Safety Assessment of Hydroxyethyl-3,4-Methylenedioxyaniline HCl as Used in Cosmetics

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All interested persons are provided 60 days from the above date to comment on this safety assessment and to identify additional published data that should be included or provide unpublished data which can be made public and included. Information may be submitted without identifying the source or the trade name of the cosmetic product containing the ingredient. All unpublished data submitted to CIR will be discussed in open meetings, will be available at the CIR office for review by any interested party and may be cited in a peer-reviewed scientific journal. Please submit data, comments, or requests to the CIR Director, Dr. Lillian J. Gill.

The 2016 Cosmetic Ingredient Review Expert Panel members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Ronald A. Hill, Ph.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; James G. Marks, Jr., M.D., Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is Lillian J. Gill, D.P.A. This safety assessment was prepared by Laura N. Scott, Scientific Writer/Analyst.

1620 L Street, NW, Suite 1200 ◇ Washington, DC 20036-4702 ◇ ph 202.331.0651 ◇ fax 202.331.0088 ◇
cirinfo@cir-safety.org

INTRODUCTION

This assessment reviews the safety of Hydroxyethyl-3,4-Methylenedioxyaniline HCl. According to the *International Cosmetic Ingredient Dictionary and Handbook (Dictionary)* this cosmetic ingredient functions as a hair colorant.[1]

The majority of the data included in this safety assessment were gathered from the European Commission Scientific Committee on Consumer Safety (SCCS),[2] the European Union's website (EUROPA), and on the Australian Government Department of Health National Industrial Chemicals Notification and Assessment Scheme (NICNAS) website.[3] The above references are cited when data from these sources is summarized and the primary references were not readily obtainable. In one acute, oral exposure study from the SCCS report, the test substance 1-β-hydroxyethyl-3,4-methylenedioxybenzene was considered for read-across, however a justification for read-across was not provided.[2]

CHEMISTRY

Definition and Structure

Hydroxyethyl-3,4-Methylenedioxyaniline HCl (CAS# 94158-14-2) is the amine salt that conforms to the structure in Figure 1.[1]

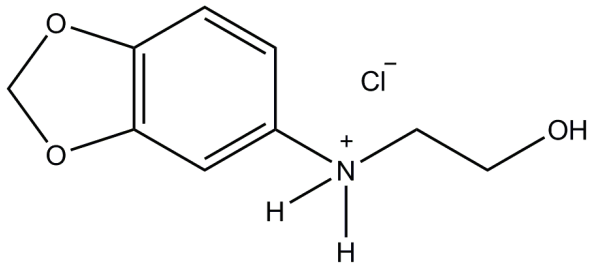


Figure 1. Hydroxyethyl-3,4-Methylenedioxyaniline HCl

Hydroxyethyl-3,4-Methylenedioxyaniline HCl is used as an oxidative hair coloring "precursor" ingredient.[2,4] In a 2009 SCCS report, an industry submission indicated that the oxidative coloring precursor and the developer (hydrogen peroxide) were mixed at ratios ranging from 1:1 to 1:3.[2] However, Hydroxyethyl-3,4-Methylenedioxyaniline HCl was also listed as a "coupler" in a different SCCS report from 2010.[5] No reaction scheme was found in the literature. In a typical formulation, a precursor is activated via an oxidant, such as peroxide. The resultant activated precursor proceeds to react with a coupler to form *in-situ* a product that is purported to be the actual dye that colors hair in these types of oxidative hair dyes.

Physical and Chemical Properties

Hydroxyethyl-3,4-Methylenedioxyaniline HCl is a beige crystal.[2] It is soluble in water, acetone/water, dimethyl sulfoxide (DMSO), and ethanol (Table 1).

Method of Manufacture

There were no method of manufacture data available in the literature for Hydroxyethyl-3,4-Methylenedioxyaniline HCl.

Impurities and Nitrosation

Impurities that may be present in Hydroxyethyl-3,4-Methylenedioxyaniline HCl include 3,4-methylenedioxy-aniline (114 to 1097 ppm in 5 batches tested), 1,3-benzodioxol (below limit of detection (35 ppm) in 5 batches tested), and 1,2-methylenedioxy-4-nitrobenzene (below limit of detection (8 ppm) in 5 batches tested).[2] The following residual solvents were not identified at a 100 ppm detection limit: methanol, ethanol, isopropanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone, and monochlorobenzene.

Hydroxyethyl-3,4-Methylenedioxyaniline HCl contains a free, secondary aromatic substituted amine group (aniline derivative), thus raising a concern about potential *N*-nitrosation. A concern in cosmetics is the conversion (nitrosation) of nitrogen-containing ingredients, such as hair dyes, into *N*-nitroso chemicals that may be carcinogenic. In one study, 85% of the approximately 209 nitrosamines tested were shown to produce cancer in laboratory animals.[6] Nitrosation can occur under physiologic conditions.[7] Depending on the nitrosating agent and the substrate, nitrosation can occur under acidic, neutral, or alkaline conditions. Atmospheric NO_2 may also participate in nitrosation in aqueous solution.[8] A recent study demonstrated the reactivity of a secondary aniline derivative (i.e., similar to Hydroxyethyl-3,4-Methylenedioxyaniline HCl) to standard nitrosation conditions (i.e., $NaNO_2$).[9] The secondary aniline derivative was efficiently *N*-nitrosated in this study. Consequently, hair dye formulations containing Hydroxyethyl-3,4-Methylenedioxyaniline HCl, and those formulations intended for admixture with this ingredient (e.g., to generate an active hair dye), should be free of nitrosating agents.

Furthermore, nitrosamines may be present in a cosmetic as an impurity of an ingredient. For example, during the safety assessment of morpholine the Cosmetic Ingredient Review CIR Expert Panel (Panel) determined that, under conditions of cosmetic use, it is highly unlikely that morpholine is totally free of carcinogenic nitrosamines. Nitrosation of morpholine to form *N*-nitrosomorpholine occurs readily. Accordingly, the Panel raised the concern about the contamination of morpholine with *N*-nitrosomorphine. In an SCCS report, concern was raised about nitrosation of the impurity 3,4-(methylenedioxy)-aniline (a primary aromatic amine) that may occur in Hydroxyethyl-3,4-Methylenedioxyaniline HCl.[2] The total content of *N*-nitroso compounds for Hydroxyethyl-3,4-Methylenedioxyaniline HCl was determined to be < 10 µg/kg (ppb) in 3 batches tested (and the SCCS concluded that such compounds should be limited to < 50 ppb). Consequently, cosmetic products containing Hydroxyethyl-3,4-Methylenedioxyaniline HCl should be free of such impurities.

Manufacturers may avoid these issues by formulating this ingredient in a way that reduces the formation of nitrosamines, and by eliminating the presence of impurities that are *N*-nitrosated or contain nitrosating agents.

