Cosmital SA

Study number : KP 165
Code of test item : **23081**

Marly, 19.10.2006 RD-EPSK/TSi

Distribution list: Archives (original)
RD-PS (copy)
Laboratory/TSi (copy)

FINAL REPORT

CUTANEOUS ABSORPTION OF

1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate (=WR23081) in a typical hair dye formulation with hydrogen peroxide and reaction partner (WR18247)

THROUGH PIG SKIN *IN VITRO*

Sponsor:

Test Facility: COSMITAL SA/RD-EPSK
Rte de Chésalles 21
CH - 1723 Marly 1

Head of Test Facility: S. Pfuhler, Toxicologist, Ph. D.
(Substitute: H.-J. Braun, Chemist, Ph. D.)

Study Director: T. P. Sieber, Chemist, Ph. D.

INDEX

page

Statement of compliance with GLP 3

Statement of Quality Assurance Unit (QAU) 4

1 GENERAL INFORMATION 5

1.1 Guidelines 5
1.2 Time schedule 5
1.3 Archiving 5
1.4 Deviation 5

2 SUMMARY 6

3 INTRODUCTION 8

4 MATERIALS AND METHODS 9

4.1 Test item 9
4.2 Test substances 10
4.3 Test-Formulations 12
4.4 Test system (Test chamber & skin samples) 12
4.5 Procedure of the absorption experiment 13
4.6 Sampling procedures 14
4.7 Sample storage 15
4.8 Analytical procedures (including LOD and LOQ) 15

5 RESULTS AND DISCUSSION 17

6 CONCLUSION 20

7 REFERENCES 21

8 ANNEXES 22

I. Statement of GLP Compliance of the Swiss Monitoring Authorities
II. Certificate of analysis of radiolabelled 2-Amino-4-hydroxyethylamino anisole sulfate
III. Formula Color cream formulation containing 1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate
IV. Certificate of analysis of unlabelled 2-Amino-4-hydroxyethylamino anisole sulfate
V. Receptor fluid fractions, overview of the results of the radioanalytical quantification (amounts of 2-Amino-4-hydroxyethylamino anisole sulfate)
VI. Formula 1 6 %

Statement of compliance with Good Laboratory Practice (GLP)

This study has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice*, adopted May 18th, 2005 [RS 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97)186/Final].

*Exception: Preliminary experiments to establish the analytical methods.

.....
Study Director
(T. P. Sieber, Ph. D.)

19.10.2006
.....
date

Statement of Quality Assurance Unit (QAU)

This study has been inspected in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted May 18th, 2005 [RS 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97)186/Final].

This final report accurately reflects the raw data obtained during the experiments.

Date:	Type and phase of inspection:	Information to Study Director:	Information to the Head of Test Facility:
07.08.2006	Study plan / valid SOPs:	07.08.2006	18.08.2006
18.08.2006	Study based:	18.08.2006	18.08.2006
	Management of test item		
23.08.2006	Facility based:	28.08.2006	28.08.2006
15.09.2006	Draft Final Report:	15.09.2006	15.09.2006
19.10.06	Final report:	19.10.06	19.10.06

Head of Quality Assurance Unit

19.10.2006
(date)

1 GENERAL INFORMATION

1.1 Guidelines

This study using an *in vitro* method with pig skin has been performed according to the OECD Guideline No. 428 for the testing of chemicals [1] and the Guidance document for the conduct of skin absorption studies [2] and the Test Guidelines of the COLIPA Task Force for „*In Vitro* Assessment of Percutaneous Absorption and Penetration of Cosmetic Ingredients" [3] comprising a Standard Protocol for *in vitro* Cutaneous Absorption/Penetration with Pig Skin.

1.2 Time schedule

Order:	21.07.2006
Study plan:	27.07.2006
Experimental starting date:	14.08.2006*
Experimental completion date:	31.08.2006
Final Report:	19.10.2006

*Preliminary experiments (non-GLP) which were necessary to establish the analytical methods, were performed prior to this date.

1.3 Archiving

All raw data and notes as described in SOP N° KP 6.1, the samples of the test item and the originals of the study plan and of the final report are stored in the archives of Cosmital SA, Rte de Chésalles 21, CH-1723 Marly, for at least 30 years (or up to the expiration date).

1.4 Deviation

Contrary to the description in the study plan, the radiolabelled 2-Amino-4-hydroxyethylamino anisole sulfate was dissolved in 200 µl water containing 0.3 % ascorbic acid and 0.4 % sodium sulfite. However, this deviation does not affect the experimental outcome of this study.

2 SUMMARY

The cutaneous absorption of 1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate (WR23081) in a typical hair dye formulation in the presence of hydrogen peroxide and a reaction partner was investigated *in vitro*, using pig skin preparations, which were continuously rinsed from underneath with physiological receptor fluid at a temperature of 32 ± 2 °C. Two independent experiments were performed with 6 diffusion cells per experiment. For calculations, the mean value of all valid skin samples (n=8) in contact with 1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate (WR23081) in a typical hair dye formulation in the presence of hydrogen peroxide and a reaction partner was used.
The integrity of each skin preparation was determined by examination of penetration characteristics with tritiated water resulting in 0.8 to 3.7 % of the applied dose found after 4 hours in the receptor fluids, which was within the limit of acceptance (≤ 2.0 %) for 8 skin samples used for determination of skin penetration. The skin samples with skin integrity values above 2 % of the applied dose (4 skin samples) were not within the limit of acceptance (vide supra) and were not taken into consideration for the calculation of the mean.

After checking the skin integrity, 400 mg of the formulation (= 100 mg/cm^2), containing 1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate, was applied to the skin samples (= 1.5 mg of WR23081/cm^2) for 30 minutes and subsequently washed off with water and shampoo. The determination of the amount of 2-Amino-4-hydroxyethylamino anisole sulfate in the washings (= amount dislodgeable from the skin surface) was performed by measuring the radioactivity by means of a scintillation counter. At 16, 24, 40, 48, 64 and 72 hours, the content of 2-Amino-4-hydroxyethylamino anisole sulfate was determined in the receptor fluid by the same method. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the radioactivity was quantified by means of a scintillation counter.

