EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

N,N-bis(2-hydroxyethyl)-*p*-phenylenediamine sulfate

COLIPA N° A50

Adopted by the SCCP
during the 8th plenary meeting of 20 June 2006

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1. BACKGROUND

Submission I for N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate was submitted in March 2003 by COLIPA[1].

The substance is currently regulated as an oxidative hair dye by the Cosmetics Directive (76/768/EC), Annex III, part 1 under entry 8 on the List of substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

According to the current submission II, submitted by COLIPA in July 2005, the N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate is used in a concentration up to 5% in oxidative hair formulations, which provides an on the scalp concentration of 2.5% of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate after mixing with hydrogen peroxide in a 1:1 ratio.

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate safe for use as an oxidative hair dye with a concentration on scalp of maximum 2.5 % taking into account the scientific data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate in oxidative hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate (INCI)

3.1.1.2.	Chemical names

2,2´-[(4-aminophenyl))imino]bis(ethanol) sulphate
Ethanol, 2,2´-[(4-aminophenyl))imino]bis-, sulphate (1:1) (salt)
2-[[(4-aminophenyl)-(2-hydroxyethyl)]amino]ethanol sulphate

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

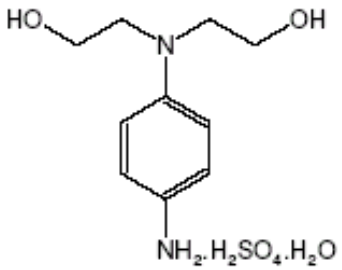
3.1.1.3. Trade names and abbreviations

RM119
COLIPA A50

3.1.1.4. CAS / EINECS number

CAS: 54381-16-7
EINECS: 259-134-5

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{10}H_{16}N_2O_2.\ H_2SO_4.H_2O$

3.1.2. Physical form

White to greyish powder

3.1.3. Molecular weight

Molecular weight : 312.34

3.1.4. Purity, composition and substance codes

Purity and impurities in various batches of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate:

Description	Batch A203G157
Chemical identification and chemical Characterisation	FTIR, NMR, and elemental analysis
HPLC profile, 254 nm (area %, without response factor)	99.9 as monohydrate
Alkalinity titre by potentiometry ($HCLO_4$), % w/w	98.7

Melting point	242.5°C
Phenyldiethanolamine	17±1 ppm
N-nitrosodiethanolamine	<20 ppb
Ash content, % w/w	<0.1
Loss on drying, % w/w	5.6

3.1.5. Impurities / accompanying contaminants

See 3.1.4

As, Sb and Hg:	<5 ppm
Cd:	<10 ppm
Pb:	<20 ppm

3.1.6. Solubility

Water*:	296.4 mg/ml
Ethanol:	0.23-0.35 mg/ml
DMSO:	416-624 mg/ml

* method for solubility measurement and report on solubility measurement are not provided

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow} : -0.771 ± 0.603 (free base), calculated

3.1.8. Additional physical and chemical specifications

Organoleptic properties:	/
Melting point:	163.8°C to 166.4°C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/

3.1.9. Stability

N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate was stable for more than a year when stored at room temperature and protected from light.
N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate in aqueous solutions (0.05 and 100 mg/ml) was stable for 30 days at -20±10°C
N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate solutions (0.1 mg/ml, 0.5 mg/ml and 40 mg/ml) in 0.2% erythorbic acid were stable for 10 days when stored at 5±3°C.

General comments on physico-chemical characterisation

- Stability of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate in marketed products is not reported.
- Log $P_{ow,}$: Calculated values can not be accepted as estimates of the true physical constants without justification, indicating that the reported values are realistic.
- N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate is a tertiary amine, and thus, prone to nitrosation.
- No data is provided on physico-chemical characterisation and purity of the test substance used in several studies

3.2. Function and uses

N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate, an ingredient of oxidative hair colouring products is used at a maximum final (on-head) concentration of 2.5%, after mixing the hair dye formulation with a hydrogen peroxide preparation typically in 1:1 proportions.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	/
Species/strain:	Rat, Tac N (SD)
Group size:	5 males per dose
Test substance:	RM119
Batch:	26290778
Purity:	not reported
Vehicle:	1% methyl cellulose
Observation:	14 days
GLP:	not in compliance

In an acute oral study the test substance (4% suspension in 1% methyl cellulose) was given to groups of 5 male rats at a dose level of 50, 100, 200 and 400 mg/kg (1.25-10 ml/kg). The LD_{50} was calculated to be 107 mg/kg bw. Clinical signs were not reported

Ref.: 3

Guideline:	/
Species/strain:	Rat, Tac N (SD)
Group size:	5 males per dose
Test substance:	RM119
Batch:	26290778
Purity:	not reported
Vehicle:	1% methyl cellulose

Observation: 14 days
GLP: not in compliance

In an acute oral study the test substance (10% suspension in 1% methyl cellulose) was given to groups of 5 male rats at a dose level of 200, 400, 800 and 1600 mg/kg (2-16 ml/kg). The LD_{50} was calculated to be 427 mg/kg bw. Clinical signs were not reported.

