular irritation potential in a GLP-compliant HET-CAM assay. Investigators applied 0.3 mL of a 50% dilution of the formulation for 20 seconds to each of four chorioallantoic membranes prepared from White Leghorn eggs, followed by rinsing. Each CAM was scored for irritation at three time points (0.5 min, 2 min, and 5 min) by evaluating hyperemia, degree of hemorrhage, coagulation, and thrombosis; the maximum possible score was 32 for each CAM. The average score was 2.5 for the 50% product dilution, indicating that the undiluted formulation would exhibit practically no eye irritation potential (CPTC 2022c).

MB Research Labs performed a GLP-compliant HET-CAM assay on a cosmetic lash serum containing 0.005% IPC. The product, diluted to 10% in saline, two positive controls (1% sodium dodecyl sulfate and 0.1 N sodium hydroxide), and a vehicle control (0.9% sodium chloride irrigation, USP (saline)) were applied to 6 CAMs each. The eggs were observed continuously for 5 minutes, and investigators documented the appearance of hemorrhage, lysis, and/or coagulation. In addition, investigators scored the eggs for severity of responses at 1 and 5 minutes after test substance application. Investigators calculated an irritation score based on time until adverse reaction and severity of adverse reaction. Based on a mean irritation score of 2.6, the cosmetic lash serum containing 0.005% IPC (at a 10% dilution) has none-to-slight ocular irritation potential (MB Research Labs 2018).

In addition, there was no evidence of eye irritation among 27 subjects in a 4-week in-use study of a formulated eyelash product containing 10% IPC, as assessed via observation and self-reports (SCCS 2022). Although this next study did not evaluate topical cosmetic use conditions, daily instillation of an eyewash containing 0.01% IPC for 3 months did not affect intraocular pressure or produce changes to the eyes upon ophthalmoscopic examination in a cohort of 23 patients with glaucoma. Mild, transient conjunctival hyperemia that resolved within 2-3 days occurred in an unspecified number of subjects,

[redacted]

but there were no other local reactions (SCCS 2022). Additional details were not available.

Eurofins performed a clinical ocular compatibility study on a cosmetic lash serum containing 0.005% IPC. The study was completed by 32 adult female volunteers. After providing informed consent, subjects were instructed to apply the product to the skin above the upper lash line of each eye once daily before bed and to record each application in a diary. Investigators performed ophthalmic exams prior to initial product use and after 1, 2, and 3 months of daily product use. Each participant responded to a consumer perception questionnaire after initial product use and after 1, 2, and 3 months of daily product use. Participants did not report any adverse events during the study and there were no subjective reports of eye irritation at the 1-, 2-, or 3-month ophthalmic examinations. After one month of product use, investigators observed palpebral conjunctival irritation in three subjects, one of whom also had bulbar conjunctival irritation;[3] after two months of product use, investigators observed palpebral conjunctival irritation in one subject; and after three months of product use, there were no observations of conjunctival irritation. All reactions were rated "1" in severity, which typically indicates a slight or mild effect. The specific rating system used in this study was not included in the laboratory report, however. There were no observed instances of lacrimation, eyelid irritation, or corneal irritation during the study. After initial product use, 8.6% of participants reported a stinging sensation and 5.7% of participants reported irritation around the eye area. Subsequent questionnaires after 1, 2, and 3 months of treatment did not ask subjects about stinging or irritation. Investigators concluded that the formulation "did not demonstrate a potential for eliciting ocular irritation" (Eurofins 2021).

Low Risk of Skin Irritation or Sensitization

Based on the results of three HRIPTs, cosmetic lash serums containing up to 0.005% IPC are neither irritating nor sensitizing to the skin. The first HRIPT was completed by 56 healthy volunteers (Eurofins CRL 2019). Four additional subjects withdrew from the study for reasons unrelated to the test substance. Each volunteer received 9 semi-occlusive induction applications of a cosmetic lash serum containing 0.0044% IPC on the same site on the upper back. Subjects removed each induction patch after 24 hours of exposure. The investigators examined each subject's skin for indications of a dermal reaction prior to placement of each induction patch using the scoring system shown below in Figure 1:

[3] The conjunctiva is a transparent membrane that covers the white part of the eye and inner surface of the eyelids. It has two segments: bulbar conjunctiva (covers the "white" of the eye) and the palpebral conjunctiva (covers the inner surface of both upper and lower eyelids).

[redacted]

Dermal Score	Description
0	No visible skin reaction
±	Barely perceptible erythema
1+	Mild erythema
2+	Well defined erythema
3+	Severe erythema and edema
4+	Erythema and edema with vesiculation

Figure 1: HRIPT Dermal Scoring System (Eurofins CRL 2019)

After a 10-21-day rest period, investigators applied a single 24-hour challenge patch, also under semi-occlusion, to a previously untested area of the skin and scored the skin at patch removal and at 24, 48, 72, and 96 hours after removal of the challenge patch. Because there were no adverse skin reactions during the study (i.e., all scores were 0), investigators concluded that the test product did not demonstrate irritation or sensitization potentials (Eurofins CRL 2019).

The second HRIPT on the same cosmetic lash serum containing 0.0044% IPC was completed by 53 healthy volunteers; three additional volunteers withdrew from the study for reasons unrelated to treatment. Each volunteer received 9 semi-occlusive induction applications of the test material to the same site on the upper back and removed each patch the day after application. Investigators evaluated the skin prior to placement of each induction patch. At least 10 days after removal of the last induction patch, investigators applied a challenge patch to an adjacent skin site. Investigators evaluated the skin the following day, upon removal of the challenge patch, and two days later. There were no skin reactions observed during the study. Accordingly, investigators concluded that the cosmetic lash serum poses no potential for skin irritation or sensitization (CPTC 2021).

Clinical Research Laboratories performed a repeat-insult patch test that was completed by 53 healthy volunteers (CRL 2018). The study was performed in accordance with good clinical practices (GCPs) and with International Council for Harmonization (ICH) standards. Three additional subjects withdrew from the study for reasons unrelated to the test substance. After providing informed consent, each volunteer received 9 semi-occlusive induction applications of a cosmetic lash serum containing 0.005% IPC (undiluted) on the same site on the upper back. Subjects removed each induction patch after 24 hours of exposure. The investigators examined each subject's skin for indications of a dermal reaction prior to placement of each induction patch.

After a 10-21-day post-induction rest period, investigators applied a single 24-hour challenge patch, also under semi-occlusion, to a previously untested area of the skin and scored the skin at patch removal and at 24 and 48 hours after removal of the challenge patch (i.e., 48 and 72 hours, respectively, after challenge patch application). Because there were no adverse skin reactions during the study (i.e., all scores were 0), investigators concluded that the cosmetic lash serum containing 0.005% IPC did not demonstrate irritation or sensitization potentials (CRL 2018).

[redacted]

CONCLUSION

A sufficient margin of safety, based on two structural surrogates, demonstrates that IPC exposure through intended use of REDACTED COMPANY 1's and REDACTED COMPANY 2's cosmetic lash serums is *de minimis* in nature and does not pose a risk of adverse systemic health effects.

In terms of localized effects, *in vitro* ocular irritation assays and HRIPTs indicate that IPC is unlikely to result in eye or skin irritation or skin sensitization, respectively, when present at low levels in REDACTED COMPANY 1's and REDACTED COMPANY 2's cosmetic lash serums.

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APPENDIX A: READ-ACROSS APPROACH

Read-across leverages available scientific data on structurally and/or mechanistically similar substances for the purpose of identifying human health hazards. As described by OECD (2014), the following attributes can be considered when identifying read-across substances:

- Common functional groups
- Common mechanism of action: pharmacokinetic and pharmacodynamic properties, key events
- Common chemical class

Importantly, members of the same chemical class are often consistently positive or negative with respect to a specific health effect (OECD 2014).

ToxServices initially identified bimatoprost, latanoprost, and travoprost as potential surrogates because they are all PGF2α analogues, have robust toxicological datasets, and were identified in the SCCS Opinion on the use of prostaglandin analogues in cosmetics (SCCS 2022).

ToxServices first assessed the structural similarity of the three analogue candidates to the target chemical, IPC. The prostaglandin backbone structure is shown below in Figure 1. The analogues are all variations of this basic structure.

