EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

2-Amino-4-hydroxyethylaminoanisole sulfate

COLIPA n° A84

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

The SCCP adopted this opinion at its 16th plenary of 24 June 2008

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 Products (SCCP), 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).

SCCP

Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

Scientific Committee members

Contact
European Commission
Health & Consumer Protection DG
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-Sc6-Secretariat@ec.europa.eu

ACKNOWLEDGMENTS

Dr. C. Chambers
Prof. V. Kapoulas
Prof. J.-P. Marty
Prof. T. Platzek (chairman)
Dr. S.C. Rastogi
Prof. V. Rogiers (rapporteur)
Prof. T. Sanner
Dr. J. van Engelen
Dr. I.R. White

External experts

Dr. M.-L. Binderup	Danish Institute for Food & Veterinary Research, Denmark
Dr. H. Norppa	Institute of Occupational Health, Finland
Prof. K. Peltonen	Finnish Food Safety Authority, EVIRA, Finland
Dr. J. van Benthem	RIVM, the Netherlands

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Opinion to be cited as: SCCP (Scientific Committee on Consumer Products), Opinion on 2-amino-4-hydroxyethylaminoanisole sulfate, 24 June 2008

TABLE OF CONTENTS

1. BACKGROUND

Submission I for 2-Amino-4-hydroxyethylaminoanisole sulphate was submitted in February 1989 by COLIPA [1].

The Scientific Committee on Cosmetology (SCC) adopted at its 46th plenary meeting on 19 of February 1991 an opinion with the final conclusion that:

"In the absence of carcinogenicity data, the SCC requires in vitro cytogenetic and in vivo UDS studies."

Again, according to COLIPA, submission II for 2-Amino-4-hydroxyethylaminoanisole sulphate was submitted in March 1992 by COLIPA.

The Scientific Committee on Cosmetology (SCC) adopted at its 54Ih plenary meeting on 10 of December 1993 an opinion stating classification A for the substance.

The substance is currently regulated in Annex III, part 2, under entry 39, on the list of substances provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission III for this substance was submitted in July 2005 by COLIPA. According to this submission, 2-Amino-4-hydroxyethylaminoanisole sulphate is used as an oxidative hair colouring agent (precursor). The intended maximum on-head concentration is 1.5%.

The SCCP adopted at its 7th plenary on 28 March 2006 an opinion stating that:

"Based on the information provided, a margin of safety of 65 has been calculated suggesting that 2-amino-4-hydroxyethylamino-anisole and its sulfate is not safe for use as a hair dye and should not be present in hair dyes or other cosmetic products. The value of the percutaneous absorption is critical to the calculation of the MOS. The data used was that provided for an in vivo test in rat skin. A skin penetration test using 2-amino-4-hydroxyethylamino-anisole sulfate and conforming to current Notes of Guidance for Safety Evaluation would be required before any re-evaluation of the substance."

In April 2007, an additional dermal absorption study was submitted by COLIPA.

2. TERMS OF REFERENCE

1. *Does SCCP consider 2-Amino-4~hydroxyethylaminoanisole sulphate safe for use as a substance in oxidative hair dye formulations with an on-head concentration of maximum 1.5 % taken into account the scientific data provided?*

2. *And/or does the SCCP have any further scientific concerns with regard to the use 2-Amino-4-hydroxyethylaminoanisole sulphate as a substance in oxidative hair dye formulations?*

[1] COLIPA – European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

Taken from SCCP/0958/05

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

2-amino-4-hydroxyethylaminoanisole sulfate (INCI)

3.1.1.2.	Chemical names

Ethanol, 2-[(3-amino-4-methoxyphenyl)amino]-, sulphate (1:1) (CA INDEX Name, 9CI)
Sulfuric acid compound with 2-(3-amino-4-methoxyanilino)ethanol (1:1) (IUPAC)
2-Amino-4-(2-hydroxyethyl)amino-anisole sulfate
1-methoxy-2-amino-4-(β-hydroxy-ethylamino)-benzene sulphate
(3-ammonio-4-methoxyphenyl)(2-hydroxyethyl) ammonium sulphate

3.1.1.3.	Trade names and abbreviations

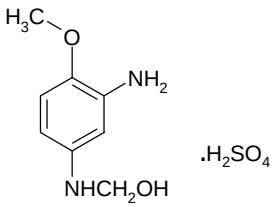
Covastyle AHEAS, HC Blue AC, Jarocol AHEA
COLIPA n° A084
Lehmannblausulfat

3.1.1.4.	CAS / EINECS number

Free base
CAS : 83763-47-7
EINECS : 280-733-2

Sulfate
CAS : 83763-48-8
EINECS : 280-734-8

3.1.1.5.	Structural formula



3.1.1.6.	Empirical formula

Formula : $C_9H_{14}N_2O_2.H_2O_4S$

3.1.2.	Physical form

Grey-blue Powder

3.1.3. Molecular weight

Molecular weight: 280.3

3.1.4. Purity, composition and substance codes

Purity and impurities in various batches of 2-amino-4-hydroxyethylamino-anisole sulfate

Description	**Batch n°**			
	57 (R96000196)	**Rob006958 (LGH 10283/1)**	**101 (R00056178)**	**57/01 (R96000195)**
NMR content, % (w/w)	93.5	91.0	94.6	93.5
HPLC purity, area% 210 nm 254 nm 304 nm	 99.6	 99.4 99.7 100	 99.3 99.8 100	 99.6 99.7 (296 nm)
HPLC content (sulphate)*, % (w/w)	-	91.3	92.3	-
Content of 4-methoxy-aniline (ppm)	<10**	<10**	<10**	<10**
Content of 4-methoxy-3-nitroaniline (ppm)	<10**	<10**	<10**	<10**
Content of 2-methoxy-5-nitroaniline (ppm)	<10**	<10**	<10**	<10**
Content of 2,4-diamino-anisole (ppm) ***	300	120	400	600
Water content, % (w/w)	10.6	10.6	10.6	5.9
Loss on drying, % (w/w)	0.2	n.d.	n.d.	0.08
Ash, % (w/w)	0.03	0.06	n.d.	0.03
Solvent residues	Methanol, ethanol, isopropanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone and monochlorobenzene were not detected at 100 ppm detection limit			

* refers to batch 57 (R96000196) as 93.1% (w/w)
** Limit of detection
*** classified carcinogenic category 2 in the EU
n.d. not done because of lack of substance

Loss on drying and water content do not correspond because of 2-amino-4-hydroxyethylamino-anisole sulphate is usually in the form of monohydrate and crystalline water content is not lost on drying.

3.1.5. Impurities / accompanying contaminants

See 3.1.4

3.1.6. Solubility

Water: 81.988 g/l (pH 2.3 saturated water solution, 20°C; pH 6.0 at <20 g/l)
Acetone/water (1:1): >5 g/l (pH 2.1)
DMSO: >90 g/l
Ethanol: <10 g/l

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 0.59 (pH 7.51, room temperature)

3.1.8.	Additional physical and chemical specifications

Organoleptic properties

Melting point:	138.4 - 146.5°C (decomposition)
Boiling point:	Not applicable
Flash point:	/
Vapour pressure:	2.0 exp – 9 hPa (20°C)
Density:	1.541 g/ml (20°C)
Viscosity:	/
pKa:	/
Refractive index:	/

3.1.9.	Stability

2-Amino-4-hydroxyethylamino-anisole sulphate on storage in dark and dry atmosphere was stable for 10 years.
Stability of 5% (w/w) aqueous solution of 2-amino-4-hydroxyethylamino-anisole sulphate, stored at room temperature and in the absence of light:
0h: 100%, 6h: 93.8%, 2 days: 95.1 %, 7 days: 79.7%

General comments on physico-chemical characterisation

- 2-Amino-4-hydroxyethylamino-anisole sulfate is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in the test material is not reported.
- The stability of 2-amino-4-hydroxyethylamino-anisole sulfate in marketed products is not reported.