USE

Cosmetic

The CIR Panel evaluates the safety of the cosmetic ingredient included in this assessment based on the expected use of, and potential exposure to, the ingredient in cosmetics. The data received from the United States Food and Drug Administraion (FDA) are collected from manufacturers through the FDA Voluntary Cosmetic Registration Program (VCRP), and include the use of individual ingredients in cosmetics by cosmetic product category. The data received from the cosmetic industry are collected by the Personal Care Products Council (Council) in response to a survey of the maximum reported use concentrations by product category. VCRP data obtained from the FDA in 2016 indicated that Hydroxyethyl-3,4-Methylenedioxyaniline HCl has 67 reported uses in hair dyes and colors (all requiring caution statements and patch tests).[10] The Council concentration of use survey data will be added to the safety assessment report when the data become available.

This ingredient is considered a coal tar hair dye for which regulations require caution statements and instructions regarding patch tests in order to be exempt from certain adulteration and color additive provisions of the United States' Federal Food, Drug, and Cosmetics Act. In order to be exempt, the following caution statement must be displayed on all coal tar hair dye products:

> Caution- this product contains ingredients which may cause skin irritation on certain individuals and a preliminary test according to accompanying directions should be made. This product must not be used for dyeing the eyelashes or eyebrows; to do so may cause blindness.

Product labels shall also bear patch test instructions for determining whether the product causes skin irritation. The CIR Expert Panel recommends, for products containing this type of ingredient, that an open patch test be applied and evaluated by the beautician and/or consumer for sensitization 48 hours after application of the test material and prior to the use of a hair dye formulation.

In 2012, a report was published regarding such self-testing for contact sensitization to hair dyes.[11] These authors concluded that, in its present form, the hair dye self-test has severe limitations. The authors issued the warning that, if the use of a hair dye self-test to predict contact sensitization becomes widespread, there is severe risk that a tool has been marketed that may cause morbidity in European consumers. An accompanying editorial performed on behalf of the European Society of Contact Dermatitis (ESCD) asserted that industry is focusing on predicting the risks from exposure to hair dyes by having millions of European consumers perform a self-test prior to each hair dying and stated that it is the opinion of the ESCD that attention must be given to reducing the risks of serious allergic reactions by improving the safety of the products themselves.[12]

The opinion issued by the European Commission SCCS on Hydroxyethyl-3,4-Methylenedioxyaniline HCl in 2009 expressed that Hydroxyethyl-3,4-Methylenedioxyaniline HCl was not considered a health risk to the consumer when the hair dye was used at a maximum concentration of 1.5% on the head, after mixing under oxidative conditions.[2,4] The SCCS also concluded that Hydroxyethyl-3,4-Methylenedioxyaniline HCl has the potential to be a strong sensitizer and, as a secondary amine, could undergo *N*-nitrosation. Thus, the SCCS opinion stated that Hydroxyethyl-3,4-Methylenedioxyaniline HCl should not be used with other nitrosating agents and the nitrosamine content restricted to < 50 ppb. Additionally, the SCCS determined that genotoxicity/ mutagenicity studies in finished hair dyes should be conducted in accordance with Scientific Committee on Consumer Products and Non-Food Products Intended for Consumers (SCCNFP/SCCP) opinions and Notes of Guidance.

According to the European Commission, Hydroxyethyl-3,4-Methylenedioxyaniline HCl is allowed for restricted cosmetic use as regulated in the Annex III List of Substances which Cosmetic Products Must Not Contain Except Subject to the Restrictions Laid Down (European Union Reference #246), as stated above.[1,4] The European Commission stipulates that oxidative hair dye ingredients, including Hydroxyethyl-3,4-Methylenedioxyaniline HCl, must be labeled as such:

> "The mixing ratio must be printed on the label. Hair colorants can cause severe allergic reactions. Read and follow instructions. This product is not intended for use on persons under the age of 16. Temporary 'black henna' tattoos may increase your risk of allergy. Do not color your hair if you have a rash on your face or sensitive, irritated and

damaged scalp, you have ever experienced any reaction after coloring your hair, you have ever experienced any reaction to a temporary 'black henna' tattoo in the past."[1]

Other international restrictions placed on Hydroxyethyl-3,4-Methylenedioxyaniline HCl include the Association of South East Asian Nations Cosmetic Directive Annex III - Part 1 (List of substances which cosmetic products must not contain except subject to restrictions and conditions laid down and the New Zealand Cosmetic Products Group Standard-Schedule 5), Table 1 (Components cosmetic products must not contain except subject to restrictions and conditions laid down).[3] In Australia there are no known restrictions for Hydroxyethyl-3,4-Methylenedioxyaniline HCl as reported by NICNAS, although it is recommended that consumers use products containing the ingredient as instructed by the label; the assessment suggested control measures for industry to minimize risk associated with dermal exposure to Hydroxyethyl-3,4-Methylenedioxyaniline HCl. The NICNAS assessment mentions a recommendation to be included in Schedule 6 of the Poisons Standard 2015-Standard for the Uniform Scheduling of Medicines and Poisons that recites that the concentration of use should be appropriately limited for use in hair dye products. Recommended classification and labeling in Australia with regard to the ingredient for the Hazardous Substances Information Systems criteria include "harmful if swallowed" and "may cause sensitization by skin contact." According to the Globally Harmonized System of Classification and Labeling of Chemicals, Hydroxyethyl-3,4-Methylenedioxyaniline HCl is "harmful if swallowed" and "may cause an allergic skin reaction."

The ECHA website listed substance information submitted by industry for 2-(1,3-benzodioxol-5-ylamino)ethanol hydrochloride (another name for Hydroxyethyl-3,4-Methylenedioxyaniline HCl).[13] The ingredient was noted to be a serious health hazard; the property of concern specified for this ingredient was skin/respiratory sensitizer.

TOXICOKINETIC STUDIES

Dermal Penetration

In Vitro

A percutaneous absorption study was conducted in accordance with Organization for Economic Co-operation and Development Test Guideline (OECD TG) 428 and Good Laboratory Practice (GLP).[2] To the back and flank of porcine skin (Schweizer Edelschwein, female), 1.5 mg/cm^2 (1.5%) of Hydroxyethyl-3,4-Methylenedioxyaniline HCl (99.8% purity), as part of an oxidative hair dye formulation (400 mg formulation consisting of 1.5% Hydroxyethyl-3,4-Methylenedioxyaniline, 1.75 mg "reaction partner" with no further details provided, and 3% hydrogen peroxide), was applied to 4 cm^2 skin mounted on a diffusion Teflon-chamber. A phosphate buffer containing sodium chloride and antibiotics was pumped through the receptor compartment at 5 ml/h. Test substance was washed off the skin 60 min post-application, twice with water (4 ml) followed by a shampoo formulation (4 ml) and then twice again with water. The portion of dye in the washing solutions was quantitatively determined by high-performance liquid chromatography (HPLC). At 16 and 24 hours post-application, fractions of receptor compartment fluid were collected, concentrated and analyzed. When the experiment was completed, the skin above the basal layer was mechanically separated from the basal layer skin down to the upper dermis by heat treatment. These skin compartments were analyzed and the amount of dye present was quantitated by HPLC.

