EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

Hydroxyethyl-3,4-methylenedioxyaniline HCl

COLIPA n° A98

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 5th plenary meeting
of 8 December 2009

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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ACKNOWLEDGMENTS

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This opinion has been subject to a commenting period of four weeks after its initial publication. All comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged.

Keywords: SCCS, scientific opinion, hair dye, A98, hydroxyethyl-3,4-methylenedioxyaniline HCl, directive 76/768/ECC, CAS 94158-14-2, EINECS 303-085-5

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on hydroxyethyl-3,4-methylenedioxyaniline HCl, 8 December 2009

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1. BACKGROUND

Submission I for Hydroxyethyl-3,4-methylenedioxyaniline hydrochloride was submitted in February 1989 by COLIPA[1] according to COLIPA.

The Scientific Committee on Cosmetology (SCC) adopted at its 48th plenary meeting the 4 of October 1991 an opinion with the conclusion that:

"Aminol has moderate acute toxicity by the oral route. Limited studies suggest that dermal absorption from hair dye formulations can be up to 2 %. There was no evidence of skin irritancy in animals using a 5 % solution of aminol, and only a mild effect in some humans using a hair dye formulation containing hydrogen peroxide and 2 % aminol and using an occlusive dressing for 24 hours. A 2 % solution produced no significant eye irritation in animals. In a 90-day oral study the No Effect Level was 20 mg/kg with evidence of bone marrow toxicity at 275 mg/kg, and lethality at 550 mg/kg. Aminol has been examined in a range of mutagenicity studies in vitro (gene mutation in Salmonella and mouse lymphoma cells, metaphase analysis of lymphocytes for clastogenicity) with negative results. Negative results were also obtained in in vivo assays for sister chromatid exchange and micronucleus induction in bone marrow. No adverse effects were reported in oral teratogenicity studies in rats at up to 1000 mg/kg or rabbits up to 250 mg/kg. An adequate test for the sensitization potential of aminol is required."
Submission II for Hydroxyethyl-3,4-methylenedioxyaniline hydrochloride was submitted in May 1993 by COLIPA according to COLIPA.

The Scientific Committee on Cosmetology (SCC) adopted at its 60th plenary meeting the 23 of June 1995 an opinion with the conclusion that:

"*Aminol has a sensitizing potential."*

The substance is currently regulated in Annex III, part 2, entry 44 on the list of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission III for Hydroxyethyl-3,4-methylenedioxyaniline hydrochloride was submitted by COLIPA in July 2005. According to this submission the substance is used as an oxidative hair colouring agent (precursor). The intended maximum on-head concentration is 1.5%. The oxidative colouring agent and the developer are mixed at ratios between 1:1 to 1:3 (g dye + g hydrogen peroxide). It is common practice to apply up to 100 g of the finished mixed product for a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at monthly intervals.

The Scientific Committee on Consumer Products (SCCP) adopted during the 7th plenary meeting of 28 March 2006 the opinion (SCCP/0951/05) with the following conclusion:
"*The SCCP is of the opinion that the information submitted is insufficient to assess the safe use of the substance. Hydroxyethyl-3,4-methylenedioxyaniline HCl is a secondary amine and thus prone to nitrosation. It should therefore not be used in combination with nitrosating substances.*
Before any further consideration, the following information is required:

- *The nitrosamine content should be given.*
- *Clarification on the problems with the stability of the test substance used in the prenatal developmental toxicity study (ref. 31).*

- *Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP opinions and in accordance with its Notes of Guidance.*

This hair dye, like many other hair dyes, is a skin sensitiser."

The current submission is the response to the opinion (SCCP/0951/05)

2. TERMS OF REFERENCE

1. *Does SCCS consider Hydroxyethyl-3,4-methylenedioxyaniline hydrochloride safe for use as an oxidative hair dyes with an on-head concentration of maximum 1.5% taken into account the scientific data provided?*

2. *And/or does the SCCS have any concerns with regard to the use of Hydroxyethyl-3,4-methylenedioxyaniline hydrochloride in any hair dye formulations?*

3. OPINION

Taken from SCCP/0951/05

3.1. Chemical and Physical Specifications

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

Hydroxyethyl-3,4-methylenedioxyaniline HCl (INCI)

3.1.1.2.	Chemical names

Ethanol, 2-(1,3-benzodioxol-5-ylamino)-, monohydrochloride (CA INDEX Name, 9CI)
2-(1,3-benzodioxol-5-ylamino)ethanol hydrochloride (IUPAC)

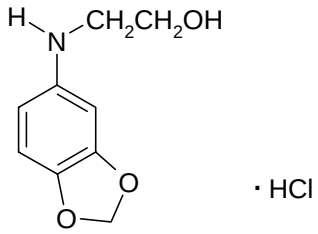
3.1.1.3.	Trade names and abbreviations

Aminol
COLIPA n° A98

3.1.1.4.	CAS / EC number

CAS: 94158-14-2
EC: 303-085-5

3.1.1.5.	Structural formula



· HCl

3.1.1.6.	Empirical formula

Formula: $C_9H_{11}NO_3.HCl$

3.1.2.	Physical form

Beige crystals

3.1.3.	Molecular weight

Molecular weight: 217.65

3.1.4.	Purity, composition and substance codes

Purity and impurities in various batches of Hydroxyethyl-3,4-methylenedioxyaniline HCl

Description	Batch n°				
	474 D (R00064585)	CH:5611/92	FF II/39	007 (R0075037)	9/93 (= 9/93 Barrel 195; 9/93 (EMS); R96007257)
NMR content, % (w/w)	99.8	99.6	99.2	99.9	98.1
HPLC purity, area% **210 nm** **254 nm** **285nm**	 99.9 99.8 100	 99.85 100 99.96	 99.80 99.91 99.86	 99.7 99.5 99.7	 99.5 99.7 99.8
HPLC content, % (w/w)	101.0*	100.6*	100.5*	99.0[a]	97.6[a]
Content of 3,4-(Methylenedioxy)-aniline (ppm)	114	360	1097	715	633
Content of 1,3-benzodioxol (ppm)	<35**	<35**	<35**	<35**	<35**
Content of 1,2-(Methylenedioxy)-4-nitrobenzene (ppm)	<8**	<8**	<8**	<8**	<8**
Chloride content	16.2	16.2	n.d.	16.2	16.2
Water content, % (w/w)	0.09	0.01	n.d.	0.06	0.09
Loss on drying, % (w/w)	0.03	0.03	n.d.	0.05	0.02
Ash, % (w/w	0.06	0.02	n.d.	0.01	0.02
Solvent residues	Methanol, ethanol, isopropanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone and monochlorobenzene were not detected at 100 ppm detection limit				

[a] refers to batch 474 D (R00064585)
* refers to batch 9/93 (R96007257)
** Limit of detection
n.d. not done because of lack of substance

The nitrosamine content of batches 5611/92, 9/93 (R96007257 and R00064585) was determined as total content of N-nitroso compounds (ATNC).

Hydroxyethyl-3,4-methylenedioxyaniline HCl	ATNC [µg/kg]	Nitrite [µg/kg]
5611/92	< 10	< 10
9/93 (R96007257)	< 10	< 10
474D (R00064585)	< 10	< 10

The ATNC content was <10 µg/kg for the tested batches.

Ref.: 37

Comment
3,4-(Methylenedioxy)-aniline (Annex II, n° 1248) is an aromatic amine with a free amino group. A 100-1100 ppm content of 3,4-(Methylenedioxy)-aniline impurity in Hydroxyethyl-3,4-methylenedioxyaniline HCl may be of concern.

3.1.5. Impurities / accompanying contaminants

See point 3.1.4. "Purity, composition and substance codes"

3.1.6. Solubility

Water: 408 g/L at 20°C (EC method A.6), pH 1.5; for solutions at pH 6: <20 g/L
Acetone/water (1:1): >100 g/L (pH 7.0)

DMSO: >100 g/L
Ethanol: 15 - 40 g/L

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 0.412 (pH 4.65; 36°C) EC method A.8

3.1.8. Additional physical and chemical specifications

Organoleptic properties: /
Melting point: 162-165°C (decomposition)
Boiling point: not applicable
Flash point: /
Vapour pressure: 7.3 exp – 8 hPa (20°C)
Density: 1.4269 g/ml (20°C)
Viscosity: /
pKa: /
Refractive index: /

3.1.9. Stability

Approximately 10% (w/w) solution of Hydroxyethyl-3,4-methylenedioxyaniline HCl in water and acetone:water was stable (variation <10%) for the one week study period, when stored in dark at room temperature.