3.2. Function and uses

2-Amino-4-hydroxyethylamino-anisole sulfate is used at a final concentration of 1.5% in oxidative hair dye formulations after mixing the developer containing oxidative agent.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCP/0958/05

Guideline:	/
Species/strain:	Rat, strain Wistar Mouse, strain CF 1
Group size:	Rat: 5 dose/sex; mouse: 10 dose/female
Test substance:	1-methoxy-2-Amino-4-ß-hydroxyethylamino-benzene-sulphate
Batch:	/
Purity:	/
Dose:	Rat: 250, 375, 500, 625 and 750 mg/kg bw Mice: 250, 375, 500, 625, 750 and 875 mg/kg bw

Vehicle: 10% aqueous preparation
Observation: 14 days
GLP: not in compliance

Doses selected for this study were based on pre-tests. 5 rats/sex were tested at a single dose of 875 mg/kg bw, all of them died. In female mice (10 per dose 250, 875 and 1500 mg/kg bw), a median lethal dose of less than 875 mg/kg bw was found. The animals received a single dose of test substance by gastric gavage at 5 dose levels ranging from 250 to 750 mg/kg bw in rats and at 6 dose levels ranging from 250 to 875 mg/kg bw in mice. The animals were observed daily for clinical signs and mortalities for 14 days. Bodyweights were recorded weekly and macroscopic abnormalities were recorded at autopsy. No histological examinations were performed.

Results
At dosages of 375 mg/kg bw and above, deaths in mice occurred within 48h, whilst in rats the deaths occurred mainly after 4-5 days. Clinical signs noted were tonic spasm, piloerection, higher respiration rate in both rats and mice, but it was unclear if these occurred at all doses.
Surviving animals appeared normal thereafter. Weight gain was normal for the strain used. Autopsy of the animals surviving up to day 14 were reported to be normal.
Animals died between the first 24 hours and 6 days after dosing. The mortality rates are summarised for the different groups. At necropsy, no macroscopic organ changes/damages were noted.

Table 1: Mortality in rats and mice after a single oral application

Species	**Dose [mg/kg bw]**					
	250	**375**	**500**	**625**	**750**	**875**
Mice (female)	0/10	2/10	6/10	5/10	9/10	10/10
Rats (female)	0/5	2/5	2/5	4/5	5/5	-
Rats (male)	0/5	3/5	4/5	5/5	5/5	-

Based on the observed mortality, the following LD_{50} figures were calculated by the method of Spearman-Kärber:

LD_{50} rat, female: 588 mg/kg bw
LD_{50} rat, male: 475 mg/kg bw
LD_{50} mouse, female: 538 mg/kg bw

Conclusion
Based on these findings, 1-methoxy-2-Amino-4-ß-hydroxyethylamino-benzene-sulphate is considered as moderately toxic to rats and mice with LD_{50} values in the range of 475 to 588 mg/kg bw.

Ref.: 15

Acute oral toxicity study in mice (1990)

Guideline: OECD 401 (1987)
Species/strain: Mouse, BOR: NMRI White
Group size: 5 dose/sex
Test substance: Lehmannblausulphat

Batch: /
Purity: /
Dose: 125, 250, 500, 750 and 1000 mg/kg once by oral gavage
Vehicle: deionised water
Observation: 14 days
GLP: in compliance

A preliminary range finding test with 2 female mice of 2000 mg/kg bw indicated that the median lethal dose was less than 2000 mg/kg bw as both died within 24 hours.
The test substance was administered as 1.25 – 10% dilution in deionised water at 5 dose levels from 125 to 1000 mg/kg bw. Mortality and clinical signs were checked daily for the 14-day observation period.

Results
The test substance caused dose related clinical signs to CNS, coordination, reflexes and autonomic functions increasing in severity up to 72 hours after dosing. Weight gains were reduced in all surviving animals.
Animals died within 24-72 hours after dosing. The mortality rates for the dosages are summarised below. At necropsy no macroscopic organ changes/damages were noted.

Table 2: Mortality in mice after a single oral application

Species	**Dose [mg/kg bw]**				
	125	**250**	**500**	**750**	**1000**
Mice (female)	1/5	1/5	3/5	5/5	4/5
Mice (male)	1/5	2/5	4/5	3/5	5/5

Based on the observed mortality, the following LD_{50} figures were determined at 14 days according to Finney:

LD_{50} mouse, female: 351 mg/kg bw
LD_{50} mouse, male: 333 mg/kg bw
LD_{50} mouse male + female: 327 mg/kg bw

Conclusion
Lehmannblausulphat under the conditions of this study showed an LD_{50} of 327 mg/kg bw.
Ref.: 16

Comment
The studies are not up to modern standards but the results give an indication of the LD_{50} in both rats and mice.

3.3.1.2. Acute dermal toxicity

No data

3.3.1.3. Acute inhalation toxicity

No data

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Taken from SCCP/0958/05

Guideline: OECD 404
Species: New Zealand White rabbit
Group: 3 (sex not indicated)
Test substance: Lehmannblausulfat
Batch: /
Purity: /
Dose: 0.5 g of hair dye formulation (Koleston 2000) containing 3% Lehmannblausulfat
Route: dermal
Application: single administration, 4h, semi-occlusive
GLP: in compliance

0.5 g of a commercial hair dye formulation containing 3% Lehmannblausulfat was applied to shaved areas (about 6cm²) of the back of 3 New Zealand White rabbits. After 4h semi-occlusion, the test item was washed off with water. Animals were examined for signs of erythema and oedema at 30 min, 60 min, 24, 48 and 72 hours post exposure period and thereafter up to 14 days.

Results
Slight erythema (score 1 and 2) and slight oedema (score 1) were observed at several times but the signs had resolved within 7 days. The individual 24/48/72 hours mean scores for erythema and oedema were 1.33, 1.0; 1.67, 1.0 and 1.33, 1.0.

Conclusion
Under the described test conditions, the commercial hair dyes product Koleston 2000 containing 3% Lehmannblausulfat caused mild transient irritation to the skin of the rabbit.
Ref.: 17

Comment
This test is not applicable for safety evaluation. The test substance by itself should have been tested. However, it will be assumed that Lehmannblausulfat is slightly irritant to the skin of the rabbit.

Guideline: /
Species: Guinea pig, SPF white
Group: 10 females
Test substance: 1-methoxy-2-amino-4-(β-hydroxy-ethylamino)-benzene sulphate
Batch: /
Purity: /
Dose: 1% test substance suspended in 10% gum arabic
Route: dermal
Application: triple application, 20 min each, for two consecutive days
GLP: not in compliance

A 1% preparation of 1-methoxy-2-amino-4-(β-hydroxy-ethylamino)-benzene sulphate, suspended in 10% gum Arabic, was brushed onto 3 x 4 cm areas of the right and left clipped flanks of 10 female albino guinea pigs and then washed off after 20 minutes. The procedure was repeated three times daily for 2 days. The treatment period was followed by a 3 day observation period.

Results
2 of the 10 animals showed slight erythema during the second day of treatment only.

Conclusion
Under the condition of the test, a 1% preparation of 1-methoxy-2-amino-4-(β-hydroxy-ethylamino)-benzene sulphate was not irritant to the skin of the guinea pig.

Ref.: 18

Comment
This study is not acceptable for evaluation. The test does not conform to a guideline, GLP assurance is not provided. The test substance is of unknown specification and was tested at a concentration below that which it is intended to be used at.

3.3.2.2. Mucous membrane irritation

Taken from SCCP/0958/05

Guideline:	OECD 405
Species:	New Zealand White rabbit
Group:	3 (sex not indicated)
Test substance:	Lehmannblausulfat
Batch:	/
Purity:	/
Dose:	0.1ml of hair dye formulation (Koleston 2000) containing 3% Lehmannblausulfat
Route:	ocular
Application:	single administration, without rinsing
GLP:	in compliance

0.1 ml of a hair dye formulation (Koleston 2000) containing 3% Lehmannblausulfat was introduced into the conjunctival sac of the left eye of 3 New Zealand White rabbits, the right eye served as the control. Both eyes were examined at 1, 24, 48 and 72 hours post application.