Ref.: 4

In reference 2, an acute oral study was reported in male rats (5 animals/dose, strain not specified). The test substance (RM119, no purity was reported) was administered in 10% DMSO at 100, 200, 400 and 800 mg/kg. The LD_{50} was calculated to be 246 mg/kg.

In reference 5, an acute i.p study was reported in male rats (5/dose, strain not specified). The test substance (RM199, no purity, no batch number) was administered in 10% DMSO at 16, 31, 62, 125, 250 and 500 mg/kg. The LD_{50} was calculated to be between 16 and 31 mg/kg.

Conclusion
The oral LD_{50} in 2 (non-guideline) studies after oral administration ranged between 100 and 400 mg/kg bw (the differences between these 2 studies are not clear (mortality at administration of 0.5 ml/kg). After i.p. administration the LD_{50} was between 16 and 31 mg/kg bw.

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

Guideline: /
Species/strain: New Zealand white rabbit
Group size: 6 (2 males and 2 females)
Test substance: RM 119
Batch: 262 90778
Purity: not stated
Doses: 500 mg
Vehicle: / (illegible)
GLP: not in compliance

The skin irritation potential of RM 119 was evaluated in rabbits. A single application of 500 mg of an aqueous slurry of the test substance to shaved intact and abraded skin for 24 hours produced no evidence of irritation at any of the reading times (24 and 72 hours post application).

Conclusion

The test substance was considered to be a non-irritant to the skin.

Ref.: 8

3.3.2.2. Mucous membrane irritation

Guideline: /
Species/strain: rabbit
Group size: 6
Test substance: N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch: 262 90778
Purity: not stated
Doses: 100 mg
Vehicle: /
GLP: not in compliance

The irritant effect of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate was studied in rabbits. The 100 mg of test substance was instilled into the rabbit eye and reactions were checked for 4 days. Positive reactions were obtained in all 6 rabbits.

Conclusion
N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate is irritant for the rabbit eye.

Ref.: 6

Guideline: /
Species/strain: rabbit
Group size: 6
Test substance: N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch: 262 90778
Purity: not stated
Doses: 0.1 ml
Concentration: 1% w/v in propylene glycol
GLP: not in compliance

The irritant effect of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate was studied in rabbits. The 0.1 ml of substance was instilled into the rabbit eye and reactions were checked for 4 days. No positive reactions were obtained in all 6 rabbits.

Conclusion
N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate is non- irritant for the rabbit eye.

Ref.: 7

Comment
The test concentration is lower than the requested use concentration.

3.3.3. Skin sensitisation

Guideline: /
Species/strain: albino guinea pigs of the Hartley strain
Group size: 10
Test substance: RM-119 (N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate)
Batch: 26290778
Purity: 3% solution
Doses: 0.5 ml
Vehicle: Schultz Hamburg Vehicle II
Dosing period: 4 weeks
GLP: not in compliance

An area of approximately 25 cm² on the left flank of each animal was clipped free of hair with a small animal electric clipper. A 3% solution of the test material in Schultz Hamburg Vehicle II was prepared fresh daily. A 0.5 ml aliquot of the appropriate solution was applied to the clipped left flank of each animal and gently spread with a glass rod to cover an area of about 6.5 cm². Each animal was dosed five days per week for three weeks. Three additional doses were given the fourth week for a total of 18 doses. Two weeks after the final application of each the test materials the opposite (right) flank of each animal was clipped free of hair. A single 0.5 ml application of the test material was made to this site to serve as a challenge. This challenge site was observed and scored for irritancy by the method of Draize at 24, 48, and 72 hours after application of the test material.
The challenge sites were depilated, gently washed and the animal was allowed to rest for several hours prior to the first scoring (this procedure allowed for removal of residual hair and dye and facilitated evaluation of the challenge reactions).