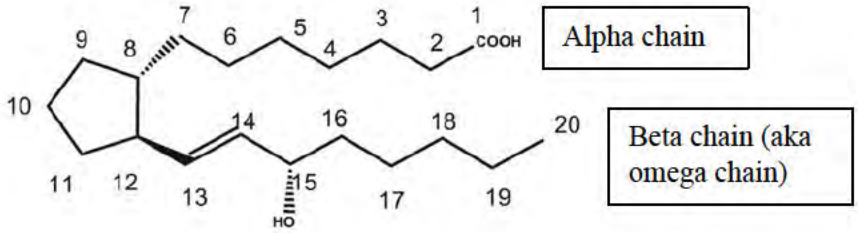


Figure A-2: Basic Structure of Endogenous Prostaglandins (SCCS 2022)

Available information on each potential surrogate's elemental composition, chemical functional groups, and selected physicochemical properties is shown below in Table A-1. The surrogate candidates are similar with respect to size, charge, and functional groups, with one notable exception: IPC, latanoprost, and travoprost are esters (RCOOR group), while bimatoprost is an amide (RCONHR'). In contrast to the other prostaglandin analogues (collectively termed prostanoids), bimatoprost is considered a prostamide due to the presence of an amide rather than an ester at the C1 position on the alpha chain (Ishida et al. 2006).

[redacted]

Table A-2: Physiochemical Properties of IPC and Surrogate Candidates (PubChem 2023, SCCS 2022)				
Property	**Isopropyl Cloprostenate (IPC)**	**Latanoprost**	**Travoprost**	**Bimatoprost**
CAS	157283-66-4	130209-82-4	157283-68-6	155206-00-1
MF	$C_{25}H_{35}ClO_6$	$C_{26}H_{40}O_5$	$C_{26}H_{35}F_3O_6$	$C_{25}H_{37}NO_4$
MW	466.9985	432.597	500.5505	415.57
Charges	0	0	0	0
Water solubility	0.047 mg/L @ 25°C (calculated)	12.9 mg/L	7.59 mg/L	18.7 mg/L
Partition coefficient	5.15 (calculated)	3.98	4.6	3.2
Individual Elements				
C	25	26	26	25
H	35	40	35	37
O	6	5	6	4
Ca	0	0	0	0
F	0	0	3	0
Cl	1	0	0	0
N	0	0	0	1
Na	0	0	0	0
S	0	0	0	0
Functional Groups				
RNH_2	0	0	0	0
R_2NH	0	0	0	0
R_3N	0	0	0	0
RCONHR'	0	0	0	1
$ROPO_3$	0	0	0	0
ROH	3	3	3	3
RCHO	0	0	0	0
RCOR	0	0	0	0
RCOOH	0	0	0	0
RCOOR	1	1	1	0
ROR	1	0	1	0
RCCH	0	0	0	0
RCN	0	0	0	0
RINGS	2	2	2	2
AROMATIC	1	1	1	1

Molecular formula (MF), molecular weight (MW), charges, atom counts, functional group counts, and ring counts. Physiochemical properties evaluation methods described in Dreyer et al. 2018.

To compare the structure of IPC to the three potential surrogates and evaluate whether these molecules are closely related in structure, ToxServices used ChemMine tools (ChemMine 2020) and OECD Toolbox (OECD 2020). Generally, chemical similarity analysis involves structural descriptors and similarity coefficient calculations, with the Tanimoto coefficient being widely used due to its utility and predictive accuracy (Chen and Reynolds 2002). The Tanimoto coefficient ranges from 0 to 1, with 0 being the least

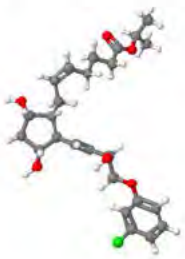
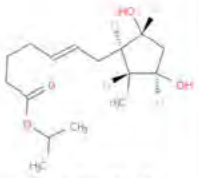
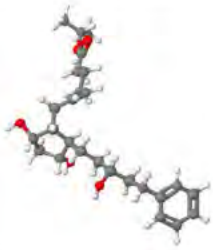
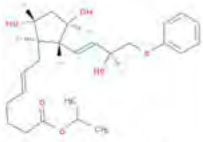
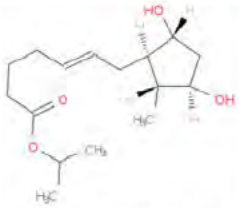
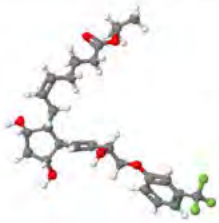
[redacted]

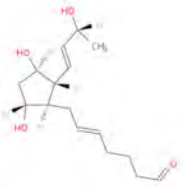
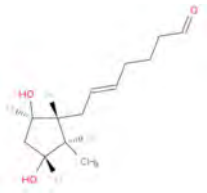
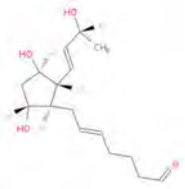
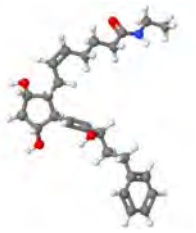
similar and 1 being the most similar. A Tanimoto coefficient of 0.8 or greater is often used to group similar chemicals.

ToxServices compared chemicals in a pairwise manner using ChemMine's Similarity Workbench. For each pair, ChemMine calculated an atom pair Tanimoto similarity score (AP Tanimoto) and a maximum common substructure Tanimoto similarity score (MCS Tanimoto) (Chen and Reynolds 2002, Cao et al. 2008). Similarly, ToxServices compared chemicals in a pairwise manner using OECD Toolbox to calculate an atom pair Tanimoto (Jaccard) similarity score.

The results of the structural similarity analyses are shown in Table A-2. Based on Tanimoto scores the from two software programs, IPC and travoprost display the greatest structural similarity.

Table A-3: Structural Similarity Analysis of Four Prostaglandin Analogues

	Isopropyl Cloprostenate	**Latanoprost**	**Travoprost**	**Bimatoprost**
SMILES	CC(C)OC(=O)CCC\C=C/C[C@H]1[C@@H](O)C[C@@H](O)[C@@H]1\C=C\[C@@H](O)COc2cccc(Cl)c2	CC(C)OC(=O)CCC\C=C/C[C@H]1[C@@H](O)C[C@@H](O)[C@@H]1CC[C@@H](O)CCc2ccccc2	CC(C)OC(=O)CCC\C=C/C[C@H]1[C@@H](O)C[C@@H](O)[C@@H]1\C=C\[C@@H](O)COc2cccc(c2)C(F)(F)F	CCNC(=O)CCC\C=C/C[C@H]1[C@@H](O)C[C@@H](O)[C@@H]1\C=C\[C@@H](O)CCc2ccccc2
Isopropyl Cloprostenate		**ChemMine** **AP Tanimoto: 0.623** **MCS Tanimoto: 0.465** **MCS Size: 20** **MCS Min: 0.645** **MCS Max: 0.625** **OECD Toolbox** **Tanimoto (Jaccard): 0.537**	**ChemMine** **AP Tanimoto: 0.742** **MCS Tanimoto: 0.861** **MCS Size: 31** **MCS Min: 0.969** **MCS Max: 0.886** **OECD Toolbox** **Tanimoto (Jaccard): 0.811**	**ChemMine** **AP Tanimoto: 0.549** **MCS Tanimoto: 0.476** **MCS Size: 20** **MCS Min: 0.667** **MCS Max: 0.625** **OECD Toolbox** **Tanimoto (Jaccard): 0.512**
Latanoprost	 See Note 1		ChemMine AP Tanimoto: 0.534 MCS Tanimoto: 0.435 MCS Size: 20 MCS Min: 0.645 MCS Max: 0.571 OCED Toolbox Tanimoto (Jaccard): 0.500	ChemMine AP Tanimoto: 0.596 MCS Tanimoto: 0.356 MCS Size: 16 MCS Min: 0.533 MCS Max: 0.516 OCED Toolbox Tanimoto (Jaccard): 0.564
Travoprost	 See Note 1	 See Note 1		ChemMine AP Tanimoto: 0.471 MCS Tanimoto: 0.444 MCS Size: 20 MCS Min: 0.667 MCS Max: 0.571 OCED Toolbox Tanimoto (Jaccard): 0.477

Bimatoprost	 See Note 1	 See Note 1	 See Note 1	

Note 1: Image represents the common substructure found in the two compounds being compared, based on an analysis of similarity in ChemMine (ChemMine 2020).
AP Tanimoto and Tanimoto (Jaccard)– similarity between corresponding atom pairs based on Tanimoto coefficient. MCS Tanimoto – similarity of the maximum common substructure (MCS) based on the Tanimoto coefficient. Structural similarity evaluation supported methods described in Dreyer et al. 2018.

MECHANISTIC SIMILARITY AMONG IPC AND POTENTIAL ANALOGUES

In addition to structural similarity, similarity in toxicologic or pharmacologic mechanism of action can serve as the basis for surrogate identification (OECD 2014).