Results
In all animals conjunctival redness (score 1) was present at 24 hours and persisted to 48 hours in two of the three animals. No corneal or iris effects were observed. The individual mean scores for 24/48/72 hours for conjunctival redness were 0.67, 0.33 and 0.67.

Conclusion
A hair dye formulation (Koleston 2000) containing 3% Lehmannblausulfat caused transient and mild irritation to the conjunctivae of rabbits.

Ref.: 19

Comment
This study is not acceptable for evaluation because the test substance was tested as part of a product and not by itself. However, it will be assumed that Lehmannblausulfat is slightly irritant to the eye of the rabbit.

Guideline:	/
Species:	Guinea pig, Pirbright White (SPF)
Group:	10 females
Test substance:	1-methoxy-2-amino-4-(β-hydroxy-ethylamino)-benzene sulphate
Batch:	/
Purity:	/

Dose: 0.1ml of 1% test substance in water
Route: ocular
Application: single administration, without rinsing
GLP: not in compliance

0.1 ml of a 1% aqueous solution of 1-methoxy-2-amino-4-(β-hydroxy-ethylamino)-benzene sulphate was instilled into the conjunctival sacs of the right eyes of 10 female guinea pigs. The left eyes were untreated and served as controls. Observations were made at 30 minutes, 1, 2, 3, 4, 6 and 7 hours; at 24 hours fluorescein staining was used to improve the observations.

Results
The test preparation caused conjunctival redness and discharge in 5 of the animals at 30 minutes, redness was observed in 2 animals at 7 hours but had cleared by the 24 hour reading.

Conclusion
Under the conditions of the test, 1% aqueous solution of 1-methoxy-2-amino-4-(β-hydroxy-ethylamino)-benzene sulphate caused transient conjunctival irritation to the eyes of guinea pigs.

Ref.: 20

Comment
Despite the deficiencies in the study, it should be accepted as indicating a mild irritant potential. A 1.5% solution should have been used.

3.3.3. Skin sensitisation

Taken from SCCP/0958/05

Local Lymph node assay (LLNA)

Guideline: OECD 406
Species: Mouse, strain CBA/Ca
Group: 5 females per test concentration
Test Substance: Lehmann-Blau
Batch: 57
Purity: 97.7%
Dose levels: 0, 0.25, 0.5, 1 and 2% in DMSO w/v
Route: dermal, once daily for 3 consecutive days
GLP: in compliance

25 µl of negative control (DMSO, the vehicle), 0.25, 0.5, 1 and 2% of Lehmann-Blau in DMSO w/v was applied to the surface of the ear of five female CBA/Ca mice per group for three consecutive days. After application, the ears were dried with a hair dryer for five minutes.
As the positive control, p-phenylenediamine (PPD) in DMSO at the same dilutions was used in parallel.

On day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 25 µCi of [H^3] methyl thymidine. Approximately 5 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 determined by means of liquid scintillation counting as disintegrations per minutes (dpm). The mean dpm per treated group was determined and the stimulation index (SI) – the test item compared to the concurrent vehicle control – calculated.

Results
No concentration dependent increase in the mean SI values (1.29, 1.03, 1.12, 1.42) could be detected for the 4 consecutive concentrations of Lehmann-Blau in DMSO.
The sensitivity of the test system was shown by the positive control, PPD, for which the SI were 5.47, 12.39, 19.12 and 7.07 respectively for the 4 consecutive concentrations.

Conclusion
There was no indication of skin sensitisation by Lehmann-Blau at up to 2% in DMSO. An EC3 value could not be calculated.

Ref.: 22

Comment
The study was not performed correctly since the induction concentrations used were too low. These doses are not suitable for a robust hazard assessment.

3.3.4. Dermal / percutaneous absorption

Taken from SCCP/0958/05

Guideline:	/
Species:	Rat, Sprague-Dawley: OFA, SPF
Group:	3 per sex and dose
Test substance:	^{14}C ring labelled 1-methoxy-2-amino-4-(β-hydroxy-ethyl)-aminobenzene dihydrochloride
Batch:	/
Purity:	radiochemical purity 97%
Doses/Applications:	Application area 3 x 3 cm^2. Three preparations used: - Commercial formulation without hydrogen peroxide. Equal to 0.83mg/cm^2 of free base - Commercial formulation with hydrogen peroxide. Equal to 0.83mg/cm^2 of free base - 3.47% aqueous solution. Equal to 0.83mg/cm^2 of free base
Schedule:	single cutaneous application of 30 minutes.
GLP:	not in compliance

^{14}C ring labelled 1-methoxy-2-amino-4-(β-hydroxy-ethyl)-aminobenzene dihydrochloride was applied to the skin of groups of three male and female Sprague-Dawley rats (body weights about 205g). The application area was 3 x 3 cm^2 with a contact time of 30 minutes. The test substance was present at a concentration of 0.75% in a commercial hair dye without hydrogen peroxide and at 0.75% in a commercial hair dye with hydrogen peroxide. In addition, a 3.46% aqueous solution was applied. For all three applications, the mean dose was 0.83mg/cm^2 of free base.

After 30 minutes the hair dye formulations were scraped off and these sites, as well as those where only the aqueous preparation was applied, rinsed with about 100ml of a 3% shampoo solution. After rinsing the areas were covered with gauze and an air permeable plastic cone to prevent licking of the treated areas during the 72 hour observation period.

Urine and faeces were collected daily.

Animals were killed 72 hours after application. Application sites, blood, and various organs were collected and analysed for radioactivity. The radioactivity in the carcasses was determined after complete removal of the skin.

Results
Mean recovery rates of 95.1, 97.7, and 99.9% were found for the commercial formulations without or with hydrogen peroxide, and with the aqueous solution, respectively.

The majority of the applied dose (93.6 to 98.9%) was recovered in the washing solutions.

The amount of radioactivity remaining at the cutaneous application site was 4.73 and 12.53 µg/cm^2 (equal to 1.51% and 0.57% of the applied dose) for the formulations without and with hydrogen peroxide, respectively. For the aqueous solution, the figure was 6.23 µg/cm^2 (equal to 0.75% of the applied dose).

Based on the amounts detected in urine, faeces and the carcasses, absorption rates of 1.06 µg/cm^2 (equal to 0.128% of the applied dose), 0.27 µg/cm^2 (equal to 0.033% of the applied dose) and 1.97 µg/cm² (equal to 0.237% of the applied dose) were obtained for the formulations without and with hydrogen peroxide and the aqueous formulation, respectively.

The absorbed test substance was mainly excreted via the urine for the formulations without hydrogen peroxide (0.097% of the applied dose) and with hydrogen peroxide (0.020% of the applied dose), as well as for the aqueous solution (0.180% of the applied dose). Only small amounts were found in the faeces: 0.027%, 0.008% and 0.047% respectively.

The remaining ^{14}C ring labelled 1-methoxy-2-amino-4-(β-hydroxy-ethyl)-aminobenzene detected in the carcasses at the end of the 72 hour observation period was close to or below the limit of detection of 0.005 to 0.008% of the applied dose for all three groups. The concentrations in the organs were very low (below or close to the detection limit).

Taking the mean amounts in urine, faeces, residual carcass and the total content of the dye remaining in the skin as bioavailable, absorption rates of 5.8 µg/cm² (equal to 0.70%) and 12.8 µg/cm² (equal to 1.54%) were found for the commercial formulations without and with hydrogen peroxide. The respective figure for the aqueous solution was 8.2 µg/cm² (equal to 0.99%).

Conclusion
The total amount found in the skin was considered as bioavailable. A penetration rate of 12.8 µg/cm² of 1-methoxy-2-amino-4-(β-hydroxy-ethyl)-aminobenzene free base, is equivalent to 19.7 µg/cm² for 2-amino-4-hydroxyethylamino-anisole sulfate, which should be used to calculate the systemic exposure dose.