The majority of the test substance could be found in the rinsing solutions (1.341 ± 0.051 mg/cm^2). Small amounts of 2-Amino-4-hydroxyethylamino anisole sulfate could be found in the upper skin (2.352 ± 0.824 µg/cm^2), in the lower skin (0.303 ± 0.219 µg/cm^2) and in the fractions of the receptor fluid collected within 72 hours (0.409 ± 0.223 µg/cm^2).

The mass balance of the test substance resulted in values of 92.20 to 103.31 % recovery for all (8) skin samples with acceptable integrity (as measured by the penetration characteristics of tritiated water).

The results of the cutaneous absorption experiment are summarised in table 1 (for 2-Amino-4-hydroxyethylamino anisole sulfate).

Table 1: Summary of the cutaneous absorption of 2-Amino-4-hydroxyethylamino anisole sulfate

Amount of WR 23081 in:	µg/cm² (mean ± S.D, n=8)			%* (mean ± S.D, n=8)		
Receptor fluid (72 hours)	0.409	±	0.223	0.028	±	0.015
Lower skin (72 hours)	0.303	±	0.219	0.021	±	0.015
Upper skin (72 hours)	2.352	±	0.824	0.162	±	0.057
Rinsing solution (after 30 min.)	1340.92	±	50.57	92.69	±	3.46
Total balance (recovery)**	1446.71	±	7.86	96.59	±	3.53

* Corrected for individual applied dose; ** Total is corrected for losses on tips

With respect to the receptor fluid, only small amounts of 2-Amino-4-hydroxyethylamino anisole sulfate were detectable within the first fractions collected during 72 hours (see Annex V), indicating that no further 2-Amino-4-hydroxyethylamino anisole sulfate is available from a potential reservoir of the skin compartments.

Under the assumption that a depot effect is absent, a maximum amount of **0.712 ± 0.313 µg/cm²** of **2-Amino-4-hydroxyethylamino anisole sulfate** is considered as **biologically available** (n=8, three donors; receptor fluid + lower skin; 0.409 µg/cm² + 0.303 µg/cm²).

Taking into account the recommendations in the guidelines [1 & 2] and the acceptance criteria defined in the study plan (at least eight valid chambers) the study is **valid.**

3 Introduction

The purpose of the present experiment was to determine the *in vitro* penetration of 2-Amino-4-hydroxyethylamino anisole sulfate (WR23081) through dermatomed pig skin over a period of 72 hours for quantitative risk assessment from skin contact with nominal 1.5 % w/w formulation of 2-Amino-4-hydroxyethylamino anisole sulfate in the presence of hydrogen peroxide and reaction partner 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate (A154, WR18247). The experimental conditions, the quantity applied, the vehicle used, and the time of exposure imitated use conditions for humans when applying a typical hair dye formulation.

In vitro methods have been used for many years to measure the transport of dermally applied compounds across full- or split-thickness animal or human skin to a receptor fluid reservoir. It is possible to estimate *in vivo* absorption of a test item by extrapolating from suitable *in vitro* data [1 to 6]. Among several animal species, pig skin from flank and back represents a good model for human skin [7]. Skin viability is not a prerequisite for the studies since the permeability properties of skin are usually maintained after excision from the body and appropriate storage in a freezer for several months. Furthermore, penetration is driven by passive diffusion and there is no evidence for active transport.

To assess the performance and reliability of the test system, the reproducibility of the method is being verified at appropriate time intervals using mannitol as a reference chemical to measure the cutaneous absorption. The experiments with mannitol as positive control are performed with random skin samples from each pig. The continuously increasing set of penetration data from previously performed studies allows to monitor the intra- and inter-individual variability of the test results among the donors (pig skin samples), monitoring the robustness of this biological test system.

With respect to the present data, the intra-individual variability is higher than the inter-individual variability. The mean value found for the penetration of mannitol through pig skin preparations calculated for all samples is 0.76 ± 0.49 % of the applied dose (n=12, water was used as vehicle, data not shown).

The principal diffusion barrier has been identified as the stratum corneum, the integrity of which is controlled in each experiment by ^3H_2O penetration characteristics.

4 MATERIALS AND METHODS

4.1 Test item

Color cream formulation with 3 % 2-Amino-4-hydroxyethylamino-anisole sulfate and equimolar amounts of reaction partner (WR18247)

Identity:	Color cream with Pyrazol DHE and Lehmannblau
Batch N°:	DTF68081
Recipe N°:	85903070
Appearance of cream formulation:	Beige cream
Aggregate state at room temperature:	Paste-like
Storage:	At room temperature
Supplier:	F. Biolley, RD-IPC, COSMITAL SA
Preparation Date:	26.07.2006
Expiration Date:	26.01.2008

The formula is given in Annex III.

4.2 Test substances

Labelled 2-Amino-4-hydroxyethylamino-anisole sulfate

Additional information is given in the corresponding certificate of analysis (Annex II).

Identity:	[ring-U-^{14}C] Lehmann blue
Batch N°:	CFQ14700 Batch 1
Storage:	- 20° C in the absence of moisture, light and air
Supplier:	GE Healthcare, Amersham
Date of Analysis:	30th May 2006
Chemical Identity:	HPLC profile and retention time of the labelled product is identical with that of the standard compound
Radiochemical Purity:	98.8 %
Orientation of radiolabelling:	[U-^{14}C] Uniform labelling of the aromatic ring system
Method of Preparation:	Prepared from [ring-U-^{14}C] phenyl acetate

Special handling

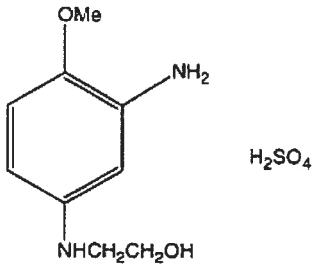
In order to prepare an aliquot of the labeled test substance, the solid product was dissolved in 200 µl of H_2O, containing 0.3 % ascorbic acid and 0.4 % sodium sulfite.
The concentration of the resulting solution is 10 mCi/ml. For each of the two experiments an amount of 40 µl (= 0.2 mCi = 440'000'000 dpm) was used.