Prostaglandins are a group of endogenous bioactive molecules. These molecules play important roles in tissue homeostasis/normal physiology and are also activated during inflammation. IPC, latanoprost, travoprost, and bimatoprost are synthetic analogues of prostaglandin F2α (PGF2α). PGF2α mediates female reproductive functions, cardiovascular function, and pain, and is upregulated during inflammation. Binding to its cognate receptor activates G-protein coupled intracellular signal transduction pathways, resulting in increased intracellular calcium (Ricciotti and FitzGerald 2011).

The PGF2α analogues used as anti-glaucoma agents function as PGF2α receptor agonists. In the eye, agonist-mediated activation of the PGF2α receptor ultimately causes relaxation of muscles controlling outflow of vitreous fluid and/or remodeling of the extracellular matrix via increased matrix metalloproteinase activity. The analogues may also upregulate endogenous prostaglandin production and/or may increase ocular blood flow (Ishida et al. 2006). Several chemical-specific factors can influence the extent to which an analogue can participate in a mechanism or mode of action (and, hence, its suitability as a surrogate), such as absorption, metabolism to a biologically active form, and receptor affinity and specificity. The PGF2α analogues must first be absorbed from the application site (skin, in the case of IPC used in cosmetics, or cornea, in the case of drug uses of bimatoprost, latanoprost, and travoprost). Available data demonstrate that latanoprost and travoprost are rapidly absorbed from the cornea (U.S. FDA 1996, 2000) where they undergo *in situ* esterase-mediated hydrolysis to the active form. Although bimatoprost is also readily absorbed, in contrast to other prostanoids, bimatoprost is pharmacologically active as the intact molecule (Woodward et al. 2003).

Once activated, the analogues bind the PGF2α receptor with varying effectiveness (affinity). ToxServices searched for data on structural features that can impact binding to the PGF2α receptor, also known as the prostaglandin F receptor (FP receptor). Schuster et al. (2000) demonstrated that the carboxylic acid functional group (COOH, see Figure 1, above) is essential for FP receptor binding: modification of the carboxylic acid moiety

to an amide reduced FP receptor binding affinity by more than 2 orders of magnitude. While substitution of the hydroxyl group at position 15 did not appreciably impact FP receptor binding affinity, introduction of a phenyl group on the omega chain enhanced FP receptor binding (Shuster et al. 2000).

SUMMARY

ToxServices' assessment of structural and mechanistic properties of the surrogate candidates indicates that bimatoprost is different from latanoprost and travoprost in both aspects. A structural comparison of IPC to latanoprost, travoprost, and bimatoprost indicates that travoprost displays the greatest structural similarity to IPC. Available mechanistic data suggest that bimatoprost exerts different mechanism(s) of action than IPC and therefore is not a strong a surrogate for IPC.

ToxServices' analog comparison results are summarized in Table A-4. Based on high structural similarity, similar therapeutic potency, and available mechanism of action and metabolism information for prostaglandin analogues, ToxServices identifies travoprost as the most suitable surrogate for evaluation of IPC's potential health effects, including carcinogenicity, reproductive toxicity, and developmental toxicity.

Table A-4: Prostaglandin Analog Comparison

Potential Analog	Structural Similarity to IPC (Low, Intermediate, High)	Mechanism of Action	Metabolism	Pharmacology	Toxicology
Latanoprost	Intermediate --Based on low Tanimoto coefficient but similar functional groups.	PGF2α receptor agonist.	Rapidly hydrolyzed to the active form.	Effectively reduces IOP.	Can induce local ocular effects. May also induce developmental effects at high doses.
Travoprost	High --Based on Tanimoto coefficient and functional groups	PGF2α receptor agonist.	Rapidly hydrolyzed to the active form.	Effectively reduces IOP; similar in potency compared to IPC.	Can induce local ocular effects. May also induce developmental effects at high doses.
Bimatoprost	Low --Based on low Tanimoto coefficient and different functional groups	Less selective for PGF2α receptor; binds other receptor forms.	Parent is the active form; not readily hydrolyzed.	Greater IOP lowering potency.	Slightly higher likelihood of local ocular effects. May also induce developmental effects at high doses.

[redacted]

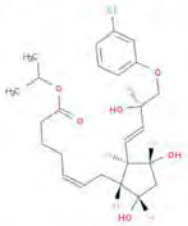
APPENDIX B: CHEMMINE STRUCTURAL SIMILARITY RESULTS

Compound Similarity

Select two compounds to compare from the grid below.

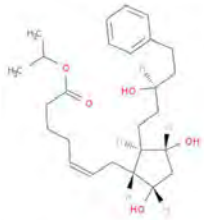
Selected Compounds

Isopropyl_cloprostenate



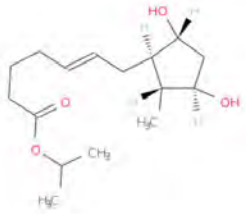
remove

Latanoprost



remove

AP Tanimoto: 0.623311
MCS Tanimoto: 0.4651
MCS Size: 20
MCS Min: 0.6452
MCS Max: 0.6250
SMILES:
C(OC(=O)CCC/C=C\C[C@H]1[C@(C[C@H]([C@@H]1C)O)O)(C)C
Isopropyl_cloprostenate

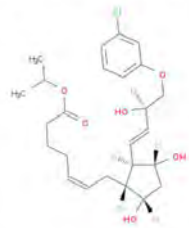


Compound Similarity

Select two compounds to compare from the grid below.

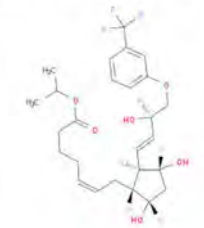
Selected Compounds

Isopropyl_cloprostenate



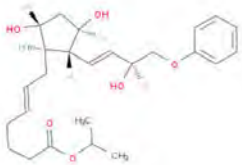
remove

Travoprost



remove

AP Tanimoto: 0.742812
MCS Tanimoto: 0.8611
MCS Size: 31
MCS Min: 0.9688
MCS Max: 0.8857
SMILES:
C(OC(=O)CCC/C=C\C[C@H]1[C@(C[C@H]([C@@H]1/C=C/[C@@H](COc1ccccc1)O)O)(C)C
Isopropyl_cloprostenate

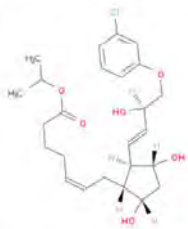


Compound Similarity

Select two compounds to compare from the grid below.

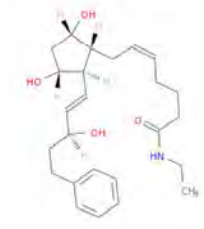
Selected Compounds

Isopropyl_cloprostenate



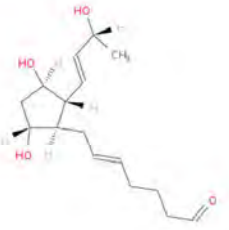
remove

Bimatoprost



remove

AP Tanimoto: 0.549085
MCS Tanimoto: 0.4762
MCS Size: 20
MCS Min: 0.6667
MCS Max: 0.6250
SMILES:
C(=O)CCC/C=C\C[C@H]1[C@@H](C[C@H]([C@@H]1/C=C/[C@@H](C)O)O)O
Isopropyl_cloprostenate

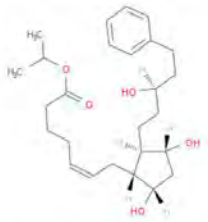


Compound Similarity

Select two compounds to compare from the grid below.

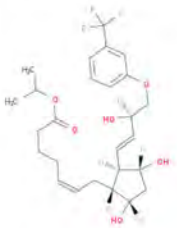
Selected Compounds

Latanoprost



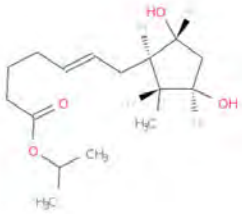
remove

Travoprost



remove

AP Tanimoto: 0.534009
MCS Tanimoto: 0.4348
MCS Size: 20
MCS Min: 0.6452
MCS Max: 0.5714
SMILES:
C(OC(=O)CCC/C=C\C[C@H]1[C@(C[C@H]([C@@H]1C)O)O)(C)C
Latanoprost

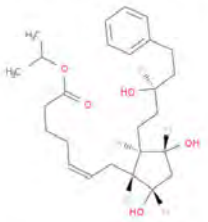


Compound Similarity

Select two compounds to compare from the grid below.