Results
Three animals died during the treatment period with respiratory conditions or diarrhoea.
The Draize irritation scores for the challenge with RM-119; positive reactions were noted in 5 of the 7 surviving animals. All scores were 1 for erythema except for a single score of 2 at 72 hours. The animals treated with RM-119 exhibited moderate irritation after several applications during the dosing period.

Conclusion
Under the conditions of this test procedure, it may be concluded that the test material is a sensitizer.

Ref.: 9

Comment
The study is considered inadequate. It did not follow current guidelines.

Guideline: /
Species/strain: albino guinea pigs Hartley
Group size: 15 females
Test substance: N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate, purified KB 5706-36
Batch: 2360981

Purity:	not stated
Doses:	0.5 ml of a 3% solution
Vehicle:	Schultz Vehicle # II
GLP:	not in compliance

The purpose of this study was to determine the potential of purified N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate to cause skin sensitization in the albino guinea pig and to explore the possibility of cross sensitivity to PPD. The method employed was the Schultz Method Modified (Hamburg 24:5, 1976).

A 0.5 ml aliquot of the test solution was applied to the clipped left flank of each animal and spread with a glass rod to cover an area of about 6.5 cm². Each animal was dosed five days per week for three weeks. Three additional doses were given the fourth week for a total of 18 doses. The test area was shaved when necessary to assure contact of the test material with the skin. The animals were observed daily and detailed observations and body weight changes were recorded weekly during the dosing period.
Two weeks after the final application of the test material the opposite (right) flank of each animal was clipped free of hair as above. A single 0.5 ml application of the test material prepared as above was made to this site to serve as a challenge. This challenge site was observed and scored for erythema and oedema by the method of Draize at 24, 48 and 72 hours after application. Five additional female guinea pigs received a single application of the test material, at the time of the challenge, to act as controls to assure that any reaction noted was not due to skin irritation.
All test animals were challenged a 2nd time on the upper left flank 2 weeks after the initial challenge. 12 days after the second challenge, each animal was challenge on the dorsal mid-line with PPD and observed for 72 hours. After the 48 hour reading of PPD all animals were challenged a 3rd time with N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate on the upper right flank.
Five additional female guinea pigs used as controls for the first challenge were used also for the 2nd and 3rd challenge with N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate and the single challenge of PPD.

Results
All test animals developed slight skin irritation after several applications of the test material. A reddish brown discoloration of the skin was noticed. One animal developed well defined redness after 1 week followed by eschar and thickening of the skin. One animal died prior to challenge. Death was due to acute pericarditis. The number of positive reactions given for challenges 1, 2 and 3 with N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate were 6, 9 and 7 respectively. Challenged animals with slight redness were considered to have skin irritation reactions since several control animals developed slight redness at time of application. The challenge reaction with PPD was negative. One animal developed well defined redness after the challenge application with PPD. This single animal appeared to be highly reactive to all treatments.

Conclusion
Under the conditions of this test procedure it may be concluded that a dose of 3% N,N-bis(2-hydroxyethyl)-p-phenylenediamine does induce delayed hypersensitivity of the skin in guinea pigs. It may also be concluded that a single 3% challenge of PPD does not induce a cross sensitivity reaction in these same animals.

Ref.: 10

Comment
The study is considered inadequate. It did not follow current guidelines.

Guideline: /
Species/strain: albino guinea pigs Hartley
Group size: 300
Test substance: N,N-bis(2-hydroxyethyl)-ppd
Batch: S4050588
Purity: Doses: 0.5 ml 3% solution
Vehicle: Schultz Vehicle # II
GLP: not in compliance

The method employed was the Schultz Method Modified (SP-12R04) (Hamburg 24.5, 1976).

A 0.5 ml aliquot of the test solution was applied to the clipped left flank of each animal and spread with a glass rod to cover an area of about 6.5 cm². Each animal was dosed daily, five days per week for three weeks for a total of 15 doses. Each animal was observed after the initial induction dose and scored according to the Draize method. Each animal acted as its own control to assure that the challenge reaction noted was not due to skin irritation. The number of reactors for each test material was recorded. The reacting test materials were ranked in order of reactivity.

Results
The number of reactors and the primary irritation index was:

	# of reactors	PII
N,N-bis(2-hydroxyethyl)-ppd	5/15	0.38
ppd	15/15	2.42

Conclusion
Under the conditions of this test procedure it may be concluded that a 3% concentration of N,N-bis(2-hydroxyethyl)-ppd and a 3% concentration of PPD does induce delayed hypersensitivity of the skin of guinea pigs.

Ref.: 11

Comment
The study is considered inadequate. It did not follow current guidelines.