Unlabelled 2-Amino-4-hydroxyethylamino-anisole sulfate

Additional information is given in the corresponding certificate of analysis (Annex IV).

Chemical name (INCI):	2-Amino-4-hydroxyethylamino-anisole sulfate
Chemical name (other):	2-Amino-4-(2-hydroxyethyl)amino-anisole-sulfate
Wella raw material N°:	23081
COLIPA N°:	A084
Batch N°:	57 (R96000196)
Appearance:	Pale grey powder
Storage conditions:	At room temperature, protected from light and moisture
Expiry date:	08.01.2008
Purity:	99.6 area% (HPLC at 254 nm)
Solubility in receptor fluid:	92.6 mg/ml (pH = 7.30)
Stability in receptor fluid:	82 % recovery after 3 days (50 mg/ml, in the presence of 0.3 % ascorbic acid and 0.4 % sodium sulfite)
LogP (www.logp.com)	-0.05 (free base)

Structure of the compound:



4.3 Test Formulation

Color cream formulation with 1.5 % of 2-Amino-4-hydroxyethylamino-anisole sulfate (WR23081) in the presence of hydrogen peroxide and reaction partner (WR18247)

At the beginning of the experiment, the test item was prepared as follows and applied to the skin samples.

Components	Batch N°	Sample amount
Color cream formulation with 3 % 2-Amino-4-hydroxyethylamino-anisole sulfate and equimolar amounts of reaction partner	DTF68081	1.5 g
Solution of partially radiolabelled 2-Amino-4-hydroxyethylamino-anisole sulfate	CFQ14700 Batch 1	40 µl**
Welloxon Perfect 6%	F359382*	1.5 g

*as indicated on the package (formula see Annex VI); ** see 1.4 Deviation

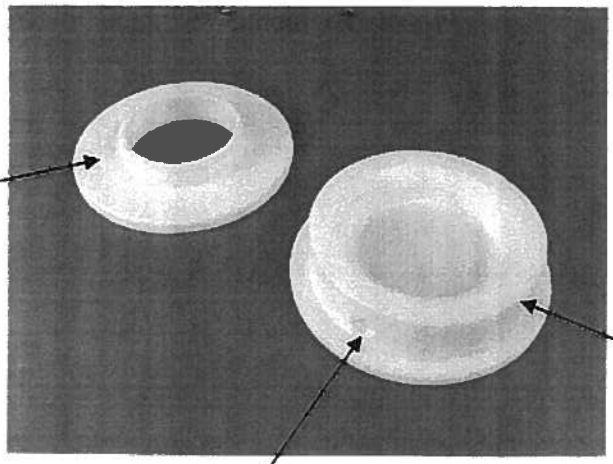
4.4 Test system (Test chamber & skin samples)

The experimental set-up as well as the preparation and storage of the skin used in this experiment have been published in detail [4 to 6].

4.4.1 Test chamber

Six permeation chambers were used (Teflon-chambers with 9.1 cm^2 surface, in-house development, see figure 1):

Figure 1: Teflon-chamber used in this study



4.4.2 Skin samples

Split thickness skin samples from back and flanks (approximatively 1000 µm thick), were prepared using a dermatome on different dates and from different donors (see table 2) and stored at – 20 °C until use. The preparations are composed of the stratum corneum, the stratum germinativum and part of the dermis containing blood vessels. Prior to the penetration experiment, the thickness of each skin sample was determined by means of a micrometer. Exact descriptions of the donor and body part of which the specific skin samples originate are given in table 2.

Table 2: Summary of the pig skins used within this penetration study

Donor	Date of dissection	body part	sex	body weight	skin sample	
1	18.07.2005	rear back	male	110 kg	6,8	1st series (14.09.2006)
2	19.07.2005	rear back	female	112 kg	10,12	
3	10.04.2006	front back	male	120 kg	2,4	
1	18.07.2005	front flank	male	110 kg	6,8	2nd series (21.09.2006)
2	19.07.2005	rear back	female	112 kg	10,12	
3	10.04.2006	front back	male	120 kg	2,4	

4.5 Procedure of the absorption experiment

At the beginning of the experiment, thawed skin samples were mounted - dermal side down - in permeation chambers. During the whole experiment, the chambers were kept at 32 ± 2 °C and 20.5 to 34.9 % (first series), 24.9 – 32.2 % (second series) relative humidity in an incubator. The experiment was performed using a flow-through system, with a constant flow of the receptor fluid[a)] of 5 ml/h. The test substance is soluble in all matrices at the concentrations used (92.6 mg/ml in receptor fluid), thus solubility is not acting as a barrier to absorption. After the integrity test with tritiated water by scintillation counting of one hour fractions over a time period of four hours, twelve skin samples were covered with 400 mg of an oxidative hair dye formulation containing 1.5 % of 2-Amino-4-hydroxyethylamino anisole sulfate on 4 cm^2 in two consecutive series. The formulations were removed after 30 minutes, followed by extensive washing in five steps (2x 4 ml of water, 1x 4 ml of shampoo [5)], 2x 4 ml of water).

a) 0.14 M NaCl [1)], 2 mM K_2HPO_4 [2)], 0.4 mM KH_2PO_4 [3)], 100 IU penicillin [4)]/ml, 76 IU streptomycin [5)]/ml, 0.3 % Ascorbic acid[6)], 0.4 % Sodium sulfite[7)] and 3 % of Ethanol[8)], pH = 7.3

1) NaCl, Merck, N° 1.06404, analytical grade; 2) K_2HPO_4 x 3 H_2O, Merck, N° 5099, analytical grade; 3) KH_2PO_4, Merck, N° 4873, analytical grade; 4) Penicilline G, sodium chloride, 1667 U/mg, Seromed A321-42; 5) Streptomycin sulfate, 779 U/mg, Seromed A331-26; 6.) L-(+)-Ascorbic acid, VWR Int., N° 20150.184, Normapur; 7) Sodium sulfite, Wella Batch N° R0032572; product N°: 21146; 8.) Ethanol, Merck, N° 1.11727, HPLC grade

b) "Salon shampoo, Energy, Duftshampoo, Formulation N° 7355034030, Batch N°: D0046163, Art.-N°:112, diluted 1 + 6 with H_2O bidest.