Additional data on the stability of Hydroxyethyl-3,4-methylenedioxyaniline HCl (10%) solution were provided in an amendment to the Teratogenicity study submitted in March 2007. In a study in compliance with GLP, Hydroxyethyl-3,4-methylenedioxyaniline HCl (10%) solutions of each concentration (5, 25, 75, 100 mg/mL) were analysed in duplicate 0, 2, 7 days after preparation. HPLC was used for quantification. The variation of the recovery of Hydroxyethyl-3,4-methylenedioxyaniline HCl (10%) solutions was smaller than 10%. Therefore, the solutions are stable for one week, when stored in the dark at room temperature. According to the applicant the observed instability of the test item in the Teratogenicity study (ref 31, 35) is assumed to have been a result of incorrect sample handling (use of glass flasks with a significant supernatant of air, which favour oxidation reactions of the test substance).

Ref. 36

General comments on physico-chemical characterisation

- 3,4-(Methylenedioxy)-aniline (Annex II, n° 1248) is an aromatic amine with a free amino group. A 100-1100 ppm content of 3,4-(Methylenedioxy)-aniline impurity in Hydroxyethyl-3,4-methylenedioxyaniline HCl may be of concern.

- Hydroxyethyl-3,4-methylenedioxyaniline HCl is a secondary amine and thus prone to nitrosation. It should therefore not be used in combination with nitrosating substances. The nitrosamine content should be less than 50 ppb.

- Stability of Hydroxyethyl-3,4-methylenedioxyaniline HCl in a typical hair dye formulation is not reported

3.2. Function and uses

Hydroxyethyl-3,4-methylenedioxyaniline HCl is used at a final concentration of 1.5% in oxidative hair dye formulations, after mixing with the developer containing hydrogen peroxide.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCP/0951/05

Guideline:	Not indicated (but resembles OECD401)
Species/strain:	Rat, Wistar Crl: Wi/Br Mouse, CF1
Group size:	Rats: 5/sex/dose Mice: 10 females/dose
Test substance:	1-β-hydroxyethyl-3,4-methylenedioxybenzene suspended to 10% in a 10% gum Arabic solution
Batch:	not indicated
Purity:	not indicated
Dose:	Rats: 1000, 1500, 2000 and 2500 mg/kg bw Mice: 500, 750, 1000 and 1250 mg/kg bw
Observation:	14 days
GLP:	not in compliance

The test substance was administered by oral gavage to groups of rats and mice at 4 different dose levels ranging from 1000-2500 mg/kg bw in rats and from 500 to 1250 mg/kg bw in mice.
Clinical signs and mortality were checked daily for a period of 14 days following the single administration of the test item. Body weights were recorded and all animals were submitted to a gross necropsy at the end of the observation period.

Results
The test substance evokes the following toxicity symptoms at the dosages tested: decreased activity immediately after the application, staggering, piloerection and exitus (only as statement in the result section, no actual data reported). Animals died within 2-72h after substance administration. At necropsy, no macroscopic organ changes were noted.

Conclusion
Based on observed mortality, for 1-β-hydroxyethyl-3,4-methylenedioxybenzene (purity unknown) the following LD_{50} values were calculated using the method of Spearman-Karber:

LD_{50} rat (female): 1550 mg/kg bw
LD_{50} rat (male): 1650 mg/kg bw
LD_{50} mouse (female): 850 mg/kg bw

Ref.: 16

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Taken from SCCP/0951/05

Guideline:	/
Species/strain:	Guinea pigs, strain Pirbright White (SPF)
Group size:	15 females
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	/
Purity:	/
Route:	dermal
Concentration:	5%, dissolved in water
Application:	Repeated open application on the clipped flank region once daily for five consecutive days
GLP:	not in compliance

A 5% aqueous solution of hydroxyethyl-3,4-methylenedioxyaniline HCl was applied onto an area of 12 cm² of the clipped flank region of 15 female albino guinea pigs, once daily for 5 consecutive days. The skin was not covered. The animals were restrained in order to avoid contact with the treated area during application. The skin was evaluated according to the Draize-scheme for erythema and oedema 5 hours after each application and once on the third day after the last application.

Results
No skin reactions at all were observed at any observation time point.

Conclusion
A 5 % solution of hydroxyethyl-3,4-methylenedioxyaniline HCl revealed no indication of irritating properties to guinea pig skin under the conditions of the experiment.

Comment
Although the experiment was not guideline compliant and the purity of the test substance not described, the results indicate that the test substance is not overtly irritant to skin.

Ref.: 17

3.3.2.2. Mucous membrane irritation

Taken from SCCP/0951/05

Guideline:	/
Species/strain:	Guinea pig, strain Pirbright White (SPF)
Group size:	10 females
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	/
Purity:	/
Route:	Ocular
Dose level:	2% dissolved in water
Application:	Single application, no rinsing
GLP:	not in compliance

0.1 ml of a 2 % aqueous solution of hydroxyethyl-3,4-methylenedioxyaniline HCl was applied into the conjunctival sac of the right eye of 10 female guinea pigs; the left eye served as control. The eyes were not rinsed, and were evaluated according to the Draize scoring system 0.5, 1, 2, 3, 4, 6, and 7 h after application. A further reading by means of fluorescein-instillation took place at 24 h.

Results
A slight conjunctival redness and oedema as well as corneal opacity were noted in two animals up to 3 hours after instillation. No other eye irritating effects were noted at any observation time in any of the 10 tested animals.

Conclusion
Under the conditions of the experiment, 2 % aqueous solution of hydroxyethyl-3,4-methylenedioxyaniline HCl caused transient irritation to the eyes of guinea pigs.

Ref.: 18

Comment
Although the guinea pig is not the recommended animal to investigate eye irritating properties *in vivo* and the experiment was not guideline compliant and the purity of the test substance not described, the findings indicate that the intended maximum on-head concentration of hydroxyethyl-3,4-methylenedioxyaniline HCl in hair dye formulations (1.5 %) should have a low potential to cause eye irritation in humans.

3.3.3. Skin sensitisation

Taken from SCCP/0951/05

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species/strain:	Mouse, strain CBA/J
Group size:	5 females per dose
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	9/93 (EMS)
Purity:	99.7 (HPLC, 254 nm)
Route:	Dermal
Concentrations:	0.5, 1.5, 5.0 and 10.0 % (w/v) in DMSO and in {aqua/acetone (1:1)} / olive oil (3:1)
GLP:	in compliance

The skin sensitising potential of hydroxyethyl-3,4-methylenedioxyaniline HCl was investigated in female CBA/J mice by measuring the cell proliferation in the draining lymph nodes after topical application on the ear.
25 µl of 0 (vehicles only), 0.5, 1.5, 5 and 10 % of hydroxyethyl-3,4-methylenedioxyaniline HCl in DMSO or in a mixture (1:1) of aqua/acetone and olive oil (3:1) (equal to the maximum solubility) were applied to the surface of the ear of five female CBA/J mice per group for three consecutive days. After application, the ears were dried by means of a hair dryer for about 5 minutes. A positive control, p-phenylenediamine (PPD) at 1 % in DMSO, was investigated in parallel under identical test conditions.
Animals were checked for morbidity/mortality at least once daily. Observation for clinical signs was done daily before and at least once after dosing. Body weight was determined at day -1 and at day 5.
At day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 23.5 µCi of [H^3] methyl thymidine. Approximately five hours later, the mice were killed by CO_2-inhalation and the draining auricular lymph nodes removed and weighed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the

radioactivity was determined (incorporation of [H^3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
Based on the data provided in the certificate of analysis, a sufficient solubility and stability of the test item in the solvents used (DMSO, acetone/water) is assumed.
Slight to severe skin desquamation on the ears was noted in animals treated with hydroxyethyl-3,4-methylenedioxyaniline HCl at 1.5 % in DMSO at day 5. This effect was associated with erythema in one animal. This effect was interpreted as being due to the low pH value of the hydroxyethyl-3,4-methylenedioxyaniline HCl solution.
The mean stimulation indices were affected in a dose-dependent manner by the treatment with hydroxyethyl-3,4-methylenedioxyaniline HCl if applied in DMSO. Mean stimulation indices of 6.4, 5.0, 8.0 and 12.4 were obtained for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively. An EC3 value (equal to the concentration inducing a stimulation index of 3) was not calculated, since all stimulation indices were above 3. However, an EC3 value well below 0.5 % can be deduced from these findings.
In the second vehicle (aqua/acetone/olive oil), the indices were 4.3, 3.6, 3.3 and 4.4 for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively. Again, an EC3 value was not calculated, since all stimulation indices were above 3, but as for DMSO an EC3 < 0.5 % can be deduced from the data obtained.
The responses noted in both groups are considered positive and indicate a skin sensitising potential of hydroxyethyl-3,4-methylenedioxyaniline HCl.
The positive control (PPD, 1 % in DMSO) caused a stimulation index of 12.5, which demonstrated the sensitivity of the test system used.