Test results regarding the integrity of the skin (using tritiated water) indicated that penetration rates of the applied amount ranged from 0.9% to 1.4%. The total recovery of the applied amount was 19.8%. The authors speculated that the low recovery may have been in part caused by the reaction partner. The amount of Hydroxyethyl-3,4-Methylenedioxyaniline HCl that remained on the skin surface was 19.5% of the applied amount. By 24 hours the applied amount recovered was 0.047% in the upper skin, 0.0067% in the lower skin, and 0.3% in the receptor compartment fluid. The authors determined that the skin penetration rate was 5.8 µg/cm^2 (i.e., the bioavailable amount, calculated by adding the portions in the skin compartments and the receptor fluid) in a 24-hour exposure duration.

Animal

A GLP study evaluating the percutaneous absorption of ^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labelled) was conducted in Sprague Dawley (Him:OFA, SPF) rats (n=3/sex/concentration).[2] To a 9 to12 cm^2 area of skin clipped free of hair, the test substance (^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl) was applied to anesthetized animals as follows: 3.33% test substance in a water solution; 1% test substance in a formulation with hydrogen peroxide (also containing *p*-toluenediamine, resorcinol, and *m*-aminophenol in this application); 1% test substance in a formulation without hydrogen peroxide. At 30 minutes post-application the test substance was removed and the skin washed with a shampoo formulation (100 ml) and warm water until no color was seen in the rinse solution. The skin was then covered with a gauze patch that was secured in place with adhesive tape (animals were fitted with an air permeable plastic cone to prevent them from licking the treatment area) for the 72-hour study duration carried out in metabolism cages (see Table 2 for metabolism results). Animals were killed 72 hours after application. Results indicated that the washing solutions contained 95.9% to 97.1% of the applied radiolabeled concentration. The skin contained 1.68% of the applied radioactivity from the formulation containing water, 0.56% from the formulation containing hydrogen peroxide, and 0.34% from the formulation without hydrogen peroxide.

Absorption, Distribution, Metabolism, Excretion

Absorption, distribution, metabolism, and excretion (ADME) studies are summarized below; details are presented in Table 2.

Studies were conducted to evaluate the ADME of Hydroxyethyl-3,4-Methylenedioxyaniline HCl via dermal and oral exposure routes. Experiments were performed in which 1% to 3% ^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labeled) was applied to rat skin (clipped free of hair) for 30 minutes and then removed by washing.[2] The applied radioactivity was eliminated mainly in urine and to a lesser extent in feces; 0.05% to 0.345% of the applied radiolabel was recovered in urine and feces (combined) by 72 hours post-application. Dermal absorption ranged from 0.59 µg/cm^2 to 14.8 µg/cm^2 and no substantial bioaccumulation was observed. The biological half-life of ^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl was determined from the blood analysis to be 1 hour.

Experiments evaluating the effects of ^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labeled) in orally exposed rats (1 to 100 mg/kg single dosage) yielded results indicating that > 95% of the administered radioactivity was absorbed.[2,14] Blood, urine, and feces samples were collected up to 24 to 96 hours post-dosing. The radioactivity detected in urine and feces were 78% and 14%, respectively, of the administered radioactivity;[14] bioaccumulation was not observed.[2] Blood analysis showed the half-life of the test substance to be 1.5 hours.[2] The highest blood concentration detected following oral exposure was 70-fold greater than the highest blood concentration after dermal exposure.

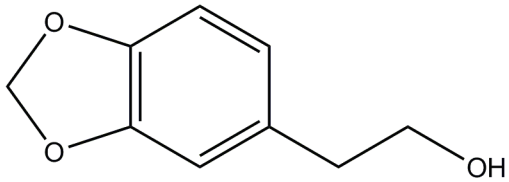
TOXICOLOGICAL STUDIES

Acute Toxicity

Animal

Oral

A non-GLP study in rats and mice was conducted to evaluate 1-β-hydroxyethyl-3,4-methylenedioxybenzene (Figure 2), which is an analog of Hydroxyethyl-3,4-Methylenedioxyaniline HCl.



1-beta-hydroxyethyl-3,4-methylenedioxybenzene

Figure 2. 1-β-hydroxyethyl-3,4-methylenedioxybenzene

1-β-Hydroxyethyl-3,4-methylenedioxybenzene (10% suspension in 10% Arabic gum solution, purity unknown) was administered by gavage to Wistar Crl: Wi/Br rats (n=5/sex/dosage) at the following single dosages: 1000, 1500, 2000, and 2500 mg/kg.[2] The test substance was also administered by gavage to CF1 mice (10 females/dosage) at the following single dosages: 500, 750, 1000, and 1250 mg/kg. The animals were observed for 14 days post-dosing and then necropsied. Immediately following dosing, observations of decreased activity, staggering, piloerection and exitus were noted. Animal deaths were reported 2 to 72 hours post-administration. No macroscopic organ changes were observed at necropsy. The calculated LD_{50} values were as follows: 1550 mg/kg for female rats, 1650 mg/kg for male rats, and 850 mg/kg for female mice.

Subchronic Toxicity

Animal

Oral

A 90-day study conducted in accordance with OECD TG 408 and GLP in Wistar HanBrl:WIST (SPF) rats (n=10/sex/dosage) was performed to evaluate the effects of Hydroxyethyl-3,4-Methylenedioxyaniline HCl (99.8% purity) following daily oral exposure, by gavage, at the following dosages: 0, 20, 100, and 350 mg/kg/day using a purified water vehicle.[2] One male died (350 mg/kg/day) on day 85; the study authors expressed that the death was unlikely to be treatment-related because no clinical symptoms were reported. In the 100 and 350 mg/kg/day groups, both sexes showed reduced locomotion. A treatment-related increase in mean absolute and relative reticulocyte counts were noted in males at 350 mg/kg/day after 13 weeks, and this effect was associated with splenic extramedullary hematopoesis found during histopathology. Reduced red blood cell count and hematocrit and hemoglobin measurements, as well as elevated mean absolute and relative reticulocyte counts, were observed in females (350 mg/kg/day). There was a decrease in thymus weights, an increase in ovary and spleen weights, extramedullary hematopoiesis in the spleen, and corpus luteum hypertrophy in the ovaries of females (350 mg/kg/day). Elevated clinical biochemistry parameters, increased liver and kidney weights, increased urine volume, hepatocellular hypertrophy, and renal tubular damage were reported in both sexes (350 mg/kg/day). At the 100 mg/kg/day dosage rate, bilirubin and phospholipid levels (clinical biochemistry parameters) were elevated in females, increases in absolute and relative liver weights and hepatocellular hypertrophy were noted in males, and increases in blood cholesterol and urinary volume occurred in both sexes. A no-observed-adverse-effect-level (NOAEL) of 20 mg/kg/day was reported.

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY (DART) STUDIES

A teratogenicity study was conducted in accordance with OECD TG 414 and GLP to evaluate Hydroxyethyl-3,4-Methylenedioxy-aniline HCl (purity 99.8%) in rats (HanBrl: WIST, SPF quality).[2] The test substance in bi-distilled water was administered by gavage to pregnant rats (n=22/ group) on day 6 to day 20 of gestation at the following dosage rates: 0, 50, 250, and 1000 mg/kg/day. All animals were killed at day 21 of gestation and necropsies were performed.