4.6 Sampling procedures

- Spatula: After application of the final formulation, the spatulas were dipped into 1 ml of ethanol. This solution was diluted with 4 ml of water and 15 ml of scintillation cocktail and the radioactivity was quantified with a scintillation counter.

- Receptor fluid: The receptor fluid was sampled after 16, 24, 40, 48, 64 and 72 hours according to the protocol in the study plan. An aliquot of the collected receptor fluid fraction (i.e. 5 ml) was transferred to scintillation vials and 15 ml of scintillation cocktail were added. The radioactivity was quantified with a scintillation counter.

- Skin: ☒ upper skin separated from lower skin
  ☐ complete

- Separation of the skin compartments "heat-method": After disassembly of the skin samples from the teflon chambers, the skin samples were wrapped in aluminium foil and put on a hot surface (80 ° to 90 °C) upside down (i.e. lower skin upside) charged with a small weight of approximatively 40 g. After 15 to 60 seconds, the packed skin sample was removed from the hot surface and unwrapped. The upper skin was separated from the lower skin with forceps.

- Extraction of the skin compartments: With concentrated KOH solution. After the separation of the upper skin from the lower skin by the heat-method, each of the skin compartments was dissolved with 2 ml of aqueous KOH solution (10M) at 80 °C overnight. Two hundred microliters of the resulting solution were mixed with 200 µl of concentrated hydrochloric acid and 3 ml scintillation cocktail. The radioactivity was quantified with a scintillation counter.

- Extraction of the aluminium foil: With 5 % methanol in water. After the separation of the skin compartments, the aluminium foil was dipped into 1 ml of aqueous solution of 5 % methanol overnight. Thereafter, the aluminium foil was removed and the extract diluted with 3 ml of the scintillation cocktail. The radioactivity was quantified with a scintillation counter.

- Rinsings — Fifteen microliters of each of the rinsing fractions were mixed with 3 ml of the scintillation cocktail and the radioactivity was quantified with a scintillation counter.

4.7 Sample storage

All samples (application formulation, rinsings, receptor fluid fractions and skin compartment extracts) were stored at –20 °C, if not processed immediately. Samples used for the determination of the stability in receptor fluid were stored at room temperature until analysis.

Reagents used:
Methanol: Biosolve, HPLC grade; KOH (potassium hydroxide): Merck, pro analysi; Ethanol: Merck, HPLC grade; Scintillation cocktail: Packard BioScience; Ultima Gold XR 6013119; HCl (hydrochloric acid): Merck, fuming 37 %; Water: MilliQ.

4.8 Analytical procedures (including LOD and LOQ)

The analytical determination of the test item is performed in the formulation, the rinsings, the receptor fluid, the extracts of the separated skin compartments, as well as on the aluminium foil used for the separation of the skin compartments and on the spatula used for the application of the final formulation. The radioactivity of each of the samples was quantified with a scintillation counter. The principle of the scintillation detection is described in detail in the cutaneous permeation SOP N° 2.17.

Short description of the method

Scintillation counter : Packard Tricarb 2250CA

Sample preparation : as described in paragraph «4.6 Sampling procedures»

Limit of detection, Limit of quantification

The limit of detection of 2-Amino-4-hydroxyethylamino anisole sulfate is the value for the dpm found for the blank sample. A priori, this value is in the range of 10 to 50 dpm. However 100 dpm may be considered as significant above the background. With respect to the applied formulation containing 1.5 % of unlabelled 2-Amino-4-hydroxyethylamino anisole sulfate this would correspond to approximatively 10.14 ng of 2-Amino-4-hydroxyethylamino anisole sulfate as the absolute limit of detection.

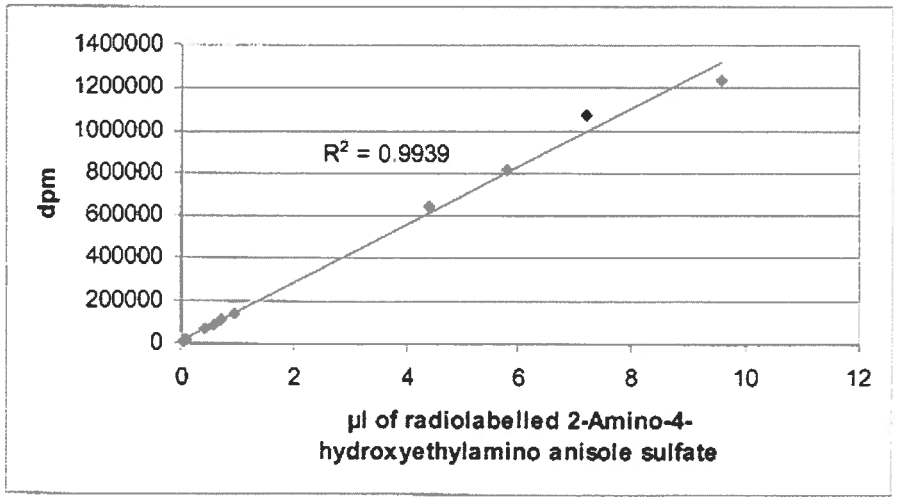
Calculation

400 mg of the final applied formulation contain 5.34 µl of a 5 mCi/ml solution of labelled 2-Amino-4-hydroxyethylamino anisole sulfate. This corresponds to an absolute amount of 26.67 µCi in 400 mg of the applied solution. These 26.67 µCi correspond to 59'200'000 dpm (1µCi = 2.22 E6 dpm). As a consideration, 100 dpm are already a significant amount above the background value. In order to interrelate the labelled to the unlabelled compound, one can define, that 6 mg of 2-Amino-4-hydroxyethylamino anisole sulfate (e.g. 1.5 % of 400 mg = 6 mg) correspond to 59'200'000 dpm (100 %). With these assumptions, 100 dpm correspond to an absolute limit of detection of 10.14 ng of 2-Amino-4-hydroxyethylamino anisole sulfate.