Ref.: 25

In a former submission, the skin penetration of 2-amino-4-hydroxyethylamino-anisole sulfate was investigated after application of a commercial hair dye formulation containing 2.2% of the dye to 5 female volunteers by a professional hair dresser for 15 min. The absorption was analysed in blood samples for 24 h by HPLC. Neither the parent compound nor the metabolites could be detected in the serum within the sensitivity limits of the method, indicating that less than 1.6 µg/cm² (equal to 925.7 µg/volunteer or 0.06% of the applied dose) became bioavailable. The study was not in compliance with GLP, and a non-standardised method and unspecified test material were used, the results obtained are unsuitable for risk assessment calculations.

Additional study, submission IV

Guideline:	OECD 428 (2004)
Date of test:	14-31 August 2006
Test system:	Excised, dermatomed (850µm) pig skin (back and flank of 1 female and 2 male pigs) on in-house developed Teflon-chambers
N° of samples:	Two independent experiments with 6 skin samples

Test substance:	1.5% 2-amino-4-hydroxyethylaminoanisole sulfate (WR23081) in a typical hair dye formulation (composition given with commercial names), in the presence of hydrogen peroxide and a reaction partner (WR18247)
Purity:	Not stated
Applied amount:	100 mg/cm², rinsed off with shampoo & water after 30 min
Receptor fluid:	physiological receptor fluid
Solubility in receptor fluid:	92.6 mg/ml (pH = 7.3) (unlabelled test substance)
Duration of study:	72 hours
GLP/QAU:	In compliance

The cutaneous absorption of 1.5% 2-amino-4-hydroxyethylamino anisole sulfate in a typical hair dye formulation in the presence of hydrogen peroxide and a reaction partner was investigated *in vitro*, using pig skin preparations, which were continuously rinsed from underneath with physiological receptor fluid. Two independent experiments were performed with 6 diffusion cells per experiment. For calculations, the mean value of all valid skin samples (n=8) was used.

The integrity of each skin preparation was determined by examination of penetration characteristics with tritiated water resulting in 0.8 to 3.7% of the applied dose found after 4 hours in the receptor fluids, which was within the limit of acceptance (≤ 2.0%) for 8 skin samples used for determination of skin penetration. The skin samples with skin integrity values above 2% of the applied dose (4 skin samples) were not taken into consideration for the calculation of the mean.

After checking the skin integrity, 400 mg of the formulation (= 100 mg/cm²) was applied to the skin samples for 30 minutes and subsequently washed off with water and shampoo. The determination of the amount of 2-amino-4-hydroxyethylamino anisole sulfate in the washings (= amount dislodgeable from the skin surface) was performed by measuring the radioactivity by means of a scintillation counter. At 16, 24, 40, 48, 64 and 72 hours, the content of 2-amino-4-hydroxyethylamino anisole sulfate was determined in the receptor fluid by the same method. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the radioactivity was quantified by means of a scintillation counter.

Results
The majority of the test substance could be found in the rinsing solutions (1.341 ± 0.051 mg/cm²). Small amounts of 2-amino-4-hydroxyethylamino anisole sulfate could be found in the upper skin (2.352 ± 0.824 µg/cm²), in the lower skin (0.303 ± 0.219 µg/cm²) and in the fractions of the receptor fluid collected within 72 hours (0.409 ± 0.223 µg/cm²).
The mass balance of the test substance resulted in values of 92.20 to 103.31% recovery for all (8) skin samples with acceptable integrity (as measured by the penetration characteristics of tritiated water).

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

Ref.: 37

Comments
- 4 of the 12 skin samples employed failed the skin integrity test with tritiated water. This appears to be a high number.

- The amount applied (100 mg/cm²) is very high compared to the normal value of 20 mg/cm².
- The "upper skin" (stratum corneum + upper stratum germinativum) was separated from the "lower skin" (lower stratum germinativum and upper dermis). This does not allow calculating the values in (i) stratum corneum (SC), (ii) epidermis without SC and (iii) dermis separately, as requested by the SCCP.
- The study authors do not consider the amount measured in the "upper skin" as biologically available. However, the "upper skin" contains a large part of the epidermis (not only the stratum corneum), therefore the mentioned value of 0.712 µg/cm² is an underestimation.
- In the individual results tables for the receptor fluid amounts, 2 out of 6 values are indicated to be "outliers". However, there is no statistical backup for this denomination. In addition, it is not mentioned whether these were the values obtained through the skin samples which failed the skin integrity test with tritiated water. In that case, it could be acceptable that they would deliver an overestimation of the real dermal absorption value, but the denomination "outlier" has a different meaning.
- Overall, it is clear that the study authors did not take into account the SCCP requirements (SCCP/0970/06). With only 8 samples in total from 3 donors (instead of the required minimum of 6 evaluable samples, from each of at least 3 donors) and an incorrect separation of the skin, the dermal absorption cannot be deduced from this study. Moreover, the compound has not been tested separately, only in the presence of hydrogen peroxide and a reaction partner.

Considering the above, the dermal absorption study is considered inadequate. A study according to the Notes of Guidance was requested in March 2006. This automatically includes the requirements of the "Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients", which were last updated in March 2006 (SCCP/0970/06). The presented study, however, does not follow these SCCP guidelines, and no justification is provided for this deviation.

3.3.5. Repeated dose toxicity

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

Taken from SCCP/0958/05

Dermal

Guideline:	OECD. 410 (1981)
Species/strain:	Guinea pig, strain SPF Pirbright White (BOR)
Group size:	5 per dose/sex
Test substance:	Lehmannblausulfat
Batch:	/
Purity:	98%
Dose levels:	0, 50, 150 and 300 mg/kg bw/day
Vehicle:	Water
Route:	Dermal
Exposure:	28 days
GLP:	in compliance

1 ml/kg bw Lehmannblausulfat (0, 5, 15 and 30 % in tap water; equivalent to 0, 50, 150 and 300 mg/kg bw/day) was applied dermally to the clipped area of skin on the animals' back (3 x 4 cm; ~ 10 percent total body surface) once daily for 28 days to guinea pigs. The test solution was applied within 1 h post preparation. Doses were adapted weekly according to the body weight gain of the animals.

Due to technical limits the maximum dose was 300 mg/kg bw/day. 30% represented the maximum concentration achievable in water and 1 ml/kg bw/day was the maximum volume to be used for application.
Treatment was continued for 28 days and since not all animals could be necropsied on the same day, treatment was continued until the day preceding necropsy.
Mortality was checked twice daily, clinical signs were recorded once daily and individual body weights were recorded weekly. Clinical laboratory investigations (haematology, blood/clinical biochemistry and urinalysis) were performed at day 0 and day 28.
All animals were subjected to a detailed necropsy and a number of organs (adrenals, heart, kidneys, liver, ovaries, spleen, testes, uterus) were weighed.
The hearts and the kidneys of the control and high dose animals were examined histopathologically. In addition, all gross lesions noted and the liver and the skin of all dose groups were examined microscopically.

Results
The stability and homogeneity of the test solutions were analytically verified and were in good agreement with the nominal doses, if analysis was done within 1 h after preparation. Thus, a sufficient stability and correctness of the dosing solutions can be assumed during the application period of 1 h.
The daily skin evaluation revealed no indications of erythema or oedema formation. The hyperplasia and hyperkeratosis noted in some animals were considered due to mechanical irritations caused by the clipping process and were not considered to be treatment-related.
No mortalities or clinical signs of toxicity were noted. The body weight was not affected by the treatment. There were no treatment-related haematological or clinical biochemistry changes. At necropsy no gross lesions were noted. Organ weights did not reveal any differences between the dose groups. No indication of systemic toxic effects was noted in a dermal 28-day toxicity study in guinea pigs up to the maximum technically achievable dose of 300 mg/kg bw/day.