There was observed instability of the test solution reported in this study (assumed to have been caused by "incorrect sample handling" contributing to oxidation of the test substance), although previous experiments showed Hydroxyethyl-3,4-Methylenedioxyaniline HCl to be stable in water at room temperature for 7 days.[2] Therefore, dosage rates were re-calculated based on analytical results to be 26, 152, and 702 (reduced to 688) mg/kg/day.

Following the first 3 days of dosing (day 9 to day 20 of gestation) the high dose of 702 mg/kg/day was reduced to 688 mg/kg/day because of severe toxicity and mortality (5 animals died or were killed because of moribund conditions).[2] In the 688 mg/kg/day group, hypoactivity, hunched posture, lacrimation, and behavioral indications of discomfort were observed. Discomfort was also seen at 152 mg/kg/day. With 152 mg/kg/day and 688 mg/kg/day, reduced food consumption and body weight were noted. At the same dosage rates, severe stomach abnormalities and effects on adrenals, spleen, and kidneys were observed. An increase in fetal resorptions occurred with the 688 mg/kg/day group only, compared to controls. The 688 mg/kg/day treatment resulted in effects on litter size and fetal body weight, but not on the sex ratio of the fetuses. In the 152 and 688 mg/kg/day groups, skeletal and visceral abnormalities were seen (at 688 mg/kg/day: hemorrhages, dilated lateral brain ventricles, thymus, cranial displacement and/or elongation; at 688 and 152 mg/kg/day: heart, aorta and pulmonary effects). At 152 mg/kg/day and 688 mg/kg/day, there was an increase in non-ossified and incompletely ossified bones of the cranium, vertebrae, ribs, sternebrae, and also of extremities and supernumerary ribs. Treatment-related effects were seen on cervical and thoracic vertebrae and on costal cartilage in the 152 mg/kg/day and 688 mg/kg/day groups.

The study authors considered the severe reproductive and developmental effects at the 688 mg/kg/day and 152 mg/kg/day dosage rates to be caused by maternal toxicity.[2] A reproductive and developmental NOAEL of 26 mg/kg/day was reported.

GENOTOXICITY STUDIES

Provided below is a summary of genotoxicity studies that are presented in detail in Table 3.

Experiments examining Hydroxyethyl-3,4-Methylenedioxyaniline HCl were conducted *in vitro*. An Ames test using *Salmonella typhimurium* was negative for genotoxicity up to 5000 µg/plate.[2] A mammalian cell gene mutation assay using mouse lymphoma cells was non-mutagenic during a 4-hour incubation with concentrations up to 1100 µg/ml with metabolic activation and up to 1650 µg/ml without activation. In a micronucleus test performed in human peripheral blood lymphocytes, there was a statistically significant, concentration-dependent increase in relevant micronucleated binucleate cells in the presence of metabolic activation. This indication of chromosomal damage resulted from a 3-hour treatment (at concentrations up to 2177 µg/ml) performed 48 hours after mitogen stimulation (cells were harvested 96 hours after mitogen stimulation); cytotoxicity (13% to 65%) was noted at concentrations ranging from 700 to 2177 µg/ml in several of the micronucleus experiments.

Palpebral closure and lethargy were observed 1 hour post-administration in an *in vivo* bone marrow micronucleus test conducted in mice that were intraperitoneally exposed to Hydroxyethyl-3,4-Methylenedioxyaniline HCl (up to 250 mg/kg).[2] No chromosomal aberrations or damage to the mitotic apparatus in bone marrow cells were observed from treatment with the test substance.

CARCINOGENICITY STUDIES

There were no carcinogenicity studies for Hydroxyethyl-3,4-Methylenedioxyaniline HCl found in the literature.

DERMAL IRRITATION AND SENSITIZATION STUDIES

Irritation

Animal

A non-GLP study was conducted in female, Pirbright White (SPF) guinea pigs (n=15) to evaluate the effect of Hydroxyethyl-3,4-Methylenedioxyaniline HCl through dermal exposure.[2] To a 12 cm^2 area of clipped flank skin, a 5% aqueous solution of Hydroxyethyl-3,4-methylenedioxyaniline HCl (purity not specified) was applied (non-occlusive) one time per day for 5 consecutive days. The skin was examined for signs of irritation five hours following each application and on the third day following the final application. The results indicated that the test substance was non-irritating.

Sensitization

Animal

A local lymph node assay (LLNA) was performed in accordance with OECD TG 429 and GLP.[2] To examine skin sensitization potential, the cell proliferation in the draining lymph nodes was measured in CBA/J mice (n=5 females/ concentration) following dermal exposure to Hydroxyethyl-3,4-Methylenedioxyaniline HCl (99.7% purity). To the ear skin of mice, 25 µl of 0 (vehicle only), 0.5, 1.5, 5, and 10% Hydroxyethyl-3,4-Methylenedioxyaniline HCl (in dimethyl sulfoxide (DMSO), or in a 3:1 mixture of aqua/acetone (1:1) and olive oil) were applied for 3 consecutive days. A hair dryer was used to dry the ears after application. On day 5, 250 µl phosphate buffered saline containing 23.5 µCi of ^{3}H methyl thymidine were injected intravenously into the animals, which were killed 5 hours later. The draining auricular lymph nodes were weighed after removal. Single cell suspensions were prepared for each mouse, trichloroacetic acid was used to precipitate cells, and radioactivity was determined.

At the 1.5% test concentration (DMSO vehicle), slight-to-severe skin desquamation on the ears was observed in all animals by day 5, and erythema was noted in one animal. The authors attributed these effects to the low pH of the test solution.[2] The stimulation indices for all the test concentrations in either vehicle were greater than 3 (dose-dependent with DMSO vehicle), and an EC3 ("equal to the concentration inducing a stimulation index of 3") was not estimated. Positive controls performed as expected. The results using either vehicle indicated that dermally applied Hydroxyethyl-3,4-Methylenedioxyaniline HCl has strong sensitization potential in mice.

QSAR

A study conducted in 2004 applied a previously published, quantitative structure-activity relationship (QSAR) model to estimate the skin sensitization potential of all European registered hair dye substances, including Hydroxyethyl-3,4-Methylenedioxyaniline HCl.[15] Topological sub-structural molecular descriptors (TOPS-MODE) were correlated with LLNA experimental data (based on LLNAs of 93 chemicals) to construct a general QSAR model, unrestrictive to chemical class. This QSAR model was not specifically designed for hair dye substances, but deemed by the study authors to be suitable for this application. The allergic contact dermatitis that may develop from the use of skin-sensitizing compounds involves the formation of an antigenic complex, consisting of a protein covalently bonded to a low-molecular-weight hapten molecule. This type of biochemical reaction is amenable to developing predictive tools, such as QSAR models. A literature review was also conducted as part of this study to retrieve relevant LLNA data (up to July 2003) and human evidence (up to August 2003) to help validate the QSAR predictions or elucidate areas in need of improvement. Hair dyes that were studied in this analysis were classified as strong/moderate, weak, or extremely weak sensitizers or non-sensitizing. Values indicating rank were assigned to each chemical studied, reflecting the predicted relative sensitization potential within each classification group. Approximately 75% of the 229 hair dye substances assessed were classified as strong/moderate sensitizers based on the QSAR model prediction. Hydroxyethyl-3,4-Methylenedioxyaniline HCl was predicted to be a moderate or strong sensitizer with an assigned numerical value of 0.2. For comparison, the highest value assigned to the hair dye substances categorized as strong/moderate sensitizers was 16.3 for Direct Red 80.