To each value measured, the amount of dpm of the blank is subtracted. Thus, each value obtained above the measured background can be considered for further calculations. With respect to the mentioned assumptions, a value of 100 dpm is supposed to be significant above the background (*vide supra*).

The calibration curve established with aliquots of the solution of the radiolabelled 2-Amino-4-hydroxyethylamino anisole sulfate was linear up to 1'200'000 dpm (See figure 2).

Figure 2: Calibration curve of radiolabelled 2-Amino-4-hydroxyethylamino anisole sulfate



5 RESULTS AND DISCUSSION

Two independent experiments were performed with 6 diffusion cells per experiment. For calculations, the mean value of all valid skin samples (n=8) in contact with 1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate (WR23081) in a typical hair dye formulation in the presence of hydrogen peroxide and reaction partner 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate (A154, WR18247) was used.

The pig skin preparations used in this study were measured by means of a micrometer, resulting in 0.85 ± 0.16 mm of thickness. The integrity of each skin preparation was demonstrated by examination of penetration characteristics with tritiated water resulting in 0.8 to 3.7 % of the applied dose found after 4 hours in the receptor fluids, which was within the limit of acceptance (≤ 2.0 %) for 8 skin samples used for determination of skin penetration. The skin samples with skin integrity values above 2 % of the applied dose (4 skin samples) were not within the limit of acceptance (vide supra) and were not taken into consideration for the calculation of the mean.

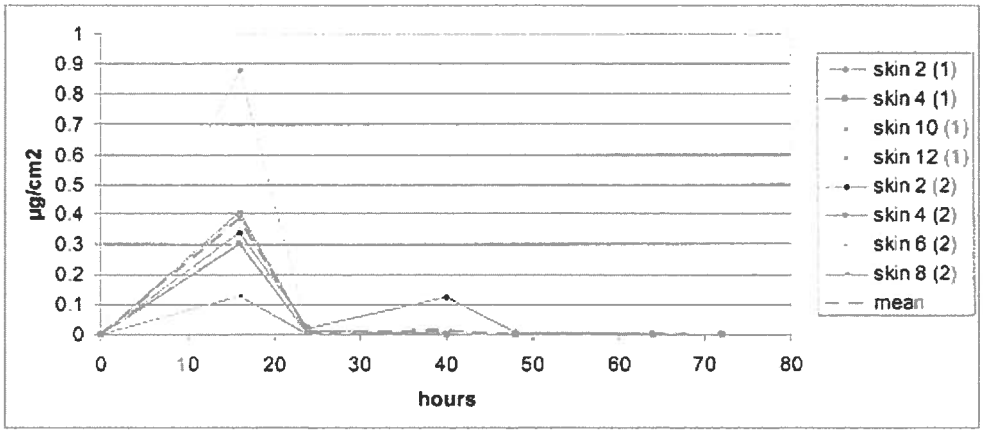
The results of the cutaneous absorption after 72 hours with 12 pig skin samples treated with 1.5 mg 2-Amino-4-hydroxyethylamino anisole sulfate/cm^2 (1.5 % of WR23081) in a typical hair dye formulation in the presence of a reaction partner are presented in table 3.

The majority of the test substance could be found in the rinsing solutions (1.341 ± 0.051 mg/cm^2). Small amounts of 2-Amino-4-hydroxyethylamino anisole sulfate could be found in the upper skin (2.352 ± 0.824 µg/cm^2), in the lower skin (0.303 ± 0.219 µg/cm^2) and in the fractions of the receptor fluid collected within 72 hours (0.409 ± 0.223 µg/cm^2).

With respect to the receptor fluid, only small amounts of 2-Amino-4-hydroxyethylamino anisole sulfate were detectable within the first fractions collected during 72 hours (see Annex V), indicating that no further 2-Amino-4-hydroxyethylamino anisole sulfate is available from a potential reservoir of the skin compartments.

The kinetics of the amount of 2-Amino-4-hydroxyethylamino anisole sulfate passing through the skin barrier is shown in figure 3

Figure 3: 72 hours cutaneous absorption after 30 min. application of 1.5 % (1.5 mg/cm^2) of 2-Amino-4-hydroxyethylamino anisole sulfate in a typical hair dye with hydrogen peroxide and reaction partner A154. Amounts of 2-Amino-4-hydroxyethylamino anisole sulfate found in the receptor fluid after specified time points.



The mass balance of the test substance resulted in values of 92.20 to 103.31 % recovery for all (8) skin samples with acceptable integrity (as measured by the penetration characteristics of tritiated water).

Under the assumption that a depot effect is absent, a maximum amount of **0.712 ± 0.313 µg/cm^2** of **2-Amino-4-hydroxyethylamino anisole sulfate** is considered as **biologically available** (n=8, three donors; receptor fluid + lower skin; 0.409 µg/cm^2 + 0.303 µg/cm^2).

Taking into account the recommendations in the guidelines [1 & 2] and the acceptance criteria defined in the study plan, (at least eight valid chambers) the study is **valid.**

Table 3

72 hours cutaneous absorption of 1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate in a typical hair dye formulation in the presence of hydrogen peroxide and reaction partner A154. Details of the results including mass balance data and 4 hours permeation of tritiated water (skin integrity test). Summary of both experimental series.