Conclusion
Hydroxyethyl-3,4-methylenedioxyaniline HCl induced a biologically relevant immune response in local lymph nodes after dermal application to the mouse ear with both vehicles tested. Hydroxyethyl-3,4-methylenedioxyaniline HCl is evaluated to be a strong skin-sensitiser under the described test conditions in DMSO and acetone/water (1:1) mixed with olive oil (3:1), with an EC3 value below 0.5 %.

Ref.: 20

Comment
Three further skin sensitisation tests, two Maximisation tests according to Magnusson and Kligman and a Buehler-Test, have been performed with hydroxyethyl-3,4-methylenedioxyaniline HCl. As these tests have several limitations, they are not discussed but confirm the sensitising potential of the substance.

3.3.4. Dermal / percutaneous absorption

Taken from SCCP/0951/05

Percutaneous absorption *in vitro*

Guideline:	OECD guideline no. 428 (2004)
Tissue:	Porcine back or flank skin (frozen/thawed; thickness: ≤ 1000 µm)
Method:	Diffusion Teflon-chambers
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	474D
Purity:	99.8%
Concentration:	1.5 mg/cm², tested as part of an oxidative hair dye formulation
No. of chambers:	6 (five for the formulation containing the dye stuff and one for the blank formulation)
GLP:	in compliance

The skin absorption of hydroxyethyl-3,4-methylenedioxyaniline HCl, tested at the maximum concentration intended for hair colorants (1.5 %), was investigated with pig skin (Schweizer Edelschwein, female, 110 kg) prepared from the back and the flanks. 1.5 mg/cm² of the dye was applied once to the skin in a commercial oxidative hair dye formulation (400 mg of formulation containing dye plus 1.75 mg reaction partner (described only by the code WR23005) and 3 % hydrogen peroxide, applied to 4 cm² skin).

The integrity of the skin was monitored at the beginning of the experiment using tritiated water.

A diffusion Teflon-chamber was used. The receptor solution (physiological phosphate buffer containing NaCl and antibiotics) was pumped through the receptor chamber at a rate of 5 ml/h.
Sixty minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, then once with 4 ml shampoo formulation and again twice with water. The washing solutions were combined and the amount of dye was determined by HPLC.

Fractions of the receptor fluid were collected after 16 and 24 hours, concentrated and analysed immediately. At termination of the experiment, the skin was heat-treated and the skin above the basal layer was mechanically separated from the skin from the basal layer down to the upper dermis. Both skin compartments were extracted separately and the dye content was quantified by means of HPLC.

Results
Based on the data available for solubility and stability in water-based systems and on the results from the pre-experiments for determination of the recovery from the different fractions/tissues, the substance was expected to be stable for the given test procedure and at the detected levels. The limit of quantification of the applied method was 5 ng/HPLC-injection.

The integrity of each skin sample was demonstrated with tritiated water, resulting in penetration rates of 0.9 to 1.4 % of the applied dose.

The total recovery of the applied dose was low (19.8 ± 2.5 %), which can be partially explained by the presence of a reaction partner. The reaction of hydroxyethyl-3,4-methylenedioxyaniline HCl with the coupler leads to the occurrence of reaction products, which may not be detected under the HPLC conditions applied. However, as no proof is available for this assumption, the low recovery is considered to limit the validity of the study.

The majority of the applied dose of hydroxyethyl-3,4-methylenedioxyaniline HCl remained on the skin surface, representing 19.5 ± 2.1 % of the applied dose (≥ 97% of the recovered amount). After 24 hours, 0.7 ± 0.2 µg/cm² (equal to 0.047 % ± 0.014 % of the applied dose) was recovered in the upper skin and 0.1 ± 0.1 µg/cm² (equal to 0.0067 ± 0.0067 % of the applied dose) in the lower skin. 5.0 ± 1.0 µg/cm² (equal to 0.3 ± 0.1 % of the applied dose) was recovered in the receptor fluid. Based on a worst case assumption (100% of the dye remaining in the skin becomes bioavailable), an amount of 5.8 ± 0.6 µg/cm² hydroxyethyl-3,4-methylenedioxyaniline HCl is considered to become bioavailable under use conditions.

Conclusion
Under the described test conditions (single application for 24 hours duration, oxidative formulation), a skin penetration rate of 5.8 µg/cm² is obtained for hydroxyethyl-3,4-methylenedioxyaniline HCl by summing up the amounts found in the receptor fluid and in the skin compartments. The observed low recovery, although partially explainable by the

presence of a reaction partner (formation of reaction products), is considered to limit the validity of this study. Therefore this study was not used for the calculation of the systemic exposure dose.

Ref.: 23

Percutaneous absorption *in vivo*

Guideline:	/
Species/strain:	Rat, strain Sprague Dawley Him:OFA, SPF
Group size:	3 per sex and treatment group
Test substance:	^{14}C-*hydroxyethyl-3,5-methylenedioxyaniline HCl* (ring-labelled)
Batch:	/
Purity:	/
Route:	Experiment A, B, C, D, dermal application Experiment E: oral administration
Dose levels:	Exp. A: Formulation without H_2O_2: 1.0 %, 10 mg/animal; 1.1 mg/cm^2 Exp. B: Formulation (also containing p-toluenediamine, resorcinol, m-aminophenol) with H_2O_2: 1.0 %, 10 mg/animal; 1.1 mg/cm^2 Exp. C: Solution in water: 3.33 %, 10 mg/animal; 1.1 mg/cm^2 Exp. D: Solution in water: 3.33 %, 10 mg/animal; 1.1 mg/cm^2 Exp. E: Solution in water: 3.33 %, 51.02 mg/kg bw
Dosing schedule:	Exp. A, B, C, D: Single dermal application for 30 min; total study period 72 h Exp. E: single oral application (gavage); total study period 72 h
GLP:	in compliance

Dermal application (experiments A - D)

^{14}C-hydroxyethyl-3,5-methylenedioxyaniline HCl was applied dermally to groups of 3 male and 3 female Sprague Dawley rats Him:OFA, SPF (body weight about 200 g). The application area was 9 cm² (except for Exp. A: 10 – 12 cm²), clipped free of hair. The test substance was applied at a concentration of 3.33 % in water (experiment C - D) and of 1% in formulation without (experiment A) and with hydrogen peroxide (experiment B) for a 30 min contact time. The mean amount of the dyestuff applied was 1.1 mg/cm² in all cases. Application was done under anaesthesia.

After the treatment period of 30 min, the test substance was removed and the skin rinsed with a shampoo solution (about 100 ml) followed by warm water until the rinsing water was free from colour. After rinsing, the area was covered with gauze, fixed by adhesive tape, and an additional air permeable plastic cone to prevent licking of the treated area during the 72 h in the metabolism cages.

Oral administration (experiment E)

51.02 mg/kg bw ^{14}C-hydroxyethyl-3,5-methylenedioxyaniline HCl was administered as a 3.3% solution in water by gavage to 3 male and 3 female rats.

In experiment D and E, blood was taken at several time points within 24 h after administration from the peri-orbital plexus under light ether anaesthesia.