OCULAR IRRITATION STUDIES

A non-GLP study was conducted in Pirbright White (SPF) guinea pigs (n=10 females) evaluating 2% (water vehicle) Hydroxyethyl-3,4-Methylenedioxyaniline HCl.[2] A single, 0.1 ml application of the test substance was instilled into the conjunctival sac of the right eye, and the left eye served as the control; the eyes were not rinsed following application. Eyes were examined 0.5, 1, 2, 3, 4, 6, and 7 hours after the instillation of the test substance. At 24 hours following treatment, a fluorescein-instillation reading was performed. Edema, slight conjunctival redness, and corneal opacity were observed in 2 animals 3 hours post-instillation. The study authors concluded that 2% Hydroxyethyl-3,4-Methylenedioxyaniline HCl caused transient irritation to guinea pig eyes.

CLINICAL STUDIES

Human

A Swedish market analysis was conducted on 122 oxidative hair dye products sold at typical retailers (i.e., grocery stores, beauty shops, hairdressing salons).[16] The hair dye products evaluated were marketed in Europe and internationally. Shades from light to dark were represented in this evaluation. Information regarding the ingredients comprising the hair dye formulations was gathered from the product label and the European Commission cosmetic ingredient database (COSING); no chemical testing was performed in this study. Of the 122 hair dye formulations examined, 120 contained ingredients known to be potent skin sensitizers. Notably, more than 80% of the hair dye products contained at least 4 potent skin sensitizers. The range of potent skin sensitizers present in light blonde shades was 0 to -8, in dark brown colors was 2 to 11, and in black dyes was 3 to 7. Hydroxyethyl-3,4-Methylenedioxyaniline HCl was one of the 37 target hair dyes, of the products examined in this analysis, identified by the study authors to be a potent skin sensitizer. It was reported to be used at the maximum authorized concentration of 3% or, when combined with hydrogen peroxide, at 1.5%, as determined by the Cosmetics Directive 76/768/EEC, consolidated version 2008-04-24. No additional information specific to Hydroxyethyl-3,4-Methylenedioxyaniline HCl was provided in this analysis.

EPIDEMIOLOGY STUDIES

Hydryoxyethyl-3,4-Methylenedioxyaniline HCl is used as an oxidative (permanent) hair dye ingredient. While the safety of individual hair dye ingredients is not addressed in epidemiology studies that seek to determine links, if any, between hair dye use and disease, such studies do provide broad information. Currently available epidemiology studies provided insufficient evidence to support a causal association between personal hair dye use and a variety of tumors and cancers.

A detailed summary of the available hair dye epidemiology data is available at http://www.cir-safety.org/cir-findings.

SUMMARY

Hydroxyethyl-3,4-Methylenedioxyaniline HCl functions in cosmetics as a hair colorant. VCRP data received in 2016 indicates that there are 67 reported uses of Hydroxyethyl-3,4-Methylenedioxyaniline HCl in hair dyes and colors. As a coal tar hair dye, this ingredient requires manufacturers to include special caution statements and patch test instructions on product labels.

Hydroxyethyl-3,4-Methylenedioxyaniline HCl contains a free, secondary aromatic substituted amine (aniline derivative), which raises a concern about possible *N*-nitrosation. Manufacturers should formulate products containing this ingredient in a manner that reduces the prevalence of nitrosamines, and eliminates the presence of impurities that are nitrosating agents or are *N*-nitrosated.

An *in vitro* percutaneous absorption study evaluated 1.5 mg/cm^2 (1.5%) Hydroxyethyl-3,4-Methylenedioxyaniline HCl as part of an oxidative hair dye formulation that was applied to 4 cm^2 porcine skin mounted on a diffusion Teflon-chamber. The test substance was washed off the skin 60 min post-application. At 16 and 24 hours post-application, fractions of receptor compartment fluid were collected, concentrated and analyzed. By 24 hours the applied amount recovered was 0.047% in the upper skin (above the basal layer), 0.0067% in the lower skin (basal layer skin down to upper dermis), and 0.3% in the receptor compartment fluid. The skin penetration rate was determined to be 5.8 µg/cm^2.

An *in vivo* percutaneous absorption experiment in rat skin was conducted to evaluate ^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labelled) in the following formulations: 3.33% pure dye in water; 1% formulation with hydrogen peroxide (also containing *p*-toluenediamine, resorcinol, and *m*-aminophenol in this application); 1% formulation without hydrogen peroxide. At 30 minutes post-application the test substance was removed and the skin washed. Results indicated that the washing solutions contained 95.9% to 97.1% of the applied radiolabeled concentration. The skin contained 1.68% of the applied radioactivity for the formulation containing water, 0.56% for formulation containing hydrogen peroxide, and 0.34% for the formulation without hydrogen peroxide.

In toxicokinetic experiments (*in vivo*), 1% to 3% ^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labeled) was applied to rat skin for 30 minutes and then removed by washing. The applied radioactive concentration detected in urine and feces (combined percentages) by 72 hours post-application were 0.05% to 0.345%. Dermal absorption rates ranged from 0.59 µg/cm^2 to 14.8 µg/cm^2 and no substantial bioaccumulation was observed.

Toxicokinetic experiments performed *in vivo* evaluated the effects of ^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labeled) in orally exposed rats (1 to 100 mg/kg single dosage). Results indicated that > 95% of the dosed radioactivity was absorbed. Blood, urine, and feces samples were collected up to 24 to 96 hours post-dosing. The dosed radioactivity detected in urine and feces were 78% and 14%, respectively; bioaccumulation was not observed. The highest blood concentration detected following oral exposure was 70-fold greater than the highest blood concentration after dermal exposure.

An acute, oral exposure toxicity study in rats and mice was conducted to evaluate 1-β-hydroxyethyl-3,4-methylenedioxybenzene, an analog of Hydroxyethyl-3,4-Methylenedioxyaniline HCl. 1-β-Hydroxyethyl-3,4-methylenedioxybenzene (10% suspension in 10% Arabic gum solution) was administered by gavage to rats at the following single dosages: 1000, 1500, 2000, and 2500 mg/kg. The test substance was administered by gavage to mice at the following single dosages: 500, 750, 1000, and 1250 mg/kg. Immediately following dosing, observations of decreased activity, staggering, and piloerection were noted, and animal deaths were reported at 2 to 72 hours post-administration. The calculated LD_{50} values were 1550 mg/kg for female rats, 1650 mg/kg for male rats, and 850 mg/kg for female mice.