Ref.: 26

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

Taken from SCCP/0958/05

15-weeks repeated dose study in Wistar rats

Guideline:	According to OECD guideline no. 408 (1998)
Species/strain:	Rat, strain Wistar HanBrl: WIST (SPF)
Group size:	15 per dose/sex
Test substance:	2-amino-4-hydroethoxyethylamino-anisole sulfate
Batch:	57
Purity:	99.5 area% (HPLC, 254 nm)
Dose levels:	0, 15, 50 and 200 mg/kg bw by gavage
Vehicle:	Bi-distilled water
Dosing schedule:	Once daily, 7 days/week for 108/109 days
GLP:	in compliance

Dose volume in treated and control groups was 10 ml/kg body weight. Clinical signs, outside cage observations, food consumption and body weights were recorded weekly pre-test and throughout the treatment period. Ophthalmoscopic examinations were performed at pre-test and at the end of the treatment period. Investigations included in the functional observational battery and of locomotor activity and grip strength were performed during week 15.
At the end of the dosing period, blood samples were taken for the examination of haematology and clinical biochemistry. In addition, in 5 animals per sex and dose, thyroid hormones as TSH, and free and total T3 and T4 were determined in week 15. Urine samples were collected for urinalyses at the end of the treatment period. All animals were killed,

necropsied and examined post mortem. Organ weights of several organs were recorded. Histopathology of organs and tissues from all control, high dose animals and all gross lesions from all animals was performed. In addition, thyroid, spleen, kidneys and pituitary were investigated for the mid and low dose groups.

Results
A sufficient solubility, homogeneity and stability can be assumed, since the dosing concentrations of 2-amino-4-hydroethoxyethylamino-anisole sulfate in the vehicle (water) were analytically verified. The mean concentrations of the test samples were 88.3 to 101.4%, 90.4 – 96.6% and 83.4 – 100.0% of the nominal values for the three dose groups, confirming proper dosing for the entire study period.
All animals survived until scheduled necropsy. A blue discolouration of the urine was noted in all animals of the mid and high dose groups and in females of the lowest test dose of 15 mg/kg bw/day.
No clinical toxicological signs were seen either during daily and weekly observations or during the functional observational investigations.
No treatment-related differences in the mean fore- and hind-limb grip strength values and in the mean locomotor activity were noted at any dose level. No changes were observed in the ophthalmoscopic examinations or in the mean daily food consumption during the study.
No treatment related effects were noted on body weight and/or body weight gain. The marginally lower body weights noted from day 29 of treatment onwards in the males showed no clear dose-response and were not considered to be toxicologically relevant. The analysis of thyroid hormones revealed no differences to controls at any dose level.

The following adverse effects were noted at the different dose levels:

200 mg/kg bw/day
Indication of a slight anaemia with a compensatory reticulocytosis was seen in both sexes, evident as lower red blood cell counts, lower haemoglobin, elevated methaemoglobin, lower haematocrit levels and elevated reticulolycte counts and reticulocyte maturity indices.
The effects on clinical biochemistry parameters (reduced creatinine, elevated triglyceride sodium and chloride concentrations), and urinalysis (slight proteinuria and increased bilirubin and nitrite) observed in both sexes may indicate changes in the liver metabolism and, to a lesser extent, changes in the kidneys.
Marginally elevated thyroid-to-brain weight ratios in both sexes and elevated mean absolute and relative liver, kidney and spleen weights in females were recorded.
Histopathological and morphological test item-related changes were recorded in thyroid (follicular cell enlargement due to storage of brown fine-granular pigment, both sexes), pituitary (slight hypertrophy of chromophobic cells in males), kidneys (pigment storage, tubulus swelling with necrosis of tubulus cells and basal membrane thickening) and spleen (increased mean grade of extra medullary haemopoiesis, both sexes).

50 mg/kg bw/day
Similar effects but of less severity as described at 200 mg/kg bw/day were noted for the mid dose groups, but predominately in females only.
Indication of a slight anaemia with a compensatory reticulocytosis as described for the high dose was noted in females only.
Elevated plasma sodium and chloride levels in females were also seen.
Histopathological and morphological changes similar to those of the high dose group were observed in thyroid (follicular cell enlargement due to storage of brown fine-granular pigment, both sexes), pituitary (slight hypertrophy of chromophobic cell in males) and kidneys (pigment storage, tubular swelling with necrosis of tubular cells and basal membrane thickening).

15 mg/kg bw/day
No adverse effects were noted.

Conclusion
Based on the adverse effects noted at 50 mg/kg bw/day, evident as slight anaemia and morphological and histological changes in the thyroid, the kidneys and the pituitary, a "no observed adverse effect level" (NOAEL) of 15 mg/kg bw/day of 2-amino-4-hydroethoxyethylamino-anisole sulfate was derived from this subchronic oral toxicity study in rats.

Ref.: 27

In another 90 day repeated dose oral toxicity study, dose levels of 2, 50 and 100 (increased to 1380) mg/kg bw/day were administered to rats. The study was considered to have limitations, i.e. in study design and test substance specification, and consequently was not provided.

3.3.5.3. Chronic (> 12 months) toxicity

No data

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Taken from SCCP/0958/05

Bacterial gene mutation assay

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA102, TA1535, TA1537
Replicates:	Triplicates were investigated per test concentration
Assay conditions:	Plate incorporation and pre-incubation assay without and with S9-mix from rat livers (phenobarbital/ß-naphthoflavone induced); 2 independent experiments were performed.
Test substance:	2-Amino-hydroxyethylamino-anisole sulfate
Batch:	57
Purity:	99.6 area% (HPLC, 254 nm)
Concentrations:	0, 33, 100, 333, 1000, 2500 and 5000 µg/plate with and without metabolic activation
GLP:	in compliance

2-Amino-hydroxyethylamino-anisole sulfate dissolved in de-ionised water was tested for mutagenicity in the bacterial gene mutation assay (experiment 1: plate incorporation method, experiment 2: pre-incubation method). 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 (S9-mix from the liver of phenobarbital/ß-naphthoflavone induced male Wistar Hanlbm rats). Test concentrations were selected based on the results obtained in a pre-experiment with strains TA98 and TA100.
For control purposes, untreated, solvent (deionised 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
Reduced background growth was observed at higher concentrations (2500 and 5000 µg/ml) in the presence and absence of S9-mix in almost all strains investigated.

No biologically relevant increase in revertant colony numbers was observed in any of the five tester strains following treatment with 2-Amino-hydroxyethylamino-anisole sulfate 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
In conclusion, it can be stated that under the experimental conditions reported 2-Amino-hydroxyethylamino-anisole sulfate did not induce gene mutations in *Salmonella typhimurium* in any of the tester strains in the presence or absence of S9-mix.

Ref.: 28

Three bacterial gene mutation tests with 2-Amino-hydroxyethylamino-anisole sulfate had been presented in a former submission. However, these studies revealed limitations with regard to the experimental design and/or the specification of the test material used. However, no indication of a mutagenic potential of 2-Amino-hydroxyethylamino-anisole sulfate was noted in these tests, too.

In Vitro Mammalian Cell Gene Mutation Test

Guidelines:	OECD 476 (1997)
Species/strain:	Mouse lymphoma cell line L5178Y; $TK^{+/-}$
Replicates:	Single cultures per concentration, 8 concentrations analysed
Test Substance:	2-Amino-hydroxyethylamino-anisole sulfate
Batch:	101
Purity:	99.8 area% (HPLC, 254 nm)
Concentrations:	0, 0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/ml without and 0, 1.0, 2.5, 5, 10, 25, 50, 100 and 500 µg/ml with metabolic activation
GLP:	in compliance

2-Amino-hydroxyethylamino-anisole sulfate was tested for its mutagenic activity in the L5178Y $TK^{+/-}$ mouse lymphoma assay. In this system, the mutagenic potential is measured by examining the induction of trifluorothymidine (TFT) resistance by forward mutations at the thymidine kinase (TK) locus. The test was performed with and without metabolic activation (S9-mix from the liver of phenobarbital/ß-naphthoflavone induced male Wistar rats). Test concentrations were defined by a range-finding test (pre-test on toxicity, measuring relative suspension growth).
Culture medium was used as solvent control, while ethylmethanesulfonate (EMS, 0.7 mg/ml) and benzo[a]pyrene (B[a]P, 2.5 µg/ml) were used as positive controls without and with metabolic activation, respectively.
The incubation time was 4 hours in the presence and absence of S9-mix. Single cultures were investigated for each concentration and test group. Mutant frequency and cell survival (measured as total suspension growth) were determined.
In addition to the number of mutant colonies, the size/optical density of the colonies was determined and the ratio of small versus large colonies was calculated.