Urine and faeces were collected daily (0-24, 24-48 and 48-72 h after administration) from the metabolism cages.

Animals were killed 72 hours after the application and the application sites, blood, and numerous organs were analysed for radioactivity. The radioactivity in the remaining carcass was determined after complete removal of the skin.

Results

Studies A, B, C

Total recovery of the applied radioactivity was good with recovery rates between 96.5 and 98.1%.

The majority of the applied dose (95.9 to 97.1% of the applied amount) was recovered in the washing solutions.
The amount of radioactivity remaining at the application site (skin) for the water solution represented 1.68% of the applied dose. The respective figures for formulations with and without hydrogen peroxide were 0.56% and 0.34%, respectively.

0.314%, 0.05% and 0.345% of the applied doses were eliminated via urine and faeces within 72 h in experiment A, B and C, respectively. The lowest absorption rate was obtained in the formulation containing H_2O_2. Radioactivity was mainly excreted via urine (84 – 99% of the total amount eliminated). Elimination was fast, as 82 - 93% of the total amount eliminated was excreted within the first 24 hours.

Excretion via faeces was of less importance representing 8.5 to 16% of the absorbed dose.
The radioactivity remaining in the carcass was very low 72 hours after administration (0.005% of the administered dose for experiments A and C, 0.002% of the administered dose for exp. B). Residues in organs were mostly below the detection limit, with highest concentrations noted for kidneys. No relevant differences were noted between males and females.
Based on these results, a cutaneous absorption rate of 3.5 µg/cm² (equal to 0.318%), for the formulation without H_2O_2, 0.59 µg/cm² (equal to 0.053%) for the formulation with H_2O_2 and of 3.95 µg/cm² (equal to 0.351%) for the pure dyestuff in aqueous solution were obtained based on the bioavailable amounts (carcass, urine and faeces).
If, the mean amounts found in urine, faeces, residual carcass and in the total skin including the application site were considered as bioavailable, a cutaneous absorption of 6.74 µg/cm² (equal to 0.613%) was calculated for the commercial hair dye formulation with hydrogen peroxide. The respective figure for the formulation without hydrogen peroxide was 7.24 µg/cm² (equal to 0.658%).

Studies D and E
The highest concentration of the test compound in the blood was observed after 35 min, the first time point of sampling, after dermal as well as after oral application of the test substance (experiment D and E) and declined with an initial half-life of 1 h after cutaneous and about 1.5 h after per-oral administration. The highest concentration observed in the blood after oral application was about 70-fold higher than the highest value observed after dermal application.

Conclusion
When applied dermally to rats in a commercial hair dye formulation in the presence of hydrogen peroxide, 0.59 µg/cm² hydroxyethyl-3,5-methylenedioxyaniline HCl become bioavailable based on the amounts determined in urine, faeces and carcass. When applied in different vehicles, i.e. a formulation without hydrogen peroxide or an aqueous solution, dermal absorption is higher, with values of 3.5 µg/cm² and 3.95 µg/cm², respectively.
Excretion takes place predominantly via urine and to a minor extent via faeces. Excretion via urine is fast, as 82 to 93 % are excreted within the first 24 hours. Low tissue residue levels were noted, indicating that no relevant bio-accumulation has to be expected after dermal administration.

Ref.: 25

Comment
Based on a conservative assumption that the total amount found in the skin will become bioavailable, an amount of 6.74 µg/cm² hydroxyethyl-3,5-methylenedioxyaniline HCl was calculated from the results of the experiment with commercial hair dye formulation with hydrogen peroxide. The tested concentration was below the maximum intended concentration in oxidative hair dye formulations (1% vs. 1.5%). Therefore, for the MOS calculation, the value was multiplied by 1.5 = 10.11.

Human study

A skin absorption study in humans with hydroxyethyl-3,5-methylenedioxyaniline HCl was presented in a former submission. As material of unknown specification was used, the number of volunteers was low and unrealistic test conditions (non-oxidative formulation) were applied, the study is not further discussed.

3.3.5.	Repeated dose toxicity

3.3.5.1.	Repeated Dose (28 days) oral / dermal / inhalation toxicity

No data submitted

3.3.5.2.	Sub-chronic (90 days) oral / dermal / inhalation toxicity

Taken from SCCP/0951/05

Guideline:	OECD 408
Species/strain:	Rat, Wistar HanBrl:WIST (SPF)
Group size:	10 animals per sex and dose
Observation:	13 weeks
Test substance:	A098, Batch 474D in bi-distilled water
Purity:	99.8 area % (HPLC, 254 nm)
Dose:	0, 20, 100 and 350 mg/kg bw
GLP:	in compliance

The test substance was administered daily to groups of Wistar rats for 13 weeks, by gavage, as a solution in the vehicle (purified water), at dose-levels of 0, 20, 100 and 350 mg/kg/day.
The animals were checked twice daily for mortality and once daily for clinical signs. Detailed clinical observations were carried out weekly and a functional observation battery (including motor activity) was conducted at the end of the treatment period. Body weight and food consumption were recorded once a week during the study. Opthalmoscopy was performed before treatment in all animals and at week 13 in control and high dose animals. Haematological and blood biochemical investigations as well as urinalysis were performed at the end of the treatment period. On completion of the treatment, the animals were sacrificed and submitted to a full macroscopic examination. Designated organs were weighed and specified tissues preserved. A microscopic examination was performed on selected tissues from animals in the control and high-dose groups. When treatment-related morphologic changes were detected in organs of high-dose animals, the same organs from animals of the mid- and low dose were examined.

Results
One male from the high dose group died on day 85 of treatment. According to the study authors it was unlikely to be related to the test item treatment. No treatment, dose-related clinical symptoms were observed.
In both males and females of the 100 and 350 mg/kg bw dose-groups reductions in mean locomotor activity were noted. No effect on body weight gain and food consumption was noted. At ophthalmoscopic examination, no test related changes were noted.
In males of the high dose group, elevated mean absolute and relative reticulocyte counts were observed after 13 weeks of treatment. This effect correlated with the splenic extramedullary haematopoesis which was observed during histopathology and was therefore considered as a test substance related effect. In females, effects on blood chemistry (reduced red blood cell count, haematocrit, and haemoglobin level) and elevated mean absolute and relative reticulocyte counts, accompanied by a shift in the reticulocyte maturity index towards less mature reticulocytes was observed. Some clinical biochemistry

parameters were elevated in both sexes (bilirubin, cholesterol, phospholipids, sodium and potassium concentrations) indicating changes in the liver and in the kidneys. Those changes were accompanied by an increased liver and kidney weight and an increased urinary volume. In females, ovary and spleen weights were elevated and thymus weights were reduced.
Histopathology revealed hepatocellular hypertrophy in both sexes, as well as renal tubular damage. In females extramedullary haematopoiesis was observed in the spleen and corpus luteum hypertrophy in the ovaries.

In the 100 mg/kg dose group increased bilirubin levels were noted in females, elevated cholesterol (both sexes) and phospholipids were observed (females only). Increased urinary volume was noted. Abs. and relative liver weights in males were increased, and hepatocellular hypertrophy was observed (most pronounced in males).

At 20 mg/kg bw/day no adverse effects were observed.

Conclusion
The NOAEL in this study was 20 mg/kg bw/day.

Ref.: 26

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Taken from SCCP/0951/05

Bacterial gene mutation assay

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA102, TA1535, TA1537
Assay conditions:	Plate incorporation and pre-incubation assay without and with S9-mix from rat livers (phenobarbital/ß-naphthoflavone induced). Three plates were investigated per test concentration. Two independent experiments were performed
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	474D
Purity:	99.8 area % (HPLC, 254 nm)
Concentrations:	33, 100, 333, 1000, 2500 and 5000 µg/plate with and without metabolic activation
GLP:	in compliance

Hydroxyethyl-3,4-methylenedioxyaniline HCl, dissolved in de-ionized water, was tested for mutagenicity in the reverse mutation assay (experiment 1: plate incorporation method, experiment 2: pre-incubation method) both with and without metabolic activation (S9-mix from the liver of phenobarbital/ß-naphthoflavone induced male Wistar Hanlbm rats). The *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were exposed to the test substance at concentrations ranging from 33 µg/plate to 5000 µg/plate with and without S9-mix. Test concentrations were selected based on the results obtained in a pre-experiment with strains TA98 and TA100.
For control purposes, untreated, solvent (deionized water) and positive controls (without S9-mix: 4-nitro-o-phenylene-diamine for strains TA98 and TA1537, sodium azide for strains

TA100 and TA1535; methyl methane sulfonate for strain TA102; with S9-mix: 2-aminoanthracene for all tester strains) were evaluated in parallel.