A subchronic (90-day) oral exposure study was conducted in rats to evaluate the effects of Hydroxyethyl-3,4-Methylenedioxyaniline HCl following administration by gavage with 0, 20, 100, and 350 mg/kg/day in purified water. In the 100 and 350 mg/kg/day groups, both sexes showed reduced locomotion. Treatment-related abnormalities in red blood cells were observed in males at 350 mg/kg/day after 13 weeks, which were associated with splenic extramedullary hematopoesis found during histopathology. Abnormalities in red blood cells and organs (thymus, ovary, spleen) were observed in females (350 mg/kg/day). In both sexes elevated clinical biochemistry parameters and liver and kidney abnormalities were reported (350 mg/kg/day). At the 100 mg/kg/day dosage rate elevated bilirubin levels and phospholipids were seen in females, liver abnormalities were noted in males, and increased cholesterol and urinary volume occurred in both sexes. A NOAEL of 20 mg/kg/day was reported.

A teratogenicity study was conducted to evaluate Hydroxyethyl-3,4-Methylenedioxyaniline HCl in pregnant rats that were exposed by gavage to the following dosage rates (on days 6 to day 20 of gestation): 0, 26, 152, and 702 mg/kg/day. Following the first 3 days of dosing (days 9 to day 20 of gestation) the high dose (702 mg/kg/day) was reduced to 688 mg/kg/day because of severe toxicity and

mortality. In the 688 mg/kg/day group, hypoactivity, hunched posture, lacrimation, and behavioral indications of discomfort (discomfort also seen with 152 mg/kg/day) were observed. With 152 and 688 mg/kg/day dosage rates reduced food consumption and body weight were noted. At the same dosage levels stomach abnormalities and effects on adrenals, spleen, and kidneys were observed. An increase in fetal resorptions occurred (688 mg/kg/day group only). The 688 mg/kg/day treatment resulted in effects on litter size and fetal body weight, but not on sex ratio of fetuses. For the 152 and 688 mg/kg/day groups skeletal and visceral abnormalities were seen. The study authors considered the severe reproductive and developmental effects at the 152 and 688 mg/kg/day dosage rates to be caused by maternal toxicity. A reproductive and developmental NOAEL of 26 mg/kg/day was reported.

Hydroxyethyl-3,4-Methylenedioxyaniline HCl was negative in an Ames test (using *Salmonella typhimurium*) up to 5000 µg/plate. Results from a mammalian cell gene mutation assay using mouse lymphoma cells indicated that the ingredient was non-mutagenic during a 4-hour incubation, at concentrations up to 1100 µg/ml with metabolic activation and up to 1650 µg/ml without activation. A micronucleus test performed in human peripheral blood lymphocytes showed a statistically significant, concentration-dependent increase in relevant micronucleated binucleate cells in the presence of metabolic activation. This indication of chromosomal damage resulted from a 3-hour treatment (at concentrations up to 2177 µg/ml) performed 48 hours after stimulation with a mitogen. No chromosomal aberrations or damage to the mitotic apparatus in bone marrow cells were observed in an *in vivo* bone marrow micronucleus test conducted in mice intraperitoneally exposed to Hydroxyethyl-3,4-Methylenedioxyaniline HCl (up to 250 mg/kg).

There were no carcinogenicity studies for Hydroxyethyl-3,4-Methylenedioxyaniline HCl found in the literature.

Hydroxyethyl-3,4-Methylenedioxyaniline HCl (5% aqueous solution) was not irritating when applied (non-occlusive) one time per day for 5 consecutive days to guinea pig skin clipped free of hair.

Hydroxyethyl-3,4-Methylenedioxyaniline HCl (up to 10% in DMSO or in a 3:1 mixture of aqua/acetone (1:1) and olive oil) was shown to have strong sensitization potential in mouse skin in an LLNA test.

A study was conducted applying a previously published QSAR model (based on TOPS-MODE and LLNA experimental data) to estimate the skin sensitization potential of all European registered hair dye substances in 2004, including Hydroxyethyl-3,4-Methylenedioxyaniline HCl. Hydroxyethyl-3,4-Methylenedioxyaniline HCl was predicted to be a moderate or strong sensitizer.

An ocular irritation study was conducted in guinea pigs to evaluate 2% (water vehicle) Hydroxyethyl-3,4-Methylenedioxyaniline HCl. A single, 0.1 ml application of the test substance was instilled into the conjunctival sac of the right eye while the left eye served as the control; the eyes were not rinsed following application. Edema, slight conjunctival redness, and corneal opacity were observed in 2 animals, 3 hours after treatment. The study authors concluded that 2% Hydroxyethyl-3,4-Methylenedioxyaniline HCl caused transient irritation to guinea pig eyes.

In a Swedish market analysis (no chemical testing was conducted in this study) Hydroxyethyl-3,4-Methylenedioxyaniline HCl was one of 37 target hair dyes in 122 products examined., Hydroxyethyl-3,4-Methylenedioxyaniline HCl was identified by the study authors to be a potent skin sensitizer. It was reported to be used at the maximum authorized concentration of 3% or when combined with hydrogen peroxide at 1.5%.

The most recent, comprehensive review of available epidemiology studies concluded that there is insufficient evidence to support a casual association between personal hair dye use and a variety of tumors and cancers. A summary of the available hair dye epidemiology data is available at http://www.cir-safety.org/findings.html.

INFORMATION SOUGHT

The CIR is seeking the following information on Hydroxyethyl-3,4-Methylenedioxyaniline HCl for use in the resulting safety assessment:

1. Method of manufacture and an oxidative dye use reaction scheme, indicating the reactive intermediate;
2. Any other data relevant to the determination of safety of these ingredients as used in cosmetics.

TABLES

Table 1. Physical and Chemical Properties of Hydroxyethyl-3,4-Methylenedioxyaniline HCl

Property	Value	Reference
Physical Form	Crystals	[2]
Color	Beige	[2]
Formula Weight (g/mol)	217.65	[2]
Density (g/ml) @ 20°C	1.4269	[2]
Vapor Pressure mmHg @ 20°C	5.5 to 6.0	[2]
Melting Point (°C)	162-165	[2]
Water Solubility (pH 1.5) (g/l) @ 20°C	408 (saturated solution)	[2]
Water Solubility (pH 6.0) (g/l) @ 20°C	< 20	[2]
Other Solubility (pH 7.0) (g/l)	Acetone/water (1:1) >100	[2]
Other Solubility (g/l)	DMSO > 100	[2]
Other Solubility (g/l)	Ethanol 15-40	[2]
Log P (pH 4.65) @ 36 °C	0.412	[2]

Table 2.Toxicokinetics Studies (ADME) for Hydroxyethyl-3,4-Methylenedioxyaniline HCl