	Skin	Integrity-Test	1)		2)		3)		4)		1) + 2) + 3) + 4)	
	No (series)	^3H_2O Permeation (4 hours cumulative)	Receptor fluid (72 hours cumulative)		Lower skin (72 hours cumulative)		Upper skin (72 hours cumulative)		Rinsing solution (after 30 minutes)		Total***	
		[% Dose]	[µg/cm²]	[% Dose]**	[µg/cm²]	[% Dose]**	[µg/cm²]	[% Dose]**	[µg/cm²]	[% Dose]**	[µg/cm²]	[% Dose]
Application of 100 mg 1.5 % 2-Amino-4-hydroxyethylamino anisole sulphate in a typical hair dye formulation* per 1 cm² skin	2 (1)	0.9	0.499	0.034	0.116	0.008	1.792	0.123	1300.36	89.37	1455.02	92.63
	4 (1)	0.8	0.303	0.021	0.120	0.008	2.415	0.166	1331.27	91.64	1452.79	95.08
	6 (1)****	3.7	*1.739*	*0.120*	*0.106*	*0.007*	*2.330*	*0.160*	*1385.14*	*95.32*	*1453.21*	*98.82*
	8 (1)****	3.4	*1.620*	*0.112*	*0.324*	*0.022*	*2.968*	*0.204*	*1395.41*	*96.10*	*1452.11*	*99.73*
	10 (1)	0.9	0.322	0.022	0.198	0.014	2.800	0.193	1442.14	99.54	1448.75	103.31
	12 (1)	1.7	0.891	0.061	0.187	0.013	3.025	0.208	1374.94	94.43	1456.11	97.72
	2 (2)	1.2	0.424	0.030	0.677	0.047	1.037	0.072	1321.01	92.02	1435.51	96.67
	4 (2)	1.4	0.306	0.021	0.153	0.011	1.467	0.102	1328.52	92.30	1439.41	96.64
	6 (2)	1.7	0.398	0.028	0.399	0.028	3.264	0.227	1352.14	93.94	1439.30	98.44
	8 (2)	0.9	0.129	0.009	0.571	0.039	3.014	0.208	1277.00	88.26	1446.81	92.20
	10 (2)****	2.7	*0.460*	*0.032*	*0.190*	*0.013*	*1.775*	*0.122*	*1361.05*	*93.57*	*1454.58*	*96.86*
	12 (2)****	2.3	*1.003*	*0.070*	*0.322*	*0.022*	*1.759*	*0.122*	*1242.89*	*86.42*	*1438.20*	*90.93*
Mean		**1.80**	**0.409**	**0.028**	**0.303**	**0.021**	**2.352**	**0.162**	**1340.92**	**92.69**	**1446.71**	**96.59**
± S.D		1.01	0.223	0.015	0.219	0.015	0.824	0.057	50.57	3.46	7.86	3.53
(n)		(12)	(8)	(8)	(8)	(8)	(8)	(8)	(8)	(8)	(8)	(8)

*vehicle: (typical hair dye formulation as detailed in Annex III was mixed 1:1 with peroxide solution, see 4.3); **Corrected for individual applied dose; *** Total is corrected for losses on tips; **** Outlier: not considered for the calculation of the mean (with the exception for the integrity test)

6 CONCLUSION

The cutaneous absorption of 2-Amino-4-hydroxyethylamino anisole sulfate in a typical hair dye formulation in the presence of hydrogen peroxide and a reaction partner was measured by scintillation counting.

Two independent experiments were performed with 6 diffusion cells per experiment. For the calculation, the mean value of all valid skin samples (n=8) in contact with 1.5 % 2-Amino-4-hydroxyethylamino anisole sulfate (WR23081) in a typical hair dye formulation in the presence of hydrogen peroxide and a reaction partner 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate (A154, WR18247) was used.

After application of 100 mg/cm^2 formulation containing 1.5 % of 2-Amino-4-hydroxyethylamino anisole sulfate (= 1.5 mg of WR23081/cm^2) for 30 minutes on skin samples and subsequent rinse-off with water and shampoo, the recovered 2-Amino-4-hydroxyethylamino anisole sulfate was found predominantly in the rinsings (92.69 ± 3.46 % or 1.341 ± 0.051 mg/cm^2) and was not absorbed into the skin during the application period of 30 minutes. Small amounts of 2-Amino-4-hydroxyethylamino anisole sulfate could be found in the upper skin (0.162 ± 0.057 % or 2.352 ± 0.824 µg/cm^2), in the lower skin (0.021 ± 0.015 % or 0.303 ± 0.219 µg/cm^2) after 72 hours and in the fractions of the receptor fluid collected within 72 hours (0.028 ± 0.015 %or 0.409 ± 0.223 µg/cm^2).

Under the assumption that a depot effect is absent, a maximum amount of **0.712 ± 0.313 µg/cm^2** of **2-Amino-4-hydroxyethylamino anisole sulfate** is considered as **biologically available** (n=8, three donors; receptor fluid + lower skin; 0.409 µg/cm^2 + 0.303 µg/cm^2).

Taking into account the recommendations in the guidelines [1 & 2] and the acceptance criteria defined in the study plan, (at least eight valid chambers) the study is **valid.**

7 References

1 OECD Guideline for the testing of chemicals. Skin Absorption: *In Vitro* method, Test Guideline 428 adopted 13 April 2004.

2 OECD Environmental Health and Safety Publications, series on testing and assessment No. 28. Guidance Document for the conduct of skin absorption studies. Paris, March 2004.

3 W. Diembeck, H. Beck, F. Benech-Kieffer, P. Courtellemont, J. Dupuis, W. Lowell, M. Paye, J. Spengler, W. Steiling: Test Guidelines for *In Vitro* Assessment of Percutaneous Absorption and Penetration of Cosmetic Ingredients. Colipa Task Force Cutaneous Penetration. Food and Chemical Toxicology 37, 191 – 205 (1999).

4 M. Bracher, C. Faller and F. K. Noser: Evaluation of an *In Vitro* percutaneous permeation model with two oxidative hair dyes: Int. J. Cosmet. Sci. 9, 223-236 (1987).

5 F. K. Noser, C. Faller, M. Bracher: *In Vitro* permeation with pig skin: Instrumentation and comparison of flow-through versus static-diffusion protocol. J. Appl. cosmetol. 6, 111-122 (1988).

6 H. Beck, M. Bracher, C. Faller and H. Hofer: Comparison of *In Vitro* and in vivo skin permeation of hair dyes: Cosmetics & Toiletries 108, 76-83 (1993).

7 J. Bartek, J. LaBudde, H. I. Maibach: Skin Permeability in Vivo: Comparison in Rat, Rabbit, Pig and Man. J. Invest. Dermatol. 58 (3), 114 – 123 (1972).