Results
Normal background growth was observed up to the highest test concentration of 5000 µg/plate (highest test concentration recommended by the respective guideline) in the presence and absence of S9- mix in all strains investigated.
No toxic effects, evident as a reduction in the number of revertants, were noted in any of the five tester strains with and without S9-mix at all concentrations investigated.
No biologically relevant increase in revertant colony numbers in any of the five tester strains was observed following treatment with hydroxyethyl-3,4-methylenedioxyaniline HCl at any dose level, neither in presence nor in absence of metabolic activation.
Reference mutagens revealed a distinct increase in revertant colonies and demonstrated the sensitivity of the assay.

Conclusion
Under the experimental conditions reported hydroxyethyl-3,4-methylenedioxyaniline HCl did not induce gene mutations in *Salmonella typhimurium* in any of the tester strains in the presence or absence of S9-mix, up to a concentration of 5000 µg/plate.

Ref.: 27

Two older bacterial gene mutation assays with hydroxyethyl-3,4-methylenedioxyaniline HCl were presented in a former submission. As these studies revealed limitations with regard to the experimental design and/or the specification of the test material used they are not discussed here. However, the result of both tests was negative and thus in agreement with the negative result discussed above.

In Vitro Mammalian Cell Gene Mutation Test

Guidelines:	OECD 476 (1997)
Species/strain:	Mouse lymphoma cell line L5178Y; $tk^{+/-}$;
Assay conditions:	One culture per concentration (two parallel cultures for the negative control). Two independent experiments
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	474D
Purity:	99.8 % (HPLC, 254 nm)
Concentrations:	Exp. I: With metabolic activation (4 h incubation): 34.4, 68.8, 137.5, 206.3, 275.0, 412.5, 550.0, 825.0 and 1100.0 µg/ml Exp. I: Without metabolic activation (4 h incubation): 68.8, 137.5, 206.3, 275.0, 412.5, 550.0, 825.0, 1100.0 and 1650.0 µg/ml Exp. II: Without metabolic activation (24 h incubation): 12.5, 25.0, 50.0, 100.0, 150.0, 200.0, 250.0 and 300.0 µg/ml
GLP:	in compliance

Hydroxyethyl-3,4-methylenedioxyaniline HCl was examined for its mutagenic activity in the L5178Y $tk^{+/-}$ mouse lymphoma assay in the absence and presence of metabolic activation (S9-mix from the liver of phenobarbital and ß-naphthoflavone induced rats). A range-finding test (pre-test on toxicity, measuring total and relative suspension growth for a concentration range of 17.2 to 2200.0 µg/ml) was performed to allow a proper concentration setting for the main experiment. The highest applied concentration in the pre-test on toxicity (2200 µg/ml) was chosen with regard to the molecular weight of the test item, corresponding to a molar concentration of about 10 mM. The concentration range of experiment I with metabolic activation and of experiment II was limited by toxicity of the test item.

Deionised water was used as solvent control, while methyl methane sulphonate (MMS, 13 µg/ml) and cyclophosphamide (CPA, 4.5 µg/ml) were used as positive controls without and with metabolic activation system, respectively.
Incubation time was 4 hours in the presence and absence, 24 hours solely in the absence of S9-mix. One culture was investigated for each of the 8 to 9 concentrations and test group. Mutant frequency and cell survival (measured as total suspension growth) were determined. Apart from the number of mutant colonies, the size/optical density of the colonies was also determined and the ratio of small versus large colonies was calculated.

Results
Relevant toxic effects were observed at 412.5 µg/ml and above without S9-mix and at 206.3 µg/ml and above with S9-mix in experiment I. In experiment II, toxic effects were observed at 150 µg/ml and above.
In the first experiment without metabolic activation, the threshold of twice the mutation frequency of the corresponding solvent control was reached at 1650 µg/ml. However, toxicity was severe at this concentration and the absolute values of the mutation frequency still remained within the historical control range of negative and solvent controls. The observed increase was therefore judged not to be biologically relevant.
In none of the other treated cultures, a biologically relevant or dose-dependent increase in the number of mutant colonies was obtained if compared to the concurrent and the historical control range up to concentrations which showed severe toxicity (less than 20 % cell survival).

Conclusion
Hydroxyethyl-3,4-methylenedioxyaniline HCl did not induce biologically relevant increases in mutations at the thymidine kinase locus of L5178Y cells under the described test conditions. Therefore, hydroxyethyl-3,4-methylenedioxyaniline HCl is considered to be non-mutagenic in this *in vitro* mammalian cell gene mutation test.

Ref.: 28

In a former submission, an HPRT gene mutation test with mouse lymphoma cells was presented. However, as this study revealed limitations with regard to the experimental design and the specification of the used test material, it is not discussed here. The test result was negative.

In Vitro **Micronucleus Test**

Guideline:	OECD 487 (2004, draft guideline)
Species/strain:	Human peripheral blood lymphocytes
Assay conditions:	Two independent experiments using pooled blood from two male donors in each trial. Two cultures per concentration and positive controls, 3 to 4 concentrations of the test item analysed
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	474D
Purity:	99.8 area % (HPLC, 254 nm)
Concentrations:	Exp. I: with S9-mix: 500, 1100 and 1800 µg/ml; 3 h treatment 24 hours after mitogen stimulation without S9-mix: 200, 400 and 700 µg/ml; 20 h treatment 24 hours after mitogen stimulation Exp. II: with S9-mix: 1200, 1500, 1800 and 2177 µg/ml; 3 h treatment 48 hours after mitogen stimulation without S9-mix: 650, 750 and 950 µg/ml; 20 h treatment 48 hours after mitogen stimulation
GLP:	in compliance

Hydroxyethyl-3,4-methylenedioxyaniline HCl was examined for its ability to induce micronuclei in cultured human lymphocytes. Two independent experiments were performed with and without metabolic activation (S9-mix from the liver of Aroclor 1254 induced rats). Test concentrations were selected based on the results obtained in pre-experiments.
In experiment I, cells were treated with the test item 24 hours after mitogen stimulation with phytohaemagglutinin (PHA). In the second experiment, the treatment started 48 hours after the mitogen stimulation. The exposure times for the test item in the presence and the absence of S9-mix were 3 and 20 hours, respectively. Cytochalasin B (6 µg/ml) was added to the cultures to block cytokinesis during the recovery periods of 28 and 45 hours for the assay without and with S9-mix, respectively. Cells from Experiment I were harvested at 72 h after mitogen stimulation and those from Experiment II were harvested at 96 h after mitogen stimulation. To calculate the replication index (RI), 500 cells per replicate (1000 per concentration) were examined for proportions of mononucleate, binucleate and multinucleate cells. One thousand binucleate cells from each culture (2000 per concentration) were analysed for the occurrence/number of micronuclei.

For controls, sterile water (solvent control), 4-nitroquinoline 1-oxide (-S9 control, 2.5 and 5.0 µg/ml) and vinblastine (-S9 control, 0.08 µg/ml) and cyclophosphamide (+S9 control, 12.5 µg/ml) were evaluated.

Results
The highest test concentrations to be evaluated in experiment I were 700 µg/ml (cytotoxicity: 65 %) and 1800 µg/ml (cytotoxicity: 53 %) in the absence and presence of S9-mix, respectively.
In experiment II, the respective concentrations were 950 µg/ml and 2177 µg/ml, causing 62 % and 13% cytotoxicity, respectively (2177 µg/ml = 10 mM).
In the absence of S9-mix, the frequencies of micronucleated binucleate (MNBN) cells were similar to those observed in concurrent vehicle controls for the majority of concentrations analysed. In both experiments the frequency of MNBN cells was slightly increased at the highest concentration but within the historical vehicle control ranges.
However, in experiment II a statistically significant and biologically relevant increase in MNBN cells, revealing a clear concentration-effect relationship, was measured in the presence of S9-mix.
The positive controls showed the expected effects demonstrated the sensitivity of the test.