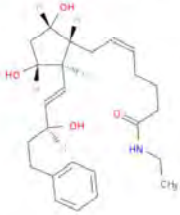
Selected Compounds

Latanoprost



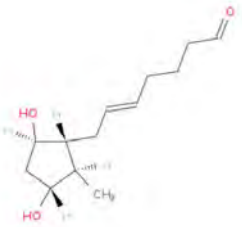
remove

Bimatoprost



remove

AP Tanimoto: 0.595745
MCS Tanimoto: 0.3556
MCS Size: 16
MCS Min: 0.5333
MCS Max: 0.5161
SMILES:
C(=O)CCC/C=C\C[C@H]1[C@@H](C[C@H]([C@@H]1C)O)O
Latanoprost

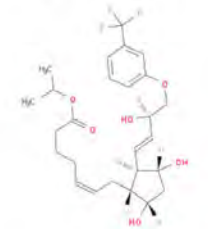


Compound Similarity

Select two compounds to compare from the grid below.

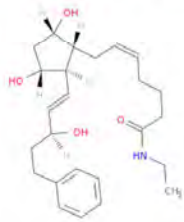
Selected Compounds

Travoprost



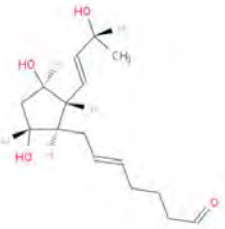
remove

Bimatoprost



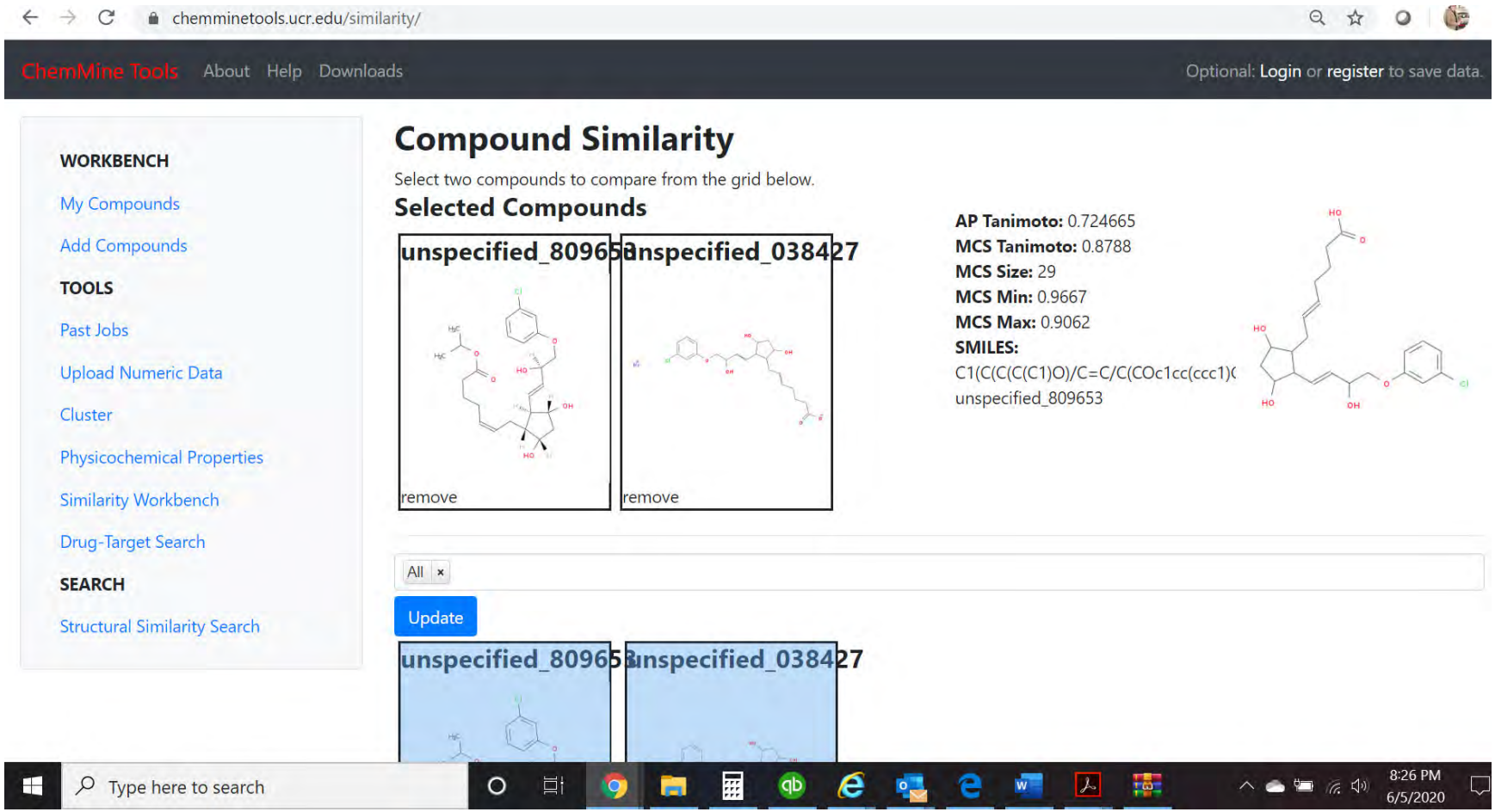
remove

AP Tanimoto: 0.471429
MCS Tanimoto: 0.4444
MCS Size: 20
MCS Min: 0.6667
MCS Max: 0.5714
SMILES:
C(=O)CCC/C=C\C[C@H]1[C@@H](C[C@H]([C@@H]1/C=C/[C@@H](C)O)O)O
Travoprost



APPENDIX C: CHEMMINE DETAILED SIMILARITY COMPARISON

Isopropyl Cloprostenate to Cloprostenol Comparison

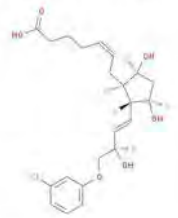


Compound Similarity

Select two compounds to compare from the grid below.

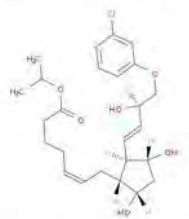
Selected Compounds

Cloprostenol



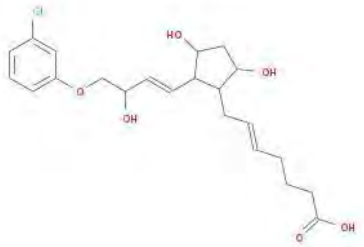
remove

Isopropyl_cloprostenate



remove

AP Tanimoto: 0.724665
MCS Tanimoto: 0.9062
MCS Size: 29
MCS Min: 1.0000
MCS Max: 0.9062
SMILES: C(COc1cccc(c1)Cl)
(/C=C/C1C(C(CC1O)O)C/C=C\CCCC(=O)O)O
Cloprostenol



[redacted]