Results
In the initial range-finding study, concentrations up to 3000 µg/ml without S9-mix were evaluated for toxicity. Pronounced toxicity was noted at a test concentration of 300 µg/ml with a relative suspension growth in the range of 2.65%.
Based on these findings, the concentrations for the main experiment were chosen to obtain at least 8 analysable concentrations covering a range of more than one logarithmic decade.
In the main experiment, a relative total growth of 20.61 % relative to the control was measured in the presence of S9-mix at the top concentration of 500 µg/ml. In the absence of S9-mix, the relative total growth was 23.09 % (relative to the control) at the highest

analysable concentration of 100 µg/ml. Precipitation was observed without metabolic activation at concentrations ≥ 50 µg/ml.
A biologically relevant increase in the number of mutant colonies (compared to the concurrent controls and the historical control range) was noted in the presence and absence of S9-mix.
A biologically relevant shift towards small colonies was evident following the treatment with 2-Amino-hydroxyethylamino-anisole sulfate in the presence and absence of S9-mix, indicating a clastogenic potential of the test item.
The positive controls demonstrated that the system was suitable to detect known mutagens, and thus indicated its sensitivity.

Conclusion
2-Amino-hydroxyethylamino-anisole sulfate induced a biologically relevant increase in mutations at the thymidine kinase locus in L5178Y cells.
Consequently, 2-Amino-hydroxyethylamino-anisole sulfate is considered to be mutagenic in this *in vitro* mammalian cell gene mutation assay.
The shift towards small colonies among the induced mutant colonies indicates a clastogenic effect rather than a potential to induce true gene mutations.

Ref.: 29

In a former submission, an older mouse lymphoma $TK^{+/-}$ gene mutation assay was presented, which also revealed a mutagenic potential.

In Vitro Micronucleus Test

Guideline:	OECD 487 (Draft; 2004)
Species/strain:	Human peripheral blood lymphocytes
Replicates:	Two cultures per concentration, 3 concentrations analysed
Assay conditions:	Single experiment using pooled blood from two female donors in each experiment
Test Substance:	2-Amino-hydroxyethylamino-anisole sulfate
Batch:	57
Solvent:	sterile water
Purity:	99.6 area% (HPLC, 254 nm)
Concentrations:	with S9-mix: 0, 25, 100 and 150 µg/ml; 3 h treatment 24 h after mitogen stimulation without S9-mix: 0, 3, 5 and 8 µg/ml, 24 h treatment, 24 h after mitogen stimulation
GLP:	in compliance

2-Amino-hydroxyethylamino-anisole sulfate was examined for its clastogenic and aneugenic potential by evaluating its ability to induce micronuclei in cultured human lymphocytes. Two independent cultures were prepared per test concentration in the presence and absence of metabolic activation (S9-mix from the liver of Aroclor 1254 induced rats). Test concentrations were selected based on the results obtained in pre-experiments on cytotoxicity.
In the main experiment, cells were treated with the test item 24 hours after mitogen stimulation (phytohaemagglutinin (PHA)). The exposure times were 3 and 20 hours for the test item in the presence and the absence of S9-mix, 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 were harvested 72 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 concentrations) were analysed for the occurrence/number of micronuclei.

The following controls were evaluated: Sterile water as solvent control, 4-nitroquinoline 1-oxide (5µg/ml) and vinblastine (0.08 µg/ml) as positive controls in the absence of S9-mix and cyclophosphamide (6.25 µg/ml) as positive control in the presence of S9-mix.

Results
At the highest analysable test concentrations of 8 µg/ml (without S9-mix) and 150 µg/ml (with S9-mix), the RI was reduced by about 68 % and 63 %, respectively.
In the absence of S9-mix, the frequencies of micronucleated binucleate (MNBN) cells were similar to the concurrent controls for all analysed concentrations and well within the range of the historical vehicle controls. At 8 µg/ml, a borderline result comprising a small increase in the frequency of MNBN cells was noted that exceeded the historical vehicle control range in one culture only. This marginal increase was seen at a high level of cytotoxicity (68 %), and is regarded as an equivocal effect.
In the presence of S9-mix, a concentration-related increase in the frequency of MNBN cells was observed, reaching a statistically significant level at the two highest test concentrations. In addition, the frequency of MNBN cells exceeded the historical control range in single cultures at the same concentrations.

Conclusion
The potential of 2-Amino-hydroxyethylamino-anisole sulfate to cause chromosomal damage was investigated in an *in vitro* micronucleus test with cultured human peripheral blood lymphocytes from female donors. The test item caused a statistically significant and biologically relevant increase in the frequency of micronuclei in the presence of S9-mix, when treatment was commenced 24 hours following mitogen stimulation. In the absence of S9-mix, an equivocal result was obtained.
Based on these results, 2-Amino-hydroxyethylamino-anisole sulfate is considered to be mutagenic in this *in vitro* micronucleus test.

Ref.: 30

In addition, several chromosomal aberration tests using human lymphocytes and CHO cells are available for 2-Amino-hydroxyethylamino-anisole sulfate which had been described in a former submission. Since these tests had limitations with regard to the test performance and/or specifications of the test material, they are not discussed here. Altogether they showed equivocal results.
An *in vitro* UDS test with a negative result was also presented in a former submission. As this test had limitations with regard to the test performance and the specification of the material tested, it is not discussed here.

3.3.6.1. Mutagenicity / Genotoxicity *in vivo*

Taken from SCCP/0958/05

Mouse bone marrow micronucleus test

Guideline:	OECD 474 (1997)
Species/strain:	Mouse, strain NMRI
Group size:	5 animals per sex, dose group and sacrifice time
Test substance:	2-Amino-hydroxyethylamino-anisole sulfate
Batch:	101
Purity:	99.8 area % (HPLC, 254 nm)
Dose levels:	0, 20, 100 and 200 mg/kg bw administered as single doses
Route:	intraperitoneal
Vehicle:	distilled water
Sacrifice times:	24 and 48 hours (high dose only)
GLP:	in compliance

2-Amino-hydroxyethylamino-anisole sulfate dissolved in distilled water was administered at doses of 20, 100 and 200 mg/kg bw to groups of five male and five female NMRI mice by intraperitoneal injection. Two groups were treated with the high dose to allow sampling after 24 and 48 hours. Single doses were administered per animal in a total volume of 10 ml/kg bw.
Negative control groups received distilled water and concurrent positive control groups received 40 mg/kg bw cyclophosphamide (CPA) dissolved in 0.9 % NaCl.
Dose selection was based on findings for toxicity from pre-experiments in which 200 and 400 mg/kg bw were administered to 3 mice per sex and dose. Deaths occurred within 24 hours after administration of 400 mg/kg bw 2-Amino-hydroxyethylamino-anisole sulfate. In addition, all animals showed signs of toxicity, such as reduced spontaneous activity, lethargy, palpebral closure, prone position at 1, 1.5 and 6 hours after administration. At 200 mg/kg bw, signs of toxicity like palpebral closure and lethargy occurred within the first hour after administration, but no effects were seen at 6 hours or any later observation time point, and no deaths occurred at this dose.
Femoral bone marrow was sampled from sacrificed mice 24 hours after dosing for all dose groups, and additionally, after 48 h for the high dose group. Bone marrow of the negative and concurrent positive control group animals was sampled at 24 h. Slides were stained with May-Grünwald/Giemsa, and evaluated for the number of polychromatic erythrocytes (PCE) with micronuclei. At least 2000 PCEs per animal were analysed. In addition, the ratio between polychromatic and total erythrocytes per animal was determined by counting at least 200 immature (polychromatic) erythrocytes per animal. Ten animals per test group were evaluated.