Conclusion
Hydroxyethyl-3,4-methylenedioxyaniline HCl caused chromosomal damage in the presence of S9-mix. In the absence of S9-mix, no biologically relevant increases in micronucleated cells were apparent, even at markedly cytotoxic concentrations. Therefore, hydroxyethyl-3,4-methylenedioxyaniline HCl is considered to be mutagenic in the presence of S9-mix in this *in vitro* micronucleus test.

Ref.: 29

An *in vitro* chromosomal aberration test with cultured human lymphocytes has been presented in a former submission. Because this study revealed limitations with regard to the experimental design and the specification of the test material used, it is not discussed here. However weak positive effects in the presence of S9-mix were measured, which is in agreement with the positive result discussed above.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Taken from SCCP/0951/05

Mouse bone marrow micronucleus test

Guideline: OECD 474 (1997)
Species/strain: Mouse, strain NMRI

Group size:	5 per sex, dose group and sacrifice time
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch no:	9/93
Purity:	99.7 area % (HPLC, 254 nm)
Dose:	25, 125 and 250 mg/kg bw administered as single doses
Route:	intraperitoneal
Vehicle:	distilled water
Dosing schedule:	single administration 24 and 48 hours (high dose only) before sacrifice
GLP:	in compliance

Hydroxyethyl-3,4-methylenedioxyaniline HCl, dissolved in distilled water, was administered intraperitoneally to groups of 5 male and 5 female NMRI mice at doses of 25, 125 and 250 mg/kg bw. For the high dose, two groups were treated to allow sampling after 24 and 48 hours. Single doses were administered in a total volume of 10 ml/kg bw to animals.
Dose selection was based on findings in the pre-experiment in which doses of 250 and 1000 mg/kg bw were administered to three female and three male mice under the same treatment procedure.
Negative control groups received distilled water and positive control groups received 40 mg/kg bw cyclophosphamide (CPA), dissolved in deionised water.
The number of polychromatic erythrocytes (PCE) with micronuclei was analysed in at least 2000 PCEs per animal. In addition, the ratio between polychromatic and total erythrocytes per animal was determined.
The animals were examined for acute toxic signs three times within the first 24 hours after treatment and the high dose group additionally 48 hours after administration.

Results
In the pre-experiment, at 1000 mg/kg bw all animals (3 male and 3 female mice) died within 24 hours. At 250 mg/kg bw toxic signs (palpebral closure and lethargy) were noted within the first hour after administration and lethargy was obvious for up to 6 hours after start of treatment. No mortality occurred. Based on these findings, doses of 25, 100 and 250 mg/kg bw were chosen for the main study.
In the main study, toxic signs like those described in the pre-experiment (250 mg/kg bw) were noted in all animals of the high dose group within the first hour after substance administration.
The ratio between PCEs and total erythrocytes was not affected by the test item at any test concentration or sampling time as compared to the ratio observed in the vehicle control. Therefore, this parameter cannot be taken to demonstrate relevant exposure of the bone marrow. However, the observed signs of systemic toxicity indicate that the test item was systemically distributed and bio-available. This assumption is further supported by an *in vivo* toxicokinetic study with rats, which indicated good bioavailability after oral administration of aqueous formulations of hydroxyethyl-3,4-methylenedioxyaniline HCl.
There was no statistically significant or biologically relevant increase in the number of micronuclei per 2000 PCEs in the mice of any of the hydroxyethyl-3,4-methylenedioxyaniline HCl treated groups compared to the respective vehicle control groups.
The positive control (CPA) induced a statistically significant increase in micronucleated PCEs, thus demonstrating the sensitivity of the test.

Conclusion
Hydroxyethyl-3,4-methylenedioxyaniline HCl did not induce micronuclei in the in vivo micronucleus test with NMRI mice after a single intraperitoneal administration at doses up to the maximum tolerated dose of 250 mg/kg bw. It can be concluded that hydroxyethyl-3,4-methylenedioxyaniline HCl does not induce chromosome aberrations or damage to the mitotic apparatus in bone marrow cells of mice under the test conditions used.

Ref.: 30

Another mouse bone marrow micronucleus test was submitted as part of a former submission. In this test, unspecified material was used. This study which also showed a negative result is not discussed here.
An *in vivo* SCE test with rats was also submitted as part of a former submission. This study was not considered valid as unspecified material was tested and the study design was not appropriate. This study which also showed a negative result is not discussed here.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	OECD 414 (2001)
Species/strain:	rat, strain HanBrl: WIST (SPF Quality)
Group size:	22 mated females per dose group
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	474D
Purity:	99.8 area % (HPLC), for details see Annex I
Dose:	50, 250 and 1000/750 mg/kg bw/d
Route:	oral, gavage
Vehicle:	bi-distilled water
Dosing schedule:	once daily from day 6 through day 20 of gestation
GLP:	in compliance

Please note: Study in draft status.

Hydroxyethyl-3,4-methylenedioxyaniline HCl was administered once daily in a constant volume of 10 ml/kg bw by oral gavage to groups of 22 pregnant HanBrI: WIST (SPF Quality) rats at doses of 0, 50, 250 and 1000/750 mg/kg bw/d from day 6 to day 20 of gestation. Successful mating was verified by vaginal smear analysis or by the occurrence of a copulation plug.
Dose selection was based on data obtained from a former teratology study and from subchronic toxicity studies.
Animals were observed twice daily for clinical signs during the entire treatment period. Body weights were recorded daily. Food consumption was measured every third day.
At day 21 post coitum, all mated females were killed under CO2-asphyxiation and a complete necropsy and a macroscopic examination of the organs was carried out. The uterus (prepared by caesarean section) was removed and the presence of resorption sites (early, late) and foetuses (live or dead) was examined. The number of implantation sites and of corpora lutea was also determined. Each live foetus was weighed, sexed and examined for gross external malformations. After adequate processing, a skeletal and a visceral examination of the foetuses were performed for about 50 % of the foetuses each. In addition, placenta and uterus weights were recorded.

Results
In the draft final study report, the homogeneity and stability of the test solutions was analytically investigated. A sufficient homogeneity (97.4 to 111. 8 % of the nominal values) was demonstrated. With regard to the stability of the test item over 7 days, deviations of –

18 to – 47 % of the nominal values were noted in the draft report, indicating that the test item was not stable over a 7 day period. This result was not in line with previous findings which demonstrated that Hydroxyethyl-3,4-methylenedioxyaniline HCl is stable in water over a period of 7 days (see certificate of analysis, (Reference: 22)). Furthermore, in a 90 day study which was performed in the same laboratory under identical conditions (solvent, concentrations, etc.) at the same period of time, the test item was shown to be stable (see chapter 4.5.2). These contradictory results have been further investigated and the results of additional stability studies were provided in a supplementary submission together with the final study report. The study report states in the section on deviations from the study plan that the stability of the test item did not meet the acceptance criteria defined in the SOP. The test item was not stable in the vehicle at room temperature for seven days. Because of the observed instability, the dose levels were re-calculated using the respective analytical results assuming that at least the minimum dose has been daily administered. .