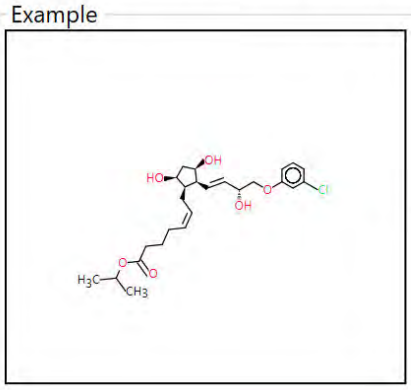
APPENDIX D: QSAR TOOLBOX STRUCTURAL SIMILARITY RESULTS

Isopropyl Cloprostenate and Latanoprost Comparison

Structure

CC(C)OC(=O)CCC\C=C/C[C@H]1[C@@H](O)C[C@@H](O)[C@@H]1\C=C\[C@@H](O)COc1cccc(Cl)c1

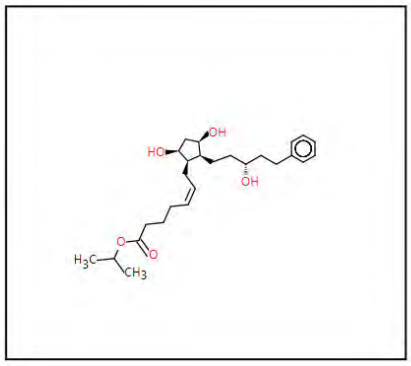
Example



A	B	C
10	9	22

Similarity = 53.659% Details

<->

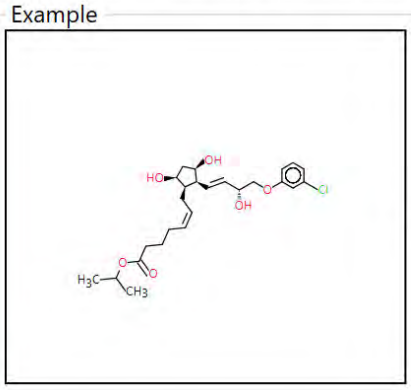


Isopropyl Cloprostenate and Travoprost Comparison

Structure

CC(C)OC(=O)CCC\C=C/C[C@H]1[C@@H](O)C[C@@H](O)[C@@H]1\C=C\[C@@H](O)COc1cccc(Cl)c1

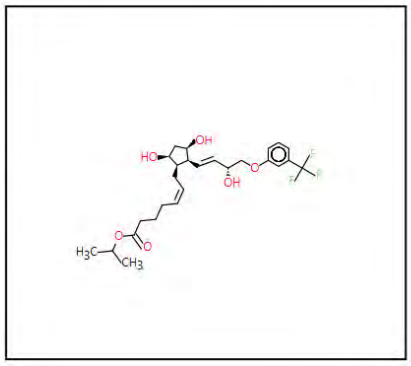
Example



A	B	C
2	5	30

Similarity = 81.081% Details

<->

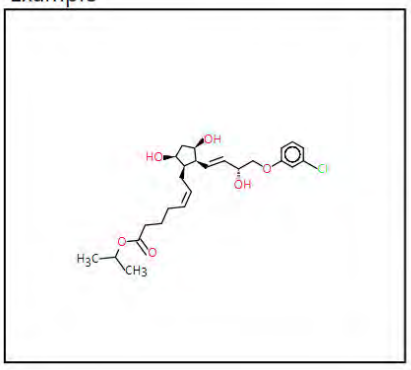


Isopropyl Cloprostenate and Bimatoprost Comparison

Structure

CC(C)OC(=O)CCC\C=C/C[C@H]1[C@@H](O)C[C@@H](O)[C@@H]1\C=C\[C@@H](O)COc1cccc(Cl)c1

Example



A	B	C
11	9	21

Similarity = 51.220% Details

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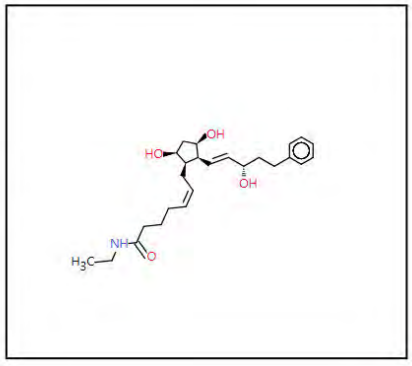
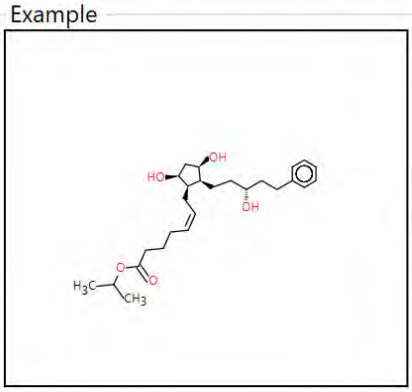


Latanoprost and Travoprost Comparison

Structure

CC(C)OC(=O)CCCC=CCC1C(O)CC(O)C1CCC(O)CCc1ccccc1

Example



A	B	C
9	13	22

Similarity = 50.000% Details

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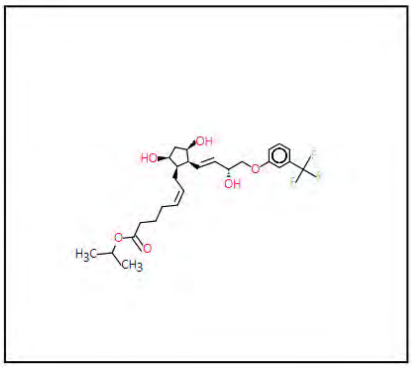
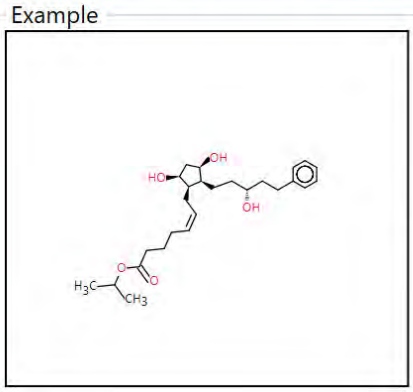


Latanoprost and Bimatoprost Comparison

Structure

CC(C)OC(=O)CCCC=CCC1C(O)CC(O)C1CCC(O)CCc1ccccc1

Example



A	B	C
9	8	22

Similarity = 56.410% Details

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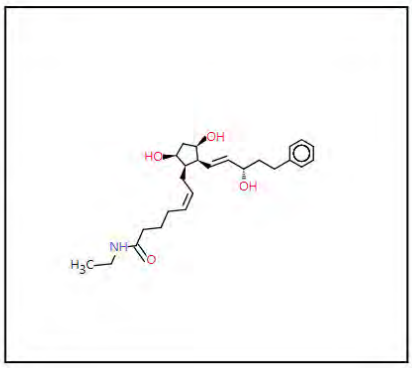
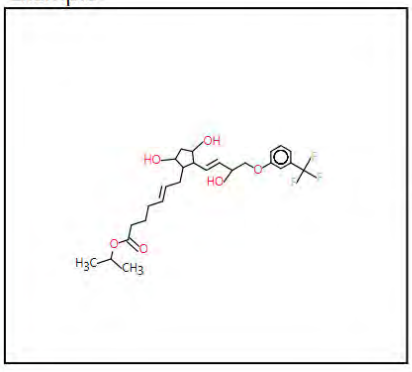


Travoprost and Bimatoprost Comparison

Structure

CC(C)OC(=O)CCCC=CCC1C(O)CC(O)C1C=CC(O)COc1cccc(c1)C(F)(F)F

Example



A	B	C
14	9	21

Similarity = 47.727% Details

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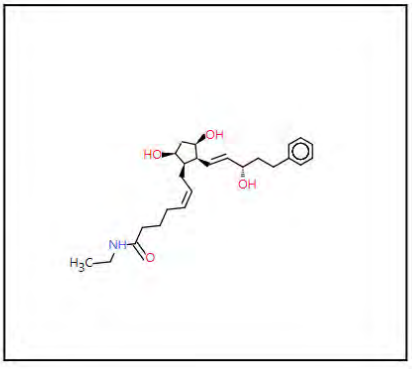


APPENDIX E: MARGIN OF SAFETY CALCULATIONS

The margin of safety (MOS) is the ratio of an acceptable intake/exposure level (such as the ADI, RfD, WEEL, or DNEL) to an estimated human exposure level or dose. Larger MOS are generally more health protective (ChemSafetyPro 2019).

For cosmetics ingredients, the MOS is calculated by dividing the highest NO(A)EL value that is lower than the lowest LO(A)EL of the cosmetic substance (identified throughout this safety assessment as a point of departure) under study by its exposure through use of the cosmetic (SCCS 2023). If the point of departure is obtained from a study in which dosing was conducted on fewer than 7 days per week, the NOAEL or LOAEL is adjusted for daily exposure, and the adjusted N(L)OAEL is used in MOS calculations. Similarly, if the point of departure is either a LOAEL or is obtained from a systemic toxicity study less than 90 days' duration, a safety factor of 3 is applied, except if the critical effect in a shorter-term study is on a reproductive or developmental endpoint, in which case the safety factor is not needed (SCCS 2023).

As outlined in SCCS (2023), for most cosmetic ingredients evaluated by the SCCS, the exposure is compared to an oral NOAEL or LOAEL. Generally, the N(L)OAEL identified in a toxicity study corresponds to the dose that has been administered orally (i.e., the external dose). However, for cosmetic ingredients, the MOS is calculated by dividing the internal (systemic) dose (designated N(L)OAELsys) by the SED. For cosmetic ingredients, SCCS considers not more than 50% of an orally administered dose to be systemically available. Thus, in the absence of data supporting a different value, 50% of the orally administered dose is the default oral absorption value. The N(L)OAELsys is derived from the N(L)OAEL by dividing by a factor of 2: N(L)OAELsys = N(L)OAEL/2). If there is information to suggest poor oral bioavailability, a default value of 10% oral absorption may be considered (SCCS 2023).

After deriving NOAELsys, the MOS is calculated as follows:

$$\text{MOS} = \frac{\text{NOAELsys or LOAELsys}}{\text{SED}}$$

$$\textbf{SED} = [\textbf{A}\ (\text{mg/day}) * \textbf{C}\ (\%)/100 * \textbf{DA}\ (\%)/100 * \textbf{DR}] / \textbf{BW}$$

The SED is a composite value that takes into account specific exposure conditions:

A is the estimated daily exposure to the cosmetic product in mg/day,
C is the concentration of the substance of interest,
DA is dermal absorption,
DR is dermal retention, and
BW is body weight.