8 ANNEXES

8.1 Annex I

The Swiss GLP Monitoring Authorities

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Home Affairs DHA
Federal Office of Public Health FOPH

Federal Department of the Environment, Transport, Energy and Communications DETEC
Federal Office for the Environment FOEN

swissmedic
Swissmedic
Swiss Agency for Therapeutic Products

Statement of GLP Compliance

According to Art. 14 paragraph 3 Ordinance on Good Laboratory Practice [SR 813.112.1]

It is hereby confirmed that

during the period of **21 – 22 March 2006**

the following test facility of **Cosmital S.A.**
Route de Chésalles 21
CH-1723 marly

was inspected by the Federal Office of Public Health with respect to the compliance with the Swiss Ordinance on Good Laboratory Practice, adopted on 18th May 2005 [SR 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted on 26th November 1997 by decision of the OECD Council [C(97)186/Final].

Test Facility	Area of expertise:
Cosmital S.A.	• **Toxicity studies (in vitro toxicology)**

The above mentioned test facility is listed in the GLP Register and is inspected on a regular basis. Based on the decision dated 07th July 2006 it can be confirmed that the above mentioned test facility is able to conduct studies according to the aforementioned area of expertise in compliance with the principles of GLP.

Swiss Federal Office of Public Health
Consumer Protection Directorate
Notification Authority for Chemicals
The Head

FEDERAL OFFICE OF PUBLIC HEALTH

Dag Kappes

Bern, 11th September 2006

Dr. Dag Kappes

8.2 Annex II

CAUTION - RADIOACTIVE MATERIAL

Product Specification

http://www.customlabelling.com
GE Healthcare UK Limited
Amersham Place Little Chalfont Buckinghamshire HP7 9NA UK
Telephone +44 (0)870 606 1921

Certificate number LRQ659685

Before using this product, please read the instructions on safe handling, storage and disposal

Amersham
[ring-U-^{14}C]Lehmann blue
Code CFQ14700 Batch 1
Pack size 37.0 MBq, 1mCi

Technical data

Specific activity determined by mass spectrometry	:	851 MBq/mmol,	23 mCi/mmol
Molecular weight	:	281.0 (at this specific activity)	

Radiochemical purity

by high performance liquid chromatography	:	98.8%

Column	:	Lichrospher 60, RP Select B 5µ (250 x 4.6mm)
Solvent A	:	0.025M potassium dihydrogen phosphate (aq) + 0.025M disodium hydrogen phosphate (aq) pH 6.9
Solvent B	:	methanol
Gradient	:	15% B isocratic for 45 minutes
Flow rate	:	1.5 ml/min
Temperature	:	40°C
UV detection	:	220 nm

Analysed on 30th May 2006

Chemical identity

The material co-chromatographs with customer supplied material in the above chromatographic system.

The mass spectrum is consistent with the proposed structure and a non-labelled reference.

The ^{1}H-nmr spectrum is consistent with the proposed structure and a non-labelled reference.

GE Healthcare

8.3 Annex III

Color Cream with Pyrazol DHE and Lehmannblau

RD-IPC no: 85903070
Batch for Mr. Sieber/ TOX

Batch no. : DTF68081
Date : 26.07.2006
Based on Formula: VDE.0018
Size: 0.4Kg
Tare : 190,0 gr

pos.	art.no.	Exp.Date	charge no	Raw material	%	g	real weight (g)
1	21645	13.04.07	B003922	LANETTE	14	64.00	64,0
2	22710	12.12.06	R00 793	DUSORAN D	4.00	16	16,0
3	20754	12.12.08	D390159	LAURYLETHERSULFAT 28	10	40.00	40,0
4	19007	21.03	R0031688	KOKOSFETTSAEURE ISETHIONAT	4.00	16.00	16,0
5	21146	21.03.2	R0032572	NATRIUMSULFIT	0.4	60	60
6	21531	22.04.08	R0033221	ASCORBINSAURE DAB	0.30	1.	2
7	21526	29.03	R00063062	REXAT	0.1	0	0,4
8	23081	10.04.	GST 7-18092	LEHMANNBLAU	3.00	12.	12,0
9	18247	08.01	R98000196	PYRAZO	2	10.48	10,48
10	21102	12.04	R0043654	AMMONIAK 2o	3.0	2.3	2,3
11	21102	12.04.07	R0043654	AMMONIAK 25%	4.55	18.20	18,2
2	21136			WASSER VOLLENTSALTZ	53.	20 .88	
				otal	100.00	400 .0	398,68

Procedure: Wax phase: Add position 1-2 in separate suita le vesse heat 80°C (pa clear)
Water-phase: Add pos tion 3-4-5-6-7-8-9-10-12. Heat 0°C (Phase ear).

Add water phase e nto ax-phase, stirred manual an coo ed with water at
At 30°C, Add position 11. ut at. with water/Art. 136

CCSMITAL SA P&G
Product Name: Color Cream with Pyrazol DHE and Lehmannblau
Formula Code No: 85903070
Batch N° DTF68081
Batch for Mr Sieber
MFG Date 26.07.06 EXP. Date 26.01.08
Dept: RD-IPC/208 Made by F.Bolley

PAGE 081 DTF68 NOTEBOOK ATTACHMENTS
SIGNATURE DATE 27.04.06

8.4 Annex IV

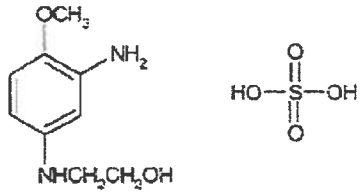
Cosmital SA

Marly, 19.02.2001 FOF/BR/DOU/os
modified: 03.08.2004
modified: 16.06.2005
modified: 05.07.2005
modified: 07.03.2006

CERTIFICATE OF ANALYSIS

Wella raw material no.: 23081 **Code:** A000157

Structure:



Molecular formula: $C_9H_{14}N_2O_2.H_2O_4S$ **Molecular weight:** 280.30
Molecular weight of free base: 182.22

name: LEHMANN BLAU

Trade name: HC BLAU AC (ROBINSON). HC BLUE AC (CLARIANT)