Because severe clinical signs of toxicity and mortality were observed at 1000 mg/kg bw, the dose was reduced to 750 mg/kg bw/d after the first three days of administration (from gestation day 9 to day 20). In total, 5 animals died/were sacrificed due to moribund conditions in this dose group.
Apart from the above described fatalities in the high dose group, the following clinical signs were observed: Hypoactivity, hunched posture, lacrimation or hardened stomach. After application, the animals pushed their heads through the bedding material, an indication for discomfort. The latter effect was also noted at 250 mg/kg bw/d.
Mean food consumption was markedly reduced (-56 % compared to controls) in the high dose group during the treatment period. During this period, the body weight gain (corrected for uterus weight) was also markedly reduced, revealing a 16 % loss (control: 11 % gain). Similar but less pronounced effects were noted at 250 mg/kg bw/d (body weight gain: 5 % less than controls, feed consumption 10 % less compared to the control).
At necropsy, severe effects in the stomach (foci in the stomach wall/perforation) were observed in all animals of the high dose group and in addition in one animal treated with 250 mg/kg bw/d. In addition, effects on adrenals, spleen and kidneys were seen in the high dose group in all dams and in the mid dose group in one dam.
No effects were noted in the low dose group (50 mg/kg bw/d).
In the high dose group, an increase in foetal resorptions was observed, leading to lower numbers of foetuses per total implantation sites if compared to the control group (83 % vs. 93 %). No such effects were observed in the mid or low dose group.
Treatment related effects with regard to litter size and foetal body weight were noted at the high dose. The sex ratio of the foetuses was not affected by the treatment.
The skeletal (including cartilage) and visceral examination of the foetuses revealed distinct treatment related effects for the mid and high dose group. Visceral findings as haemorrhages (subcutaneous, intrathoracic or intra-abdominal), dilated lateral brain ventricles, thymus, cranial displacement and/or elongation, heart, aorta and pulmonary trunk abnormalities, as well as not inflated lungs were noted in the high dose group. For the heart, aorta, pulmonary trunk and lungs similar findings were noted at 250 mg/kg bw/d, however, at a lower frequency.
The skeletal investigation of the high dose group showed a markedly increased incidence of non-ossified and incompletely ossified bones of the cranium, vertebrae, ribs, sternebrae, as well as of extremities and supernumerary ribs. Furthermore, several other skeletal malformations were observed. Again similar but less frequent and pronounced effects were noted at 250 mg/kg bw/d. Cartilage examination revealed treatment related effects on cervical and thoracic vertebrae as well as on costal cartilage at both dose levels.
The skeletal, visceral and cartilage examination did not show any treatment related effects for the low dose group.

Conclusion
Taking into account the stability data of the analytical examinations, minimal dose levels of 26, 152, and 702/688 mg/kg bw/d were achieved in dose groups 2, 3 and 4 respectively.

In this teratogenicity study, severe effects were noted on reproduction and on the development of the foetuses at the high dose group (702/688 mg/kg bw/d). At this dose level, the maximum tolerated dose was clearly exceeded as it led to pronounced maternal toxicity and several cases of death. The effects at this dose level were therefore considered to be due to the high maternal toxicity.
Similar, but less pronounced maternal and embryo/foetotoxicity were noted at the mid dose (152 mg/kg bw/d). The observed embryo-foetal effects were also considered to be related to the observed maternal toxicity. No effects were observed at the lowest dose level. Based on these results a No Observed Adverse Effect Level (NOAEL) of 26 mg/kg bw/d was derived for maternal and for embryo-foetal effects.

Ref.: 31, 35

Comment SCCS
In a supplementary submission of March 2007, additional stability data have been provided. The results of this stability study are described in section 3.1.9.
It should be noted that even the corrected NOAEL does not have any influence on the risk assessment as the corrected value for the NOAEL (26 mg/kg bw/d still lies above the NOAEL observed in the 90 day study.

3.3.9. Toxicokinetics

3.3.9.1 Toxicokinetics *in vitro*

Taken from SCCP/0951/05

Biovailability across intestinal barrier in TC-7 (human intestinal epithelial) cells

Guideline:	/
Cells:	human intestinal epithelial cell line TC-7
Test substance:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch:	9/93 Fass 195
Purity:	99.7 % (HPLC at 254 nm)
Test concentration:	50 µM in HBSS buffer containing 1 % DMSO
Incubation time:	120 min
Number of experiments:	two independent experiments
GLP:	not in compliance, but QAU-checked

The bioavailability of hydroxyethyl-3,4-methylenedioxyaniline HCl across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro*. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37°C in 24-well transwell plates with shaking for a 120 min contact time. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of HLPC-MS/MS and the apparent permeability coefficient (P_{app}) was calculated for two independent experiments. ^{14}C-mannitol (about 4 µM) was used to demonstrate the integrity of the cell monolayer. Only monolayer revealing a permeability of < 2.5 x 10^{-6} cm/sec were used. Propranolol, vinblastine and ranitidine were analysed concurrently to demonstrate the validity of the test system.

According to the laboratory's classification system, a low permeability is considered for test items revealing a P_{app} < 2 x 10^{-6} cm/sec. A P_{app} of 2 - 20 x 10^{-6} cm/sec and a $P_{app} \geq$ 20 x 10^{-6} cm/sec classify a substance to have a moderate and a high permeability, respectively. As recommended by FDA, ranitidine (50 % absorption in humans) was used as the low permeability reference compound and Propanolol (90 % absorption in humans) was used as the high permeability reference compound.

Results

The total recovery for the reference substances and hydroxyethyl-3,4-methylenedioxyaniline HCl ranged from 64 to 88 %.
The figures obtained for the reference substances propranolol (P_{app} = 34.2 x 10^{-6} cm/sec), a high permeability reference compound with about 90 % absorption in humans, and ranitidine (P_{app} = 0.3 x 10^{-6} cm/sec; revealing an absorption of about 50 % in humans) were well within the acceptance range of 20 - 45 x 10^{-6} cm/sec and 0.2 - 2 x 10^{-6} cm/sec, respectively, and demonstrated the validity of the assay.
Hydroxyethyl-3,4-methylenedioxyaniline HCl revealed a P_{app} of 83.1 x 10^{-6} cm/sec and was classified to be of high permeability.

Conclusion
With hydroxyethyl-3,4-methylenedioxyaniline HCl a mean permeability in human intestinal epithelial (TC-7) cells of 83.1 x 10^{-6} cm/sec was obtained, which classifies the test item to be of high permeability. As the absorption across the intestinal epithelial is considered to be the limiting factor for the uptake through the gastro-intestinal tract, the high permeability observed in this assay indicates very good absorption of hydroxyethyl-3,4-methylenedioxyaniline HCl after oral administration. This result is in accordance with the data obtained in the *in vivo* kinetic study in rats

Ref.: 32

3.3.9.2 Toxicokinetics *in vivo*

Taken from SCCP/0951/05

Toxicokinetics in rats

Guideline:	OECD 417 (1984), OECD 427 (Draft, 2000)
Species/strain:	Rat, strain Wistar Kyoto WKY/NR Crl BR (inbred)
Group size:	4 females per group for Mass balance studies (group 1-4) Toxicokinetics: 8 females (group 5), 6 females (group 6+7), 5 females (group 8)
Test substance I:	14C-hydroxyethyl-3,4-methylenedioxyaniline HCl (ring-labelled, specific activity 60 mCi/mmol)
Batch:	01BLY060
Purity:	100 % (Radiochemical purity, HPLC and TLC)
Test substance II:	hydroxyethyl-3,4-methylenedioxyaniline HCl
Batch no:	9/93 Fass 195
Purity:	99.7 % (HPLC at 254 nm)
Dose levels:	Group 1 + 5: 1 mg/kg bw in sterile physiological saline solution (0.9 %) for intravenous administration; pH adjusted to 7.0 – 8.5 Group 2 + 6: 1 mg/kg bw in water for low oral dose groups Groups 3 + 7: 100 mg/kg bw water for high oral dose group Group 4 + 8: 1.5 % in acetone/water 1:1 (equivalent to 10 mg/kg bw and 0.15 mg/cm2) for dermal application; pH adjusted to 7.0 – 8.5
Dosing schedule:	Single application; study duration 48 h (group 8), 72 h (groups 5-7) and 96 h (groups 1-4)
GLP:	in compliance

In this study the absorption, distribution, metabolism and excretion of ^{14}C- hydroxyethyl-3,4-methylenedioxyaniline HCl in female Wistar Kyoto rats after single oral, dermal or intravenous applications were investigated.

Oral administration
Oral exposure was performed by oral gavage at doses of 1 and 100 mg/kg bw in a volume of 5 ml/kg bw. The rats were fasted for approximately 18 h prior to and 4 h after dose administration.

Intravenous administration
Intravenous exposure was performed by i.v. injection of 1 mg/kg bw in a volume of 2 ml/kg bw into the tail-vain of restrained animals. Animals used for intravenous dosing were not fasted.