Results
There was no statistically significant or biologically relevant increase in the number of micronuclei per 2000 PCEs in the mice of any of the 2-Amino-hydroxyethylamino-anisole sulfate treated groups compared to the respective vehicle control groups.
The positive control substance (CPA) produced a marked induction of micronuclei, thus demonstrating the sensitivity of the test system used.
Both, the positive control and the vehicle control were well within the range of historical control data of the performing laboratory.
There was no treatment related change in the proportion of PCE among total erythrocytes and, therefore, this parameter does not indicate the relevant exposure of the bone marrow.

Conclusion
The study was conducted appropriately. 2-Amino-hydroxyethylamino-anisole sulfate did not induce chromosome aberrations or damage to the mitotic apparatus in bone marrow cells of mice after a single intraperitoneal administration under the test conditions used. Although there was no effect on the PCE/NCE ratio indicating bone marrow toxicity, systemic availability can be assumed after i.p. application and is further suggested by the general signs of toxicity observed in treated animals.

Ref.: 31

An older mouse bone marrow micronucleus test with the free base was presented in a former submission. The test result was negative. However, as this test revealed limitations with regard to the test performance and the specification of the material tested, it is not discussed here.

Rat Liver *In vivo / In vitro* UDS Assay

Guideline:	not indicated, but in line with OECD guideline no. 486 (1997)
Species/strain:	rat, strain Wistar/WU
Group size:	5 males per dose group and per sacrifice time
Test substance:	2-Amino-hydroxyethylamino-anisole sulfate

Batch:	Rob006958
Purity:	99.7 area% (HPLC, 254 nm)
Dose levels:	750 mg/kg bw (4 h treatment); 75 and 750 mg/kg bw (16 h treatment)
Route:	oral, gavage
Vehicle:	aqua bidestilled
Preparation time:	4 and 16 hours
GLP:	in compliance

2-Amino-hydroxyethylamino-anisole sulfate was assessed for its potential to induce DNA-damage and -repair in the *in vivo / in vitro* UDS test using rat hepatocytes.
In a range finding study, mortalities were noted at the limit dose of 1000 mg/kg bw for 2 out of 5 animals within 24 hours. All surviving animals showed clinical signs of toxicity (reduced spontaneous activity, eyelid closure, pilo-erection). At 750 mg/kg bw, all animals survived for at least 24 hours. Due to treatment with the test item, the kidneys, the urine and the liver of the animals showed dark discolouration.
Based on these findings, the limit dose of 750 mg/kg bw was chosen for the 4 h treatment and 75 and 750 mg/kg bw for the 16 h treatment. 2-Amino-hydroxyethylamino-anisole sulfate was suspended in bidistilled water and was administered by gavage to groups of male Wistar rats at single doses.
For each treatment group and time point, five rats were used. The concurrent negative control group received 10 ml/kg bw of the vehicle alone. Positive control group animals were dosed with 2-acetylaminofluorene (2-AAF) at a dose level of 100 mg/kg bw. After 4 hours (750 mg/kg bw group only) or 16 hours post dosing, the animals of the respective groups were sacrificed and liver perfusion was carried out. At least five primary hepatocyte cultures were established from each animal and exposed for 4 hours to ^{3}H-thymidine which is incorporated into the DNA if UDS occurs. Possible liver cell toxicity was examined by means of dye-exclusion (trypan blue) method. Three cultures from each animal were used for the UDS assay. In the remaining two slides, cytotoxicity and attachment efficiency were determined by means of the neutral red assay.
At least two slides per animal were evaluated for the occurrence of UDS for three animals per dose group, covering in total 100 cells/animal. Heavily labelled S-phase cells were excluded from counting. Background grains (determined in an area of cytoplasm of similar size as a nucleus) were subtracted from the grains observed for the nucleus to obtain the relevant net nuclear grains.

Results
No signs of toxicity were noted in the main study. The determined viability (trypan blue exclusion) of the isolated hepatocytes was very similar for control and treatment groups (74 - 83 % and 56 - 87 %, respectively). Similarly, the attachment efficiency was not affected by the treatment with 2-Amino-hydroxyethylamino-anisole sulfate.
No dose level of 2-Amino-hydroxyethylamino-anisole sulfate revealed UDS induction in the hepatocytes of the treated animals as compared to the vehicle control. Neither the number of nuclear grains nor the resulting net grains were increased due to the *in vivo* treatment of the animals with 2-Amino-hydroxyethylamino-anisole sulfate for 4 or 16 hours, respectively. Therefore, the net grain values obtained after treatment were consistently negative. In addition, no substantial shift to higher values was noted for the percentage distribution of the nuclear grain counts.
In vivo treatment with 2-AAF (2 acetylaminofluorene) induced distinct increases in the number of nuclear and net grain counts, indicating the sensitivity of the test method used.

Conclusion
In conclusion, it can be stated that under the experimental conditions reported, 2-Amino-hydroxyethylamino-anisole sulfate did not induce DNA damage that is detectable with the UDS test.

Ref.: 32

In addition, two *in vivo* sister chromatid exchange test in rats were performed with 2-Amino-hydroxyethylamino-anisole sulfate with conflicting outcome. As both tests reveal limitations with regard to test performance, reporting and/or specification of the test material used, they are not discussed here.

Summary mutagenicity
A battery of *in vitro* and *in vivo* genotoxicity tests was performed with 2-Amino-hydroxyethylamino-anisole sulfate covering the relevant genetic endpoints. 2-Amino-hydroxyethylamino-anisole sulfate induced mutations in an *in vitro* mammalian cell gene mutation assay (MLA) The noted shift towards small colonies indicated a clastogenic potential. This is confirmed by the positive result of the *in vitro* micronucleus test with human lymphocytes. An *in vivo* micronucleus test using intraperitoneal administration and an *in vivo* / *in vitro* UDS test using oral application were negative and indicated that the genotoxic potential seen *in vitro* is not expressed under appropriate *in vivo* test conditions.

3.3.7. Carcinogenicity

No data

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data

3.3.8.2. Teratogenicity

Taken from SCCP/0958/05

Guideline:	OECD 414 (2001)
Species/strain:	Rat, Wistar HanBrl: WIST (SPF)
Group size:	22 mated females
Test substance:	2-amino-hydroxyethylamino-anisole sulfate
Batch:	57/01 (stored RT in dark, low humidity)
Purity:	93.5 weight % (study report), 99.6 area % (HPLC, 254 nm), Submission III
Dose levels:	0, 10, 30 and 150 mg/kg bw/day, freshly prepared daily
Route:	Gavage
Vehicle:	Bi-distilled water
Dosing schedule:	Gestation Day (GD) 6 to 20
GLP:	In compliance

Dose selection was based on data obtained in a range finding study with dose levels of 10, 30 and 90 mg/kg bw/day. Treatment at 90 mg/kg bw/day resulted in slightly reduced food consumption, slightly decreased body weight gain and grey-brown discolouration of urine and bedding. At 30 mg/kg bw/day, only grey-brown discolouration of urine and bedding were observed. Urine discolouration was attributed to the test substance (and/or metabolites) but was not considered an adverse effect. At 10 mg/kg bw/day, no effects of treatment with the test item were noted. Based on these results, dose levels of 10, 30 and 150 mg/kg bw/day were selected for this study.
Animals were observed twice daily for clinical signs during the entire treatment period. Body weights were recorded daily. Food consumption was measured over 3-day periods.
At GD 21 all mated females were killed under CO_2-asphyxiation and a complete autopsy and a macroscopic examination of the organs were carried out. The intact uterus (prepared by caesarean section) was removed and the presence of resorption sites (early, late) and foetuses (live or dead) as well as the location of the foetuses in the uterus were examined.