Ref.: 12 - illegible (written by hand)

Local Lymph Node Assay (LLNA)

Guideline: /
Species/strain: mouse – CBA/Ca
Group size: 24 females
Test substance: TM#1613 (N,N-bis(2-hydroxyethyl)-p-phenylenediamine)

Batch: /
Purity: /
Doses: 0.25, 0.5, 1.0 or 2.0% (w/v)
Vehicle: DMSO
GLP: not in compliance

The study included 4 hair dyes.
CBA/Ca female mice were treated on the dorsal surface of both ears once per day for 3 days with 0.25, 0.5, 1.0 or 2.0% (w/v) of TM#1613, positive control, p-phenylenediamine (PPD), or the vehicle, DMSO. On Day 5 the mice were injected with 20 µCi of ^{3}H-thymidine. Five hours later the mice were euthanized and the draining auricular lymph nodes were removed. The lymph node cells were precipitated with 5% trichloroacetic acid (TCA) and the pellets counted in a β-scintillation counter to determine incorporation of the ^{3}H-thymidine.
The positive control, PPD, at 0.25, 0.5, 1.0 and 2.0% resulted in test/control ratios of 3.23, 4.37, 6.47 and 17.61, respectively. Ratios greater than 3.0 represent a positive response. Therefore, PPD was positive at all concentrations tested. TM#1613 was positive at all concentrations tested. The stimulation index for test substance at 0.25, 0.5, 1.0 and 2.0% concentration were 3.90, 3.76, 10.82 and 6.42 respectively.

Conclusion
The results of this assay show that TM#1613 induced a hypersensitivity response in mice.

Ref.: 13

Guideline: /
Species/strain: mice, CBA/Ca01aHsd, female
Group size: 25
Test substance: N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch: 7492CL055
Purity: 94.1% (NMR)
Doses: 0.5, 1.5 and 2.8%
Vehicle: Acetone/Aqua/Olive Oil (AAOO)
GLP: in compliance

Each mouse was treated by topical application with the selected solution (25 µl) to the entire dorsal surface of each ear once daily over three consecutive days. Five days after the first topical application, all mice were injected intravenously with ^{3}H-methyl thymidine. Approximately 5 hours after ^{3}H-methyl thymidine-injection, all mice were sacrificed and the draining "auricular lymph nodes" were excised, in order to prepare single cell suspension of the lymph node cells. The 3H-methyl thymidine - incorporation was measured in a β-counter. Determination of radioactivity was performed individually for each animal. The proliferative response of lymph node cells was calculated as the ratio of 3H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals. A stimulation index, ratio of test substance / vehicle control, was calculated for each concentration.

Results
The stimulation index at a concentration of 0.5% was 2.4
The stimulation index at a concentration of 1.5% was 1.9
The stimulation index at a concentration of 2.8% was 2.7
The stimulation index for the positive control (1% p-Phenylenediamine) was 5.3.

Conclusion
Under the conditions of the test, the EC3 values ranged from 1.9 to 2.7, which are just below the limit of a positive response (EC3=3).

Comment
The vehicle AAOO appears to be the reason for negative result, because positive results were obtained when DMSO was used as vehicle (Ref 15).
Acetone/Aqua/Olive Oil (AAOO) is not a preferred vehicle. Its use was not explained. The range of test concentrations used was narrow. Also, higher concentrations should have been used.

Ref.: 14

Guideline:	/
Species/strain:	mice CBA/Ca 01aHsd
Group size:	25
Test substance:	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch:	7492CL055
Purity:	94.1% (NMR)
Doses:	0.5, 2.5 and 5.0%
Vehicle:	DMSO
GLP:	in compliance

Each mouse was treated by topical application with the selected solution (25 µl) to the entire dorsal surface of each ear once daily over three consecutive days. Five days after the first topical application treatment all mice were injected intravenously with ^{3}H-methyl thymidine. Approximately 5 hours after ^{3}H-methyl thymidine-injection all mice were sacrificed and the draining, auricular lymph nodes" were excised, in order to prepare single cell suspension of the lymph node cells. The ^{3}H-methyl thymidine incorporation was measured in a β-counter. Determination of radioactivity was performed individually for each animal. The proliferation response of lymph node cells was calculated as the ratio of ^{3}H-methyl thymidine - incorporation into lymph node cells of test group animals relative to that recorded for control group animals. A stimulation index, ratio of test substance / vehicle control, was calculated for each concentration.

Results
The stimulation index at a concentration of 0.5% was 0.7
The stimulation index at a concentration of 2.5% was 9.1
The stimulation index at a concentration of 5.0% was 9.7
The stimulation index for the positive control (1% P-Phenylenediamine) was 10.1.
Calculated EC3 value (derived by linear interpolation) is at a concentration of 1.04 % N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate.