In the absence of substance-specific data for dermal absorption or for formulations containing dermal penetration enhancers, a dermal absorption factor of 50% is included

in the SED calculation as a conservative approach in accordance with SCCS (2023) guidelines. For leave-on, semi leave-on, and rinse-off products, a dermal retention factor of 1.0, 0.1, and 0.01, respectively, is additionally included in the SED calculations (SCCS 2023).

A sufficiently large MOS demonstrates safety of the raw material when used in the specific product application. For purposes of establishing safety, an acceptable MOS of at least 100 is required (factors of 10, each accounting for extrapolating from animal to human and interhuman variability), with additional factors added to account for items such as use of a lowest observable adverse effect level (LOAEL) (additional factor of 3), or use of a subacute study (additional factor of 3). In these cases, the acceptable MOS is 300. In contrast, if the POD is derived from a clinical or epidemiological study, the factor of 10 for animal to human extrapolation is no longer necessary, and an MOS of 10 would be acceptable.

Table E-1 identifies the point of departure, the PODsys, an SED, and subsequent MOS for IPC at a use level of 0.005% in a cosmetic lash serum.

Table E-1: Margin of Safety Calculation for Isopropyl Cloprostenate in a Cosmetic Lash Serum

Parameter	Explanation	Value	Reference
A	Estimated daily exposure to cosmetic lash serum (mg/day)	0.28	Avomeen (2020)
C	Concentration of IPC (as decimal)	0.00005	Confidential
DA	Dermal absorption (as decimal)	0.5	SCCS (2023)
DR	Dermal retention (as decimal)	1	SCCS (2023)
BW	Body weight (kg)	60	SCCS (2023)
SED	Systemic exposure dose (mg/kg/day) = C * A * DA * DR / BW	1.17E-07	Calculated
POD	Point of departure (mg/kg/day), a LOAEL from a 3-generation study with surrogate travoprost.	0.00012	U.S. FDA 2000
PODsys	No adjustment to the POD needed because the study providing the LOAEL used parenteral exposure.	0.00012	U.S. FDA 2000
MOS	MOS = PODsys / SED	**1,029**	Calculated

[redacted]

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: November 2, 2023

SUBJECT: Isopropyl Cloprostenate

Anonymous. 2023. Use instructions for a cosmetic product containing Isopropyl Cloprostenate.

HOW TO USE

Use once daily in the PM, on upper lash line only.

1. Remove makeup and cleanse face.
2. Dry eyelids and lashes completely.
3. Apply serum only along the upper lash lines. Dip the brush once per eye and wipe excess product off the brush before applying.
4. Gently wipe off excess serum from eyelids or lashes. Do not wash your face or eye area after applying. Wait ~90 seconds for serum to dry before going to sleep or applying other products near the eye area.

Do not wash your face or eye area after applying. Wait approximately 90 seconds for serum to dry before going to sleep or applying other products around your eye area. Avoid eyelids and lashes when applying any other product to the eye area, including eye cream.

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: November 2, 2023

SUBJECT: Isopropyl Cloprostenate

Anonymous. 2023. Use concentration for a cosmetic product containing Isopropyl Cloprostenate.

Isopropyl Cloprostenate is used in an eye lash serum at a concentration of 0.0075%.

Tafluprost Appendix

Summary data table

Table 1. Structures of Ethyl Tafluprostamide and Tafluprost[1]

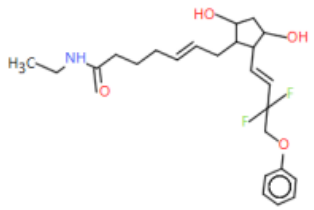
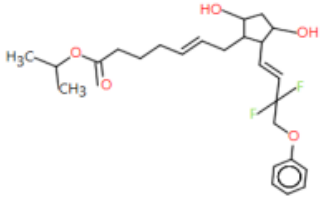
	Ethyl Tafluprostamide	Tafluprost
Structure		
CAS No.	118581-52-8	209860-87-7

Table 2. Tafluprost data summary

Study/Method of Admin.	Study Details	Results	References
ACUTE TOXICITY			
acute: oral	• test substance: tafluprost (0, 10, 30, 100 mg/kg bw); vehicle not reported • Sprague-Dawley rats (5/sex/group) • single oral dose (method of oral administration not stated); observation for 14 d	• no mortalities or marked changes in body weight, food intake, water intake, or gross pathology • one animal in the 10 mg/kg dose group displayed hunched posture, wasted appearance, chest sores, and loss of chest fur	[2,3]
acute: intravenous	• test substance: tafluprost (0, 1, and 3 mg/kg bw); vehicle not reported • Sprague-Dawley rats (5/sex/group) • single IV dose; observation for 14 d	• no signs of toxicity observed	[2,3]
acute: intravenous	•test substance: tafluprost (0, 0.0003, 0.003, 0.03 mg/kg bw); vehicle not reported • Beagle dogs (2 males/group) • single IV dose; observation for 14 d	• no mortalities or marked changes in body weight, hematology parameters, urinalysis, body temperature, or ophthalmologic effects • salivation, vomiting, moderate miosis, irregular respiration, and increased heart rate observed at ≥ 0.003 mg/kg bw • severe miosis and elevated blood pressure observed at 0.03 mg/kg bw • no effects observed at the lowest tested dose	[2,3]
REPEATED DOSE TOXICITY			
repeated dose: intravenous	• test substance: tafluprost (10, 30, or 100 µg/kg); vehicle not reported • rat (12/sex/group) (strain not specified) • 28 d IV treatment; 14 d recovery period	• no treatment-related mortality, clinical toxicity, or pathological effects observed • slightly lower hemoglobin concentrations, erythrocyte numbers, and packed cell volume observed in males at 100 µg/kg bw/d; however no significant, irreversible effects were observed • NOAEL = 100 µg/kg bw/d	[2]
repeated dose: intravenous	• test substance: tafluprost (0, 0.1, 1, and 10 µg/kg bw/d); vehicle not reported • dog (4/sex/group) (strain not specified) • 28 d IV treatment; 14 d recovery period	• no treatment-related mortality, clinical toxicity, or pathological effects observed • slight miosis, sporadic salivation, and vomiting observed in both sexes at 1 µg/kg bw/d • salivation, vomiting, miosis, increased respiratory rate, increased heart rate, and prolonged QTc interval observed in both sexes at 10 µg/kg bw/d • reversible increased alanine aminotransferase activity, increased urine volume, and decreased urinary potassium concentration observed in both sexes	[2,3]
repeated dose: intravenous	• test substance: tafluprost (98.6% purity) in 0.9% sodium chloride (0, 10, 30, and 100 µg/kg bw/d) •Crl:CD (SD) IGSBR rats (15/sex/group) •26-wk IV treatment	• 15 animals died during treatment period •hematological effects (e.g., increased mean cell hemoglobin) observed with 30 and 100 µg/kg bw/d; similar effects observed in males at the highest dose level, to a lesser extent; platelet numbers a slightly low at all dose levels in males; platelet volume and	[2,3]