The number of implantation sites and of corpora lutea was determined. Each live foetus was weighed, sexed and examined for gross external malformations. After appropriate processing, either a skeletal or a visceral examination was performed, each with 50 % of the foetuses. In addition, placenta and uterus weights were recorded.

Results
Stability, homogeneity and concentrations of the solutions of 2-amino-hydroxyethylamino-anisole sulfate in the vehicle were analytically confirmed. Dosing solutions were prepared freshly each day, as a sufficient stability for 4 hours was demonstrated. The mean concentrations of the test samples analysed were 102.0 and 99.7 %, 105.4 and 97.9 % as well as 100.3 and 102.2 % of the nominal values for the three dose groups analysed twice at the start and end of the dosing period, respectively. These values confirmed proper dosing for the entire study period.
One animal in the high dose group was found dead on GD 10, having shown poor condition during the preceding days. This was considered to be the result of a dosing error. No other treatment-related clinical observations and post-mortem findings were noted in dams. Urine of the mid and high dose group showed a dark discolouration and thus bedding was stained. At the high dose, a slightly decreased mean food consumption (about 7.4 % reduced compared to the concurrent control group) was observed for the entire treatment period. In parallel, the body weight gain was slightly reduced up to GD 16.
Reproduction data revealed no differences between treated and control groups. At gross necropsy, no treatment related effects were observed. The uterus and placenta weights, the number of corpora lutea, and implantations were similar to control in all treated groups.
There were no treatment related effects with regard to litter size, foetal mortality, foetal body weight and sex ratio. The skeletal and visceral examination of the foetuses revealed no treatment related findings. The observed variations were considered within the spontaneous variation range for the rat strain used and were not dose-dependent. No statistically significant differences in any malformation were noted compared with the concurrent control.
Conclusion
In this rat teratogenicity study reduced food consumption and body weight gain were noted in dams of the high dose group (150 mg/kg bw/day) during GD 6 to 20, indicating maternal systemic toxicity of the test item. No treatment-related effects were noted in foetuses up to the highest test dose of 150 mg/kg bw/day. Thus, a NOAEL of 30 mg/kg bw/day for maternal effects and a NOAEL of 150 mg/kg bw/day for embryo-foetal effects were deduced for 2-amino-hydroxyethylamino-anisole sulfate.

Ref.: 33

In a former dossier submission, a teratogenicity study in mated female Sprague Dawley rats was described that was performed with 2-amino-hydroxyethylamino-anisole sulfate in 1981. The test substance was orally administered from GD 6 to 15 of gestation at doses of 0, 150 and 350 mg/kg bw/day, analysis was performed on GD 19. At 350 mg/kg bw/day, the body weight gain of dams was below mean values during the entire treatment period, and compared to the concurrent control group, a trend towards an increased rate of skeletal variations was noted. Based on the findings noted, 150 mg/kg bw/day was deduced as NOAEL for both maternal toxicity and embryotoxicity. As the protocol used in this study is no longer accepted and the material used was not specified, the study is not presented here in detail.

3.3.9. Toxicokinetics

Taken from SCCP/0958/05

In vitro

Guideline: /
Cells: Human intestinal epithelial cell line TC-7

Test substance: 2-amino-hydroxyethylamino-anisole sulfate
Batch: 57
Purity: 99.6 area% (HPLC, 254 nm)
Test concentration: 50 µM in HBSS buffer containing 1 % DMSO
Incubation time: 60 min
Ref. compounds: Propranolol, ranitidine and vinblastine
Number of runs: Two independent experiments
GLP: Not in compliance, but QAU checked

The bioavailability of 2-amino-hydroxyethylamino-anisole sulfate across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37°C in 96-well transwell plates with shaking for a 60 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 4µM) was used to demonstrate the integrity of the cell monolayer. Only monolayers revealing a permeability of < 2.5 x 10^{-6} cm/sec were used. Propranolol, ranitidine and vinblastine 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. Ranitidine, which has a 50 % absorption in humans, was used as low permeability reference compound, as recommended by FDA. Propranolol (P_{app} = 25.9 x 10^{-6} cm/sec) is a high permeability reference compound with 90 % absorption in humans.

Results
Propranolol and ranitidine (P_{app} = 0.2 x 10^{-6} cm/sec) were well within the acceptance range for compounds 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.
2-amino-hydroxyethylamino-anisole sulfate showed a P_{app} of 73.3 x 10^{-6} cm/sec. This was classified as high permeability, indicating a nearly 100% absorption from the gastro-intestinal tract.

Ref.: 34

Comment
The study was not performed under GLP conditions, but a statement of the quality assurance unit of the test facility is included. There is no official guideline for this assay. However, the study was performed according to ECVAM recommendations. The generated data is considered to provide an estimation of the bioavailability of 2-amino-hydroxyethylamino-anisole sulfate after oral administration.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data provided

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data

3.3.11. Human data

No data

3.3.12. Special investigations

No data

3.3.13. Safety evaluation (including calculation of the MoS)

Not applicable

3.3.14. Discussion

Taken from SCCP/0958/05

Physico-chemical specifications
2-Amino-4-hydroxyethylamino-anisole sulfate is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in the test material is not reported. Stability of 2-amino-4-hydroxyethylamino-anisole sulfate in marketed products is not reported. The dye should not be used in combination with nitrosating substances.

Toxicity
A 15 week repeated dose study in rats provided a NOAEL of 15 mg/kg bw/day. In a teratogenicity study, no treatment-related effects were noted in foetuses up to 150 mg/kg bw/day.
A Repeated Dose (28 days) study in guinea pigs gave no indication of systemic toxic effects up to the maximum technically achievable dose of 300 mg/kg bw/day. It is readily absorbed from the gastro-intestinal tract.

Irritation
The cutaneous and mucous membrane tests for irritation are unsatisfactory but suggest mild irritant potential under the test conditions.

Sensitisation
A LLNA indicates that the substance is not allergenic at a concentration of 2% in DMSO. However, the study was not performed correctly since the induction concentrations used were too low. These doses are not suitable for a robust hazard assessment.

Percutaneous absorption
A percutaneous absorption study in rats *in vivo*, where the total amount found in the skin was considered as bioavailable, provided a penetration rate of 12.8 µg/cm^2 of 1-methoxy-2-amino-4-(β-hydroxy-ethyl)- aminobenzene free base, which is equivalent to 19.7 µg/cm^2 for 2-amino-4-hydroxyethylamino-anisole sulfate.
New study, submission IV
It is clear that the study authors did not take into account the SCCP requirements (SCCP/0970/06). With only 8 samples in total from 3 donors (instead of the required minimum of 6 evaluable samples, from each of at least 3 donors) and an incorrect separation of the skin, the dermal absorption cannot be deduced from this study. Moreover, the compound has not been tested separately, only in the presence of hydrogen peroxide and a reaction partner.

Mutagenicity/Genotoxicity

2-Amino-hydroxyethylamino-anisole sulfate induced mutations in an *in vitro* mammalian cell gene mutation assay (MLA) The noted shift towards small colonies indicated a clastogenic potential. This is confirmed by the positive result of the *in vitro* micronucleus test with human lymphocytes. An *in vivo* micronucleus test using intraperitoneal administration and an *in vivo* / *in vitro* UDS test using oral application were negative and indicated that the genotoxic potential seen *in vitro* is not expressed under appropriate *in vivo* test conditions.

4. CONCLUSION

In its opinion SCCP/0958/05 of 28 March 2006, the SCCP requested a 'skin penetration test using 2-amino-4-hydroxyethylamino-anisole sulfate and conforming to current Notes of Guidance for Safety Evaluation'.

However, the study included in the present submission does not follow these SCCP guidelines.

Consequently, opinion SCCP/0958/05 of 28 March 2006 remains unchanged.

5. MINORITY OPINION

Not applicable

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