Dermal application
The intention in this part of the study was not to simulate realistic exposure scenarios, but to obtain high penetration rates to compare metabolite profiles. Therefore acetone/water was used as vehicle.
Approximately 24 h prior to treatment, a 5 x 6 cm area of the fur on the back of the animals and a 4 x 4 cm area of the fur in the abdominal region was shaved. On the shaved skin on the back an area of 2.5 x 4 cm was marked as application site. The shaved area in the abdominal region served as negative control. Immediately prior to treatment, the application site was cleaned with a 10 % shampoo solution and water, and then dried. 10 mg/kg bw (1.5 % hydroxyethyl-3,4-methylenedioxyaniline HCl in acetone/water 1:1; 0.15 mg/cm²) was applied uniformly onto the skin of the application site. Application, exposure and removal were done under anaesthesia. After the treatment period of 30 minutes, the application site was rinsed with an aqueous shampoo formulation. During the entire study period, animals wore plastic collars to avoid licking of the treated area.
Urine and faeces were collected at the following time intervals (groups 1-4): 0-8, 8-24, 24-48, 48-72 and 72-96 h after administration, and the metabolite profiles were determined by means of HPLC.
In the toxicokinetic groups, blood was sampled at 10, 20, 40 min and at 1, 2, 4, 8, 24 and 48 hours (group 8: additionally at 72 hours) after dosing.
Animals were killed 96 hours after application. Urine, faeces, blood, tissues and numerous organs were analysed for radioactivity. The radioactivity in the remaining carcass was determined after complete removal of the skin.

Results
The average total recovery of radioactivity in the mass balance groups (1-4) was between 97 and 99 % of the applied dose.
The overall absorption after oral administration was very high (> 95 % of the administered dose). After dermal application, a significantly lower absorption rate (5 % equal to 8 µg/cm²) was obtained based on the content in the carcass, urine and faeces. When also taking the amount present at the application site into account, the potentially absorbed fraction was 8% of the applied dose, or 14 8 µg/cm².

Blood kinetics revealed a very fast absorption after oral administration for both the low and the high dose. After oral application of the test substance, the highest concentration of the test compound in the blood samples was observed after 10 min, the first time point of sampling. The absorption after dermal application was slower, but still relatively fast, as the maximum blood levels were noted after 1 h.

The most important route of excretion of ^{14}C- hydroxyethyl-3,4-methylenedioxyaniline HCl and its metabolites for all routes of administration was the urine. Excretion via faeces was significantly less important in all groups. Since the observed proportion of the radioactivity excreted through the faeces was similar after i. v. and after oral application, the amount found in the faeces after oral application is considered to represent phase II metabolites of the dye that have been excreted via the bile.

The metabolite profile in the urine samples and faeces extracts was similar between the different dosing routes. Only metabolised hydroxyethyl-3,4-methylenedioxyaniline HCl was detected in urine and faeces, thus demonstrating that the test substance is rapidly and extensively metabolised in the Wistar Kyoto rat. No conjugates were found in urine or faeces, suggesting that the metabolite itself is excreted in bile or a glucuronide conjugate is formed and excreted via bile, and is de-conjugated in the intestines.

At termination of the study, the highest residual levels of radioactivity were found in the carcass, liver, kidney and thyroid, but were very low (about 1% of the applied dose for oral and i.v. application). In the dermal group, about 5% of the applied dose was recovered in the carcass, with the majority found in the skin from the application site. The investigated organs contained no or only a very low level of radioactivity. The described low residual levels in the animals after termination of the study do not indicate bioaccumulation of hydroxyethyl-3,4-methylenedioxyaniline HCl.

Conclusion
Hydroxyethyl-3,4-methylenedioxyaniline HCl after oral administration, is rapidly and efficiently absorbed, readily distributed in the organism, extensively metabolised and quickly and almost quantitatively excreted, mainly via urine. After dermal application the absorbed amount of hydroxyethyl-3,4-methylenedioxyaniline HCl was very limited. Once absorbed, the routes and rates of elimination were similar for all three routes of administration (i.v., oral and dermal). No differences in the metabolite profile between the routes of administration or between gender were observed and no tendency for bio-accumulation was noted in this study.

Ref.: 33

3.3.10.	Photo-induced toxicity

No data submitted

3.3.11.	Human data

No data submitted

3.3.12.	Special investigations

No data submitted

3.3.13.	Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(Hydroxyethyl-3,4-methylenedioxyaniline HCl)

(oxidative conditions)

Absorption through the skin (rat, *in vivo*)	**A**	**=**	**6.74 µg/ cm^2**
X 1.5 to adjust to max. use %		**=**	**10.11 µg/ cm^2**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**5.86 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.098 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**20 mg/kg bw**
Margin of Safety	**NOAEL/ SED**	**=**	**204**

3.3.14. Discussion

Analytical data
Hydroxyethyl-3,4-methylenedioxyaniline HCl is used at a final concentration of 1.5% in oxidative hair dye formulations, after mixing with the developer containing hydrogen peroxide.
Hydroxyethyl-3,4-methylenedioxyaniline HCl is a secondary amine, and thus, it is prone to nitrosation. It should not be used in the presence of nitrosating agents. The nitrosamine content should be <50 ppb. Nitrosamine content, determined as total content of N-nitroso compounds (ATNC), was <10 µg/kg for all tested batches.
Based on additionally provided stability data (amendment to the teratogenicity study), the contradictory information in Ref. 22 and 31 were assumed to be due to incorrect sample handling.
3,4-(Methylenedioxy)-aniline (Annex II, n° 1248) is an aromatic amine with a free amino group. A 100-1100 ppm content of 3,4-(Methylenedioxy)-aniline impurity in Hydroxyethyl-3,4-methylenedioxyaniline HCl may be of concern.

Toxicity
From a 90-day oral study in rats, a NOAEL of 20 mg/kg bw/day was derived for repeated dose toxicity. After correction for decreased stability of the test substance, the NOAEL for maternal and embryo-foetal effects was 26 mg/kg bw/day in a developmental toxicity study in rats.

Irritation, sensitisation
The substance is not irritant to guinea pig skin and mildly irritant to the eyes of guinea pig. It is a strong sensitiser.

Percutaneous absorption
Due to the insufficiencies of the *in vitro* study, an *in vivo* study with rats was used for the assessment of the dermal absorption (see ref 25).
Based on a conservative assumption that the total amount found in the skin will become bioavailable, an amount of 6.74 µg/cm² hydroxyethyl-3,5-methylenedioxyaniline HCl was calculated from the results of the experiment with commercial hair dye formulation with hydrogen peroxide. The tested concentration was below the maximum intended concentration in oxidative hair dye formulations (1% vs. 1.5%). Therefore, for the MOS calculation, the value was multiplied by 1.5 = 10.11.
A further toxicokinetic study in rats included dermal application and resulted in a dermal absorption of 8 µg /cm².

Mutagenicity
A battery of *in vitro* and *in vivo* genotoxicity tests with hydroxyethyl-3,4-methylenedioxyaniline HCl of defined quality was performed which covered the relevant genetic endpoints.
Hydroxyethyl-3,4-methylenedioxyaniline HCl did not induce gene mutations in bacterial or mammalian cells in vitro. Clastogenic effects were observed at high concentrations in an in vitro micronucleus test in human lymphocytes in the presence of S9-mix. Hydroxyethyl-3,4-methylenedioxyaniline HCl, administered via the intraperitoneal route, did not induce micronuclei in vivo at doses up to the maximum tolerated dose of 250 mg/kg bw. For intraperitoneal administration, relevant bioavailability of the test compound can be assumed. Therefore, a genotoxic potential of hydroxyethyl-3,4-methylenedioxyaniline HCl is not expressed under appropriate in vivo test conditions.

Carcinogenicity
No data submitted

4. CONCLUSION

Based on the information provided, the SCCS is of the opinion that the use of hydroxyethyl-3,4-methylenedioxyaniline HCl itself as an oxidative hair dye substance at a maximum concentration on the head of 1.5% does not pose a risk to the health of the consumer, apart from its strong sensitising potential.

Hydroxyethyl-3,4-methylenedioxyaniline HCl is a secondary amine and thus prone to nitrosation. It should therefore not be used in combination with nitrosating substances. The nitrosamine content should be less than 50 ppb.

Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

6. REFERENCES

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