Chemical name: 2-Amino-4-(2-hydroxyethyl)amino-anisole-sulfate

Name (INCI): 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE

CAS-No: 83763-48-8 **EINECS/ELINCS-No:** 280-734-8

Testing material

Sample name: **Sample no:** R96000196

Batch: 57 **Study no.:** A9803/086, A2004/237, A2005/158, A2005/126

Date of entry: 08.01.96 **Expiry date:** July. 2005
Prolongation: 08.01.2006
Prolongation: 08.01.2008

Results

Aspect: pale grey powder

Odour:

Melting point:

		%C	%H	%N	%S	
Elemental analysis:	calculated:	36.24	6.08	9.39	10.75	($C_9H_{14}N_2O_2.H_2SO_4.H_2O$)
	found:	36.22	5.96	9.42	10.67	(Solvias: 01.03.2006)

Loss on drying: 0.2 weight%

Water content: 10.6 weight%

Sulfated ash: < 0.1 weight%

Mass spectrum:

pH value: 2.1

NMR spectrum: (AC-P 300MHz, DMSO-d_5)
Content: 99.1 weight% ($C_9H_{14}N_2O_2xH_2SO_4xH_2O$)
93.5 (93.1) weight% ($C_9H_{14}N_2O_2xH_2SO_4$)
3.2 ppm (t,2H,-CH_2-)
3.6 ppm (t,2H,-CH_2-)
3.8 ppm (s,3H,-CH_3)
6.6 ppm (m,1H,Ar-H)
6.65 ppm (d,1H,Ar-H)
6.9 ppm (d,1H,Ar-H)

IR spectrum:

UV/VIS spectrum: λ max: 206, 238 and 290 nm in water
ε:

GC:

HPLC: 99.6 area% at 254 nm

Identity: The ^{1}H-NMR spectra and elemental analysis confirmed the chemical identity of the test substance.

Purity: 99.6 area% (by HPLC)

Content: 99.1 weight% ($C_9H_{14}N_2O_2xH_2SO_4xH_2O$) } by
93.5 weight% ($C_9H_{14}N_2O_2xH_2SO_4$) } NMR

By-products: 0.03 weight% 4-methoxy-1,3-phenylenediaminesulfate (2,4-diaminoanisole)
4-methoxyaniline was not detectable
4-methoxy-3-nitroaniline was not detectable
2-methoxy-5-nitroaniline was not detectable
p-phenylendiamine was not detectable (LOD 10 ppm)
10.6 weight% of water
28 ppm of sodium

Solubility: 10g/l in water pH 2.8 (> 5 weight% pH 8)
1 weight% in acetone/water 1:1 (pH 2.1)
9-10 weight% in DMSO
0.2 weight% in ethanol

Stability: The substance is considered to be stable for more than 12 years, if stored dry and protected from light at room temperature.

Stability in solution:
The stability over a total period of seven days was tested by HPLC. The test stock solutions (approx. 5 weight%) were stored at room temperature and in the absence of light.
Water solution: the results (t = 0h: 100.0%; 6h: 93.8%; 2d: 95.1%; 7d: 79.7%) confirm a low degradation (G2000/003)
DMSO solution: the results (t = 0h: 100.0%; 6h: 98.8%; 2d: 92.0%; 7d: 80.5%) confirm a low degradation (G2000/003)

P. Dougoud

8.5 Annex V

Receptor fluid fractions, overview of the results of the radioanalytical quantification (amount of 2-Amino-4-hydroxyethylamino anisole sulfate)

First series

Table 4 summarises the amounts of 2-Amino-4-hydroxyethylamino anisole sulfate quantified by means of a scintillation counter in each of the receptor fluid fractions collected within the first series of chambers used for this study

Table 4: Summary of the analysis of the receptor fluid fractions. Results given in ng/cm² (first series of experiments)

sampling [hours]	skin sample Nr.					
	2	**4**	**6***	**8***	**10**	**12**
0 – 16	339	303	1718	1593	316	877
16 – 24	22	0	21	26	5	13
24 – 40	126	0	0	0	0	0
40 – 48	8	0	0	0	0	0
48 – 64	4	0	0	0	0	0
64 – 72	0	0	0	0	0	0
SUM	**499**	**303**	**1739**	**1620**	**322**	**891**

* Outlier

Second series

Table 5 summarises the amounts of 2-Amino-4-hydroxyethylamino anisole sulfate quantified by means of a scintillation counter in each of the receptor fluid fractions collected within the second series of chambers used for this study

Table 5: Summary of the analysis of the receptor fluid fractions. Results given in ng/cm^2 (second series of experiments)

sampling [hours]	skin sample Nr.					
	2	**4**	**6**	**8**	**10***	**12***
0 – 16	409	302	394	129	455	987
16 – 24	12	4	4	0	5	17
24 – 40	3	0	0	0	0	0
40 – 48	0	0	0	0	0	0
48 – 64	0	0	0	0	0	0
64 – 72	0	0	0	0	0	0
SUM	**424**	**306**	**398**	**129**	**460**	**1003**

* Outlier

8.6 Annex VI

Streng vertraulich

Rezeptnummer: 70813015900

Cr-Datum : 12.04.1999
Rezeptname : ? I 6%

Pos	Rohstoff-Nr	Rohstoffname	
1	21510	LANOL	2.00000
2	21136	WASSER, VOLLENTSALZT	50.00000
3	21529	STABIL	.05000
4	21001	DINOL	.08000
5	21127	PHOSPHORSAEURE-O 85%IG THERM. REIN	.10000
6	21136	WASSER, VOLLENTSALZT	35.36000
7	21155	WASSERSTOFFPEROXYD 50 %	12.00000
8	23466	TURPINAL SL	.01000
9	19767	ANTISCHAUMEMULSION SE 57/20	.05000
10	20990	PARFUEM 38360 G JAVELIA	.10000
11	21127	PHOSPHORSAEURE-O 85%IG THERM. REIN	.05000