Table 2. Tafluprost data summary

Study/Method of Admin.	Study Details	Results	References
		distribution slightly high in males treated with 30 and 100 µg/kg bw/d; these effects were observed in high-dose females, to a lesser extent • reduced numbers of early erythropoietic and myelopoietic cell types and increased late erythropoietic and myelopoietic cell times observed at 100 µg/kg bw/d • slight but significant increase in mean spleen weights observed in males at 100 µg/kg bw/d and in females treated with 30 and 100 µg/kg bw/d • dose-related hyperostosis and myelofibrosis in femoral and sternum bone marrow observed • increased hematopoiesis occurred as foci in liver parenchyma in all dose groups without clear dose-dependent trend for severity • dose-dependent increase in incidence and severity of femoral bone marrow hematopoiesis apparent in males • dose-dependent increase in incidence and severity of corticomedullary mineralization observed in females	
repeated dose: intravenous	• test substance: tafluprost (98.6% purity) in 0.9% sodium chloride (0, 0.1, 1, and 10 µg/kg bw/d)' • Beagle dogs (4/sex/group) • 39-wk IV treatment	• 1 animal displaying hepatic failure symptoms killed during week 19 • clinical signs (e.g., salivation, vomiting) observed at 10 µg/kg bw/d; symptoms less frequent at 1 µg/kg bw/d • slight to moderate miosis observed in high-dose animals • dose-dependent increase in heart rate evaluated shortly after dosing • slight but significant increased mean arterial blood pressure observed 30 min after dosing during weeks 4, 26, and 39 in high-dose females; mean arterial blood pressure significantly increased 5 min after dosing in week 26 in males treated with 1 and 10 µg/kg bw/d • slight but significant increase in respiratory rate observed at the highest dose 30 min after dosing (transient) • salivary gland weight increased compared to controls • minor adrenal cortical eosinophilia and acinar cell hypertrophy in salivary glands observed at 10 µg/kg bw/d	[2,3]
repeated dose: subcutaneous	• test substance: tafluprost (99.5% purity); vehicle not reported (0, 30, 10, 30 and 100 µg/kg bw/d) • Crl:CD-1(ICR)BR mice (12/sex/group) • 90-d SC treatment	• no treatment-related mortality, or clinical, hematological, or pathological toxicity observed at any dose level	[2,3]
repeated dose: subcutaneous	• test substance: tafluprost (99.5% purity); vehicle not reported (0, 3, 10, and 30 µg/kg bw/d) • Crl:CD(SD)IGSBR rats (10/sex/group) • 90-d SC treatment	• no dose-dependent effects were observed relating to mortality, clinical, or hematological toxicity • minor increase in incidence and severity of hematopoiesis in spleen observed in high-dose males and females (not statistically significant) • minor increase in incidence of severity of corticocomedullary mineralization observed in high-dose females (not statistically significant)	[2,3]
repeated dose: subcutaneous	• test substance: tafluprost (100.7% purity); vehicle not reported (0, 10, 30, and 100 µg/kg bw/d) • Crl:CD-1(ICR)BR rats (51/sex/dose) • 78-wk SC treatment	• no adverse effects observed at any dose level	[2]
repeated dose: subcutaneous	• test substance: tafluprost in isotonic sodium chloride (0, 3, 9, and 30 µg/kg bw/d) • Crj:CD(SD)IGSBR rats (60/sex/dose) • 24-mo SC treatment	• significant reduction in body weight at 30 µg/kg bw/d in both sexes • hyperostosis of the sternum and femur ins some animals and increased incidence of extramedullary hematopoiesis in the spleen of males at all dose levels	[2]

Table 2. Tafluprost data summary

Study/Method of Admin.	Study Details	Results	References
repeated dose: ocular	• test substance: ophthalmic solution containing tafluprost; vehicle not reported (0, 0.0005, 0.005, and 0.05%) • dose levels: 0, 0.15, 1.5, and 15 µg/left eye/time (2x daily) • Cynomolgus monkeys (3/sex/group) • 28-d ocular treatment	• no treatment related mortality or effects relating to clinical, pathological, or hematological toxicity observed • local changes in iris color observed in 2 animals at 1.5 µg • transient corneal precipitates, anterior chamber cells, superficial corneal opacity and erosion, positive epithelial topical ocular fluorescein staining, and red conjunctiva were occasionally observed at the 2 highest doses (some effects were also observed in untreated eyes (e.g., erosion, positive fluorescein staining, and red conjunctiva)	2,3
repeated dose: ocular	• test substance: ophthalmic solution containing tafluprost; vehicle solution of poylsorbate 80, sodium phosphate monobasic dihydrate, tetrasodium EDTA, glycerin, benzalkonium chloride, and sodium chloride (0, 0.0005, 0.005, and 0.05%) • dose levels: 0, 0.15, 1.5, and 15 µg/left eye/time (2x daily) • Cynomolgus monkeys (4/sex/group) • 13-wk ocular treatment; 28-d recovery period	• no treatment related mortality or effects relating to clinical, pathological, or hematological toxicity observed • no treatment-related effects during slit-lamp examinations or electroretinograms • reversible sunken eyelids, punctuate fluorescein staining of cornea, and tendency to reduce intraocular pressure, irreversible iris color darkening, observed at all dose levels (effects were considered to be cosmetic and not toxicologically significant)	2,3
repeated dose: ocular	• test substance: ophthalmic solution containing 0.0045% tafluprost (102.4% purity); vehicle solution containing benzalkonium chloride (0 and 100%) • dose volume 30 µl/left eye • Cynomolgus monkeys (3/sex/group) •13-wk ocular treatment	• no treatment related mortality or effects relating to mortality, clinical, pathological, or hematological toxicity observed • treatment-related eye color changes observed (considered to be cosmetic and not toxicologically significant) • slight tendency for decreased intraocular pressure observed in untreated eyes (reduced intraocular pressure within normal variation)	2
repeated dose: ocular	• test substance: ophthalmic solution containing tafluprost; vehicle solution of poylsorbate 80, sodium phosphate monobasic dihydrate, tetrasodium EDTA, glycerin, benzalkonium chloride, and sodium chloride (0, 0.0005, 0.005, and 0.05%) • dose levels: 0, 0.15, 1.5, and 15 µg/left eye/time (2x daily) • Cynomolgus monkeys (4/sex/group) •52-wk treatment	•sunken, dark iris color, blue-gray discoloration in ¾ of animals of both sexes throughout study period (all dose levels) • animals in all dose groups demonstrated increased melanocyte pigment in the iris stroma of treated eyes • all ocular effects considered cosmetic and not toxicologically significant • statistically significant decrease in intraocular pressure observed in males and females in high-dose group during week 26 • significant increase in mean thymus/brain weight noted in 2 males of intermediate and high dose groups	2,3
DEVELOPMENTAL AND REPRODUCTIVE TOXICITY (DART)			
DART: intravenous	• test substance: tafluprost (98.6% purity); vehicle: 0.9% sodium chloride (0, 10, 30, and 100 µg/kg bw/d) • dose volume: 10 ml/kg • Crl:CD(SD)IGSBR rats (24/sex/group) • treatment period: 2 wk before mating, throughout mating period, until day 6 gestation for females, or until necropsy in week 9 of treatment period for males; IV treatment	• 2 animals died on day 10 and 15 at 100 µg/kg bw/d • pale extremities observed in all dose groups (transient) • no treatment-related effects observed on estrous cycle or fertility parameters (e.g., fertility index, preimplantation loss); all parameters similar to controls • no treatment-related histopathological abnormalities of the reproductive organs observed • no abnormalities in sperm cells	2,3
DART: intravenous	• test substance: tafluprost (98.6% purity); vehicle: 0.9% sodium chloride (0, 3, 10, and 30 µg/kg bw/d) • dose volume: 10 ml/kg • Crl:CD(SD)IGSBR rats (24/females/group) • treatment period: gestation days 6-17; IV treatment	• no maternal toxicity observed at any dose level • 20% mean post-implantation loss rate in high-dose group (control: 8.6% loss) • total litter loss noted in 2 rats of high-dose group • statistically significant increase in number of late intrauterine deaths in high-dose group • fetal weight significantly decreased at 10 and 30 µg/kg bw/d compared to controls	2,3