Test Substance(s)	Species/ Strain	Sample Type/Test Population-Sex	Concentration or Dosage (Vehicle)	Procedure	Results	Reference
ANIMAL						
Dermal						
^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labelled, 100% purity)	Rat/ Wistar Kyoto WKY/ NR Crl BR (inbred)	n=4 females in Exp 1 (urine, feces, metabolite analysis performed in this mass balance experiment) n=5 females in Exp 2 (urine, feces, blood, tissues, carcass examined in toxicokinetics analysis)	Exp 1 & Exp 2: 1.5% ^{14}C-labeled (10 mg/kg, 0.15 mg/cm^2); vehicle=acetone/water, 1:1; pH adjusted to 7-8.5	A GLP study was conducted in accordance with OECD TG 417 and 427; immediately before treating the skin it was cleaned with 10% shampoo and water solution and dried; test substance (in Exp 1 & 2) was applied 1x to skin shaved free of hair on the back (2.5 x 4 cm^2); a 4 x 4 cm^2 area of shaved skin on the abdomen was used as a negative control; animals were anesthetized during application, exposure, and removal of test substance; 30 min post-application, skin was washed with an aqueous shampoo solution; in Exp 1, urine and feces were collected 5x for the first 96 h post-administration; metabolite analysis was conducted via HPLC; in Exp 2 blood was collected at 10, 20, 40 min and at 1, 2, 4, 8, 24, 48, and 72 h post-administration (urine, feces, blood, tissues, and carcass were analyzed for radioactivity-no further details provided as to when the sampling occurred); animals in Exp 1 & 2 were killed 96 h post-administration	Exp 1: Recovery of applied radioactivity was 97% to 99%; in urine and feces only the metabolized test substance was detected and no conjugates were found; authors speculated that metabolites may have been excreted in bile and any that were glucuronide conjugates may have been de-conjugated in intestines Exp 2: Absorption rate based on radioactive content in carcass, urine, and feces was 5% (8 µg/cm^2); if the radioactive content of the application site is added to the total of what was potentially absorbed the result is 8% (14.8 µg/cm^2) of applied radioactivity; maximum concentration of radioactivity in blood was observed 1 h post-application (no further details provided); study authors noted that elimination of the radioactive concentration was excreted in the greatest amounts in urine and much less in feces and that bioaccumulation was not observed	[2]
^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labelled)	Rat/ Sprague Dawley Him:OFA, SPF	n=3/sex/ concentration group	Exp 1: 1% (1.1 mg/cm^2) formulation without hydrogen peroxide (urine, feces, and carcass analysis) Exp 2: 1% (1.1 mg/cm^2) formulation with hydrogen peroxide, p-toluenediamine, resorcinol, m-aminophenol (urine, feces, and carcass analysis) Exp 3: 3.33% (1.1 mg/cm^2) pure dye in water (urine, feces, and carcass analysis) Exp 4: 3.33% (1.1 mg/cm^2) pure dye in water (blood analysis)	In a GLP study the test substance was applied (animals under anesthesia) 1 x to skin clipped free from hair as indicated in Experiments 1-4; skin surface area to which test substance was applied in Exp 1 was 10-12 cm^2 and for all other experiments was 9 cm^2; at 30 min post-application the test substance was removed and skin washed with shampoo formulation (100 ml) and then by warm water until no color was seen; skin was then covered with gauze patch and secured with adhesive and an air permeable plastic cone to prevent animals from licking treated area during the 72-hour study duration (carried out in metabolism cages); Exp 1, 2, & 3 were used to evaluate radioactivity eliminated in urine and feces and Exp 4 was used to evaluate administered radioactivity in blood; blood was sampled several times within 24 h post-dosing (animals were under light anesthesia during blood collection from the peri-orbital plexus); in all experiments animals were killed 72 h post-application	Dermal penetration results are described in the text of this assessment; applied radioactivity was eliminated within 72 h, mainly in urine and to a lesser extent in feces, in the following combined (urine + feces) amounts: 0.314% (Exp 1), 0.05% (Exp 2), and 0.345% (Exp 3); amount of applied radioactivity remaining in the carcass 72 h post-application was 0.005% for Exp 1 & 3 and 0.002% for Exp 2; cutaneous absorption rates of 3.5 µg/cm^2 (Exp 1), 0.59 µg/cm^2 (Exp 2), and 3.95 µg/cm^2 (Exp 3) were reported for bioavailable amounts in carcass, urine, and feces combined; in Exp 4, highest blood concentration (no further details provided) of test substance was observed at the first sampling (35 min post-application) then declined, half-life was determined to be 1 h	[2]

Table 2.Toxicokinetics Studies (ADME) for Hydroxyethyl-3,4-Methylenedioxyaniline HCl

Test Substance(s)	Species/ Strain	Sample Type/Test Population-Sex	Concentration or Dosage (Vehicle)	Procedure	Results	Reference
			Oral			
^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labeled)	Rat/ Sprague Dawley Him:OFA, SPF	n=3/sex	51.02 mg/kg (3.3%) pure dye in water (blood analysis)	In a GLP test performed in conjunction with the 4 dermal experiments summarized in the previous row of this table, the test substance was administered 1 x by gavage; blood was sampled several times within 24 h post-dosing (animals were under light anesthesia during blood collection from the peri-orbital plexus); animals were killed 72 h post-administration	Highest blood concentration (no further details specified) of test substance was observed at the first sampling (35 min post-application) then declined, half-life was determined to be 1.5 h; the highest blood concentration following oral exposure was approximately 70-fold greater than highest observed blood concentration following dermal application in the study summarized above (in the preceding row)	[2]
^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (ring-labeled, purity 100%)	Rat/ Wistar Kyoto WKY/ NR Crl BR (inbred)	n=4 females/experiment in Exp 1 & 2 (urine, feces, metabolite analysis performed in this mass balance experiment) n=6 females/ experiment in Exp 3 & 4 (urine, feces, blood, tissues, carcass examined in toxicokinetics analysis)	Exp 1 & 3: ^{14}C-labeled 1 mg/kg in water Exp 2 & 4: ^{14}C-labeled 100 mg/kg in water	A GLP study was conducted in accordance with OECD TG 417 and 427; single dosages were administered by gavage to fasted animals (18 h prior to and 4 h after dosing); in Exp 1 & 2 urine and feces were collected 5 x for the first 96 h post-administration; metabolite analysis was conducted via HPLC; in Exp 3 & 4 blood was collected at 10, 20, 40 min and at 1, 2, 4, 8, 24, 48, and 72 h post-administration (urine, feces, blood, tissues, and carcass were analyzed for radioactivity-no further details provided as to when the sampling occurred); all animals were killed 96 h post-administration	Exp 1 & 2: Recovery of administered radioactivity was 97% to 99%; in urine and feces, only the metabolized test substance was detected and no conjugates were found; authors speculated that metabolites may have been excreted in bile and any that were glucuronide conjugates may have been de-conjugated in intestines Exp 3 & 4: > 95% of dosed radioactivity was absorbed (no further details provided); blood kinetics showed fast absorption for low and high dosages; highest concentration of radioactivity in blood was observed 10 min post-dosing; highest residual amount of radioactivity was located in carcass, liver, kidney and thyroid but was only 1% of administered radioactive dosage; study authors noted that elimination of the radioactive dosage was excreted in the greatest amounts in urine and much less in feces, and the radioactivity in feces represented metabolites of test substance that were excreted through bile; bioaccumulation was not observed	[2]

Table 2.Toxicokinetics Studies (ADME) for Hydroxyethyl-3,4-Methylenedioxyaniline HCl