Table 2. Tafluprost data summary

Study/Method of Admin.	Study Details	Results	References
		• visceral malformations (e.g.. renal pelvic cavitation) observed in all treated groups (not dose-dependent) • skeletal malformations observed at 10 and 30 µg/kg bw/d • dose-dependent significant increase in number of litters with unossified 5th sternebrae observed at 10 and 30 µg/kg bw/d	
DART: intravenous	• test substance: tafluprost (101.5% purity); vehicle: 0.9% sodium chloride (0, 0.3, 1, 3, and 10 µg/kg bw/d) • dose volume: 3 ml/kg • Crj:CD(SD)IGS rats (22 females/group) • treatment period: gestation day 6 to lactation day 20 (35 d total); IV treatment	• no maternal toxicity observed in F0 generation; no significant differences in fertility parameters (e.g., number of implantation sites) or gestation parameters (e.g., gestation index) • poor nursing behavior in treated animals resulted in decreased F1 offspring viability at ≥ 1 µg/kg bw/d and delayed pinna unfolding • increased F1 newborn mortality and decreased body weight observed at 10 µg/kg bw/d • in F2 generation, no treatment-related effects were observed for embryonic mortality, number of corpora lutea, implantations, live F2 embryos, or preimplantation loss	[2,3]
DART: intravenous	• test substance: tafluprost (98.6% purity); vehicle: 0.9% sodium chloride (0, 0.03, 0.1, and 3 µg/kg bw/d) • dose volume: 1 ml/kg • Crl.NZW/Kbl BR rabbits (24 females/group) • treatment period: gestation days 1-19; IV treatment	• no treatment-related mortality, changes in body weight, or mean gravid uterine weight • distended urinary bladders and very low food consumption in one control female and 3 intermediate-dose animals • no treatment-related in mean number of fetuses, group mean pre- and post-implantation loss, mean fetal weight, litter weight, and placental weight • similar number of fetuses with external, visceral, and skeletal variations observed in all dose groups compared to control • statistically non-significant increased number of fetuses with abnormally pale contents in gallbladder and/or non-eruption of the incisors observed in all treated groups	[2,3]
CARCINOGENICITY			
carcinogenicity: subcutaneous	• test substance: solution containing 0.0015% tafluprost; vehicle: 0.9% sodium chloride (0, 3, 9, and 30 µg/kg bw/d) • dose volume: 3 ml/kg • Crj:SD(SD)IGSBR rats (60/sex/group) • 13-wk SC treatment	• incidence of leukemia similar in control and high-dose groups • absolute and relative adrenal weights increased significantly in males at 30 µg/kg bw/d; however, no correlation observed with histopathological evidence of adrenal tumors in any treated group • no neoplastic lesions observed during histopathological evaluation were considered to be treatment-related • no dose-related pattern was observed in tumor incidence changes	[2,3]
carcinogenicity: subcutaneous	• test substance: solution containing 0.0015% tafluprost; vehicle: 0.9% sodium chloride (0, 10, 30, and 100 µg/kg bw/d) • dose volume: 10 ml/kg • Crl:CD-1(ICR)BR mice (51/sex/group) • 18-mo SC treatment	• non-significant increased incidence rates for neoplastic lesions observed in high-dose group compared to controls	[2,3]
CLINICAL TRIALS			
clinical trial: phase II	• test substance: preservative-containing tafluprost; vehicle not reported (0.001, 0.0025, and 0.005%) • evaluation in patients with open-angle glaucoma or ocular hypertension (142 patients) • 28-d treatment period; eye drop • control patients treated with either the vehicle or latanoprost (0.005%)	The incidence of ocular adverse events (AEs) was 40.0% with 0.001% tafluprost, 50% with 0.0025%, and 43.0% with 0.005% compared to 16.7% with placebo and 40.0% with latanoprost.	[3,4]

Table 2. Tafluprost data summary

Study/Method of Admin.	Study Details	Results	References
clinical trial: phase II	• test substance: preservative-containing tafluprost; vehicle not reported (0.0003, 0.0015, 0.0025%) • evaluation in patients with open-angle glaucoma or ocular hypertension (139 patients) • 28-d treatment period; eye drop • control patients with timolol (0.05%) or latanoprost (0.005%)	There was no major difference in the incidence of ocular AEs between the doses (39.3%, 36.7%, 37.9% for 0.0003%, 0.0015%, and 0.0025% tafluprost respectively, compared to 41.4% for timolol and 32.1% for latanoprost) (conjunctival hyperemia most commonly reported adverse effect).	[3,4]
clinical trial: phase III	• test substance: unpreservative and preservative-containing tafluprost; vehicle not reported (0.0015%) • evaluation in patients with open-angle glaucoma or ocular hypertension (43 patients) • 2 treatment periods: preserved followed by unpreserved formulation or unpreserved followed by preserved formulation (separated by 4-wk washout period); 1x/daily; 4 wk treatment periods; eye drop	Ocular AEs were more frequent in the preservative free group (20 AEs in 11 subjects, 26%) compared to the preserved formulation group (seven ocular AEs in six subjects, 14%), the most common of which was conjunctival hyperemia occurred in eight compared to two subjects respectively.	[3-5]
clinical trial: phase III	• test substance: tafluprost solution (0.0015%) alone or with timolol solution (0.5%); vehicle not reported •evaluation in patients with open-angle glaucoma or ocular hypertension (489 patients) • 4 week treatment period; eye drops	No ocular or systemic effects observed.	[1]
clinical trial: phase IIIb	• test substance: preservative-free tafluprost; vehicle not reported (0.0015%) • evaluation in patients with open-angle glaucoma or ocular hypertension (190 patients) • 4-wk treatment period; eye drop	Incidence of conjunctival hyperemia w (6.45%) and eye pruritus (7.53%), eye irritation (6.45%) and conjunctivitis (9.68%)	[6]
clinical trial: phase III	• test substance: tafluprost; vehicle not reported (0.0015%) • evaluation in patients with open-angle glaucoma or ocular hypertension (185 patients) • 12 wk treatment period; eye drop • treatment with either timolol (0.5%) + tafluprost (0.0015%) or timolol (0.5%) + vehicle	There were more AEs (44.8% versus 34.8%) and more mild ocular AEs (41.7% versus 29.2%) in subjects treated with tafluprost+ timolol compared to those treated with vehicle+ timolol. The incidence of conjunctival hyperaemia and eye pruritus in the tafluprost+ timolol group was 18.8% and 14.6%, respectively, compared to 13.5% and 0% in the vehicle+ timolol group.	[3,4,7]
clinical phase: phase III	• test substance: preservative-free tafluprost; vehicle not reported (0.0015%) • evaluation in patients with open-angle glaucoma or ocular hypertension (306 patients) • 12 wk treatment period; eye drop • another group treated with preservative-free timolol (0.05%)	The adverse events of conjunctival and ocular hyperaemia (2.8 and 1.6%, respectively) were reported more frequently in the PF tafluprost group than in the preservative-free timolol group in which no conjunctival hyperaemia and 0.6% ocular hyperaemia were reported. Photophobia was reported with an incidence of 1.3% in the preservative-free tafluprost group compared with the preservative-free timolol group, which had none. Eye pruritus was reported in 6 (1.9%) patients and 3 (0.9%) patients in the tafluprost and timolol group, respectively. Serious adverse events occurred in 2 patients (0.6%) treated with preservative-free tafluprost (atrial fibrillation and myocardial infarction); not thought to be treatment related.	[3,4,6]
clinical trial: phase IIIb	• test substance: preservative-free tafluprost; vehicle not reported (0.0015%)	There were 11 subjects with 18 ocular AEs (7.0%) and 52 non-ocular AEs in 36 subjects (22.8%). Severer non-ocular adverse effects observed in 2.5% of subjects. There was a reduction in the proportion of subjects with ocular symptoms (irritation, foreign body sensation,	[3]

Table 2. Tafluprost data summary

Study/Method of Admin.	Study Details	Results	References
	• evaluation in patients with open-angle glaucoma or ocular hypertension (158 patients) • patients evaluated after switching from latanoprost (0.005%) preservative-free tafluprost (0.0015%) •12 wk treatment period; eye drop	tearing, itching, dry eye sensation) after 12 weeks of treatment.	
clinical trial: phase III	• test substance: preservative containing tafluprost; vehicle not reported (0.0015%) • evaluation in patients with open-angle glaucoma or ocular hypertension (162 patients completed full study period) • 12 mo treatment period; eye drop • another group treated with timolol 0.5%	The incidence of AEs was greater in tafluprost-treated than in timolol-treated subjects after 12 months of treatment. Tafluprost-treated subjects had more eye disorders (50.9% versus 44.0%) including conjunctival hyperaemia (18.0% versus 6.3%), eye pruritus (9.0% versus 2.6%), dry eyes (5.6% versus 3.7%), and foreign body sensation in the eyes (3.7% versus 2.1%). Systemic events that occurred more in tafluprost subjects compared to timolol were headache (13.5% versus 6.8%), nausea (3.7% versus 1.0%), hypercholesterolaemia (7.1% versus 3.7%) and cough (7.9% versus 4.2%).	3,4
clinical trial: phase III	• test substance: preservative containing tafluprost; vehicle not reported (0.0015%) • evaluation in patients with open-angle glaucoma or ocular hypertension (185 patients completed full study period) • 24 mo treatment period; eye drop • another group treated with preservative-containing latanoprost 0.005%	Overall incidence of adverse effects greater with tafluprost than with latanoprost after 24 mo of treatment. The tafluprost-treated patients reported more eye disorders (46.5% versus 43.9%), in particular conjunctival hyperaemia (9.3% versus 5.7%), eye pain (7.1% versus 2.7%), eye pruritus (3.7% versus 1.1%), growth of eyelashes (6.3% versus 4.2%), blurred vision (2.6% versus 1.1%), and visual field defect (6.7% versus 4.9%).	2-4,8
Post-marketing experience	• post-marketing experience of tafluprost eye drop users	• most common adverse effects include ocular hyperemia and eye redness • no marked systemic effects observed	4,9-12

NOAEL = no-observed-adverse-effect-level

*"Bailey, 2023" is a summary document that includes all of the information in the table above, this reference can be found in the Prostaglandins Analogues build as *data_1_ProstaglandinAnalogues_122023*

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