Test Substance(s)	Species/ Strain	Sample Type/Test Population-Sex	Concentration or Dosage (Vehicle)	Procedure	Results	Reference
^{14}C-Hydroxyethyl-3,4-Methylenedioxyaniline HCl (position of label not specified)	Rat/ Wistar CRL:WI BR	n=?	1 mg/kg (Mass balance group) 1 mg/kg (Toxicokinetics group) No further details on groups provided	Animals were fasted for 18 h before and 4 h after dosing, then food and water were freely available; a single dosage was administered; for mass balance group urine and feces were collected prior to treatment and 6 x up to 96 h post-dosing; for toxicokinetics, grouped blood samples were collected at 0.25, 0.5, 1, 4, 8, and 24 h post-dosing; animals were killed 72 or 96 h post-dosing; urine, feces, and blood samples were extracted and analyzed by LC-MS/MS; oral absorption of total radiolabeled parent compound and metabolites was calculated based on % recovered in urine following oral exposure divided by the sum of % recovered in urine and % recovered in feces following oral exposure, yielding the normalized amount excreted in urine; the fraction absorbed following oral exposure was calculated as the normalized amount in urine (after oral exposure) divided by normalized amount in urine following iv exposure (calculated similarly to calculation using the oral exposure data); calculations were performed using plasma data to estimate oral absorption (by dividing the dose normalized ^{14}C area under the curve following oral exposure by dose normalized ^{14}C area under the curve following iv exposure)	Fraction of administered radioactivity detected in urine was 77.8% ± 8.2% and in feces was 14.3% ± 4.4% of the administered radioactivity; Fraction of radioactivity absorbed following oral exposure was calculated to be 99% from total radioactivity in the urine (normalized to the amount recovered), and 91% from the normalized plasma AUCs after oral and i.v. exposures.	[14]

DMSO=Dimethyl Sulfoxide; GLP=Good Laboratory Practice; HPLC=High Performance Liquid Chromatography; LC-MS/MS=Liquid Chromatography-Mass Spectrometry/Mass Spectrometry; OECD TG=Organization for Economic Co-operation and Development Test Guideline; P_{app}=Apparent permeability values: TEER=transepithelial electrical resistance

Table 3. Genotoxicity Studies for Hydroxyethyl-3,4-Methylenedioxyaniline HCl

Species/ Strain	Sample Type/ Test Population	Concentration (Vehicle)	Procedure	Results	Reference
			IN VITRO		
Salmonella typhimurium	TA98, TA100, TA102, TA1535, TA1537	33, 100, 333, 1000, 2500, 5000 µg/plate (vehicle=de-ionized water); purity of test substance was 99.8%	Bacterial reverse mutation assay (Ames Test) was performed, with and without metabolic activation, in accordance with GLP and OECD TG 471 (Bacterial Reverse Mutation Assay); both the plate incorporation method and pre-incubation methods were used in two separate experiments using the same concentrations listed; negative, vehicle, and positive controls were used	Negative; controls performed as expected	[2]

Table 3. Genotoxicity Studies for Hydroxyethyl-3,4-Methylenedioxyaniline HCl

Species/ Strain	Sample Type/ Test Population	Concentration (Vehicle)	Procedure	Results	Reference
Mouse	Thymidine kinase locus in mouse lymphoma L5178Y cells	Exp. 1: 34.4, 68.8, 137.5, 206.3, 275, 412.5, 550, 825, 1100 µg/ml (4 h incubation, with activation); Exp. 2: 68.8, 137.5, 206.3, 275, 412.5, 550, 825, 1100, and 1650 µg/ml (4 h incubation, without activation); Exp. 3: 12.5, 25, 50, 100, 150, 200, 250, and 300 µg/ml (24 h incubation without activation) Purity of test substance was 99.8% (vehicle=de-ionized water)	Range-finding test was performed using a concentration range of 17.2 to 2200 µg/ml; Concentration ranges in Exp. 1 & 2 were limited by the toxicity of test substance; Mammalian cell gene mutation assay was performed, with and without metabolic activation, in accordance with GLP and OECD TG 476 (*In vitro* Mammalian Cell Gene Mutation Test); solvent and positive controls were used	Non-mutagenic; no biologically relevant increases in mutations were observed	[2]
Human	Peripheral blood lymphocytes	Exp. 1: 500, 1100, 1800 µg/ml (3 h treatment 24 h after mitogen stimulation, with activation); 200, 400, 700 µg/ml (20 h treatment 24 h after mitogen stimulation) Exp. 2: 1200, 1500, 1800, 2177 µg/ml (3 h treatment 48 h after mitogen stimulation, with activation); 650, 750, 950 µg/ml (20 h treatment 48 h after mitogen stimulation) Purity of test substance was 99.8% (vehicle=sterile water)	Micronucleus test was performed in accordance with OECD TG 487 and GLP; blood was pooled from 2 male donors in each test; mitogen stimulation was performed with phytohemagglutinin; 72 h and 96 h after mitogen stimulation, cells from Exp 1 and 2, respectively, were harvested; 500 cells per replicate (1000/concentration) were examined for mono-, bi-, and multi-nucleated cells used to calculate the replication index; 1000 binucleate cells from each culture were evaluated for number of micronuclei; solvent and positive controls were used	Cytotoxicity reported in Exp. 1 was 65% (700 µg/ml) and 53% (1800 µg/ml) and in Exp. 2 was 62% (950 µg/ml) and 13% (2177 µg/ml); in Exp. 2 the authors reported a clear concentration-dependent, statistically significant increase in relevant micronucleated binucleate cells in the presence of metabolic activation, indicating chromosomal damage; the same effects were not seen in the absence of metabolic activation; positive controls performed as expected	[2]
			IN VIVO		
Mouse/ NMRI	Pre-Experiment: n=3/sex/dosage Main Experiment: n=5/sex/dosage/time	Pre-Experiment: 250 to 1000 mg/kg Main Experiment: 25, 125, 250 mg/kg Purity of test substance was 99.7% (vehicle=distilled water)	A bone marrow micronucleus test was performed in accordance with OECD TG 474 and GLP; a pre-experiment was performed for dosage selection; in the main experiment, single dosage was administered intraperitoneally 24 h (all dosage levels) or 48 h (250 mg/kg) before animals were killed; at least 2000 polychromatic erythrocytes with micronuclei from each animal were analyzed; the ratio between polychromatic and total erythrocytes was determined for each animal; solvent and positive controls were used	Pre-Experiment: 3 male and 3 female mice died in 24 h (1000 mg/kg); 1st h post-administration palpebral closure and lethargy (for up to 6 h) were observed, but there was no mortality (250 mg/kg) Main Experiment: Similar toxic effects with 250 mg/kg as noted in pre-experiment at 250 mg/kg; ratio between polychromatic erythrocytes and total erythrocytes unaffected by test substance; according to the study authors, the observed systemic toxicity indicated systemic distribution and bioavailability of test substance; there was no statistically significant increase in micronuclei/2000 polychromatic erythrocytes in treated vs. control animals; positive controls performed as expected; test substance did not induce chromosome aberrations or damage to mitotic apparatus in bone marrow cells	[2]

GLP=good laboratory practice; OECD TG=Organization for Economic Co-operation and Development Test Guideline

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