Conclusion
The test item caused reactions identified as sensitization up from a concentration of 1.04 % N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate, where the stimulation index was equal to 3.0.
Based on this test, the substance can be categorised as a strong sensitiser.

Ref.: 15

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428
Species/strain:	human dermatomed skin membranes
Group size:	12 membranes from 8 donors, membrane integrity by the measurement of electrical resistance across the membrane
Test substance:	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate, monohydrate and ^{14}C-N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch:	A203G157 (unlabelled) and Lot 228-161-080 (^{14}C-labelled)
Purity:	100.4% (unlabelled) and 99.4% radiochemical purity (^{14}C-labelled)
Vehicle:	1:1 w/w mixtures with two different developers (a peroxide developer and a placebo developer), both resulting in a nominal final dose concentration of 2.5% N,N-bis(2-hydroxyethyl)-p-phenylenediamine-sulphate monohydrate.
Doses:	20 mg/cm^2, exposed area 2.54 cm^2
Receptor fluid:	Phosphate buffered saline
GLP:	in compliance

The penetration and distribution of N,N-bis(2-hydroxyethyl)-p-phenylenediamine from a nominal 5% w/w (as the sulphate monohydrate) formulation was measured *in vitro* through human skin, following the incorporation of [^{14}C]-N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate. The formulation was applied as 1:1 w/w mixtures with two different developers (a peroxide developer and a placebo developer), both resulting in a nominal final dose concentration of 2.5% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate, monohydrate.

The mixed formulations were applied to 12 human dermatomed skin membranes (nominally 400µm thick), mounted in glass diffusion cells, at a nominal rate of 20mg/cm^2. After a contact period of 30 minutes, the dose was washed from the surface of the skin using natural sponges soaked in 3% Teepol. Samples of the receptor fluid (phosphate buffered saline) were taken at recorded intervals over a 48h period, during which time the applications remained unoccluded. At the end of the experiment, the surface of the skin was washed again and layers of stratum corneum removed using a tape stripping technique. The receptor fluid samples, sponges, tape strips, residual skin and donor chambers were analysed for radioactivity, which was representative of the N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate content. Penetration rates and distribution of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate in the test system were calculated.

Results

Peroxide developer mix

The overall mean recovery of the test substance during this experiment was 97.5%. The penetrated amount of the test substance (receptor fluid + dermis/epidermis) at the end of the experiment (48h) was 0.108 ± 0.064 µg/cm^2 (range 0.037-0.252 µg/cm^2) corresponding to 0.031±0.014% dermal absorption of the applied dose.

Placebo developer mix

The overall mean recovery of the test substance during this experiment was 102%. The penetrated amount of the test substance (receptor fluid + dermis/epidermis) at the end of the experiment

(48h) was 0.188 ± 0.242 μg/cm^2 (range 0.049-0.497 μg/cm^2) corresponding to 0.054±0.07% dermal absorption of the applied dose.

Conclusions
The maximum observed dermal absorption in the presence of developer mix, i.e. 0.252 μg/cm^2, will be used for the calculation of margin of safety

Ref.: 17

3.3.5. Repeated dose toxicity

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

14 day range-finding study

Guideline:	OECD 407(1981)
Species/strain:	Rat, Crl:CD(SD)IGS BR
Group size:	5 per dose and sex
Test substance:	A50, GTS03849
Batch:	A203G157
Purity:	100.4%
Doses:	0, 50 (5), 100 (25), 200, 400 mg/kg bw/day oral gavage
Vehicle:	0.2% w/v erythorbic acid in reverse osmosis water
Dosing period:	14 days
GLP:	in compliance

Male and female Crl:CD (SD)IGS BR rats were assigned to five groups. Each group received dose preparations containing the vehicle or 50, 100, 200, or 400 mg of A50/kg of body weight/day (mg/kg/day) at a dose volume of 10 ml/kg. Due to toxicity and mortality in the two highest dose groups, animals were not dosed on Day 3. Dosing resumed on Day 4 with a reduction in dose levels in the two lowest dose groups from 50 to 5 mg/kg/day and 100 to 25 mg/kg/day (Groups 2 and 3). The animals were observed twice daily for mortality, abnormalities, and signs of pain or distress. Clinical observations, body weights, and food consumption were performed/recorded regularly.
Blood and urine was analysed for haematology, coagulation, clinical chemistry, and urinalysis. At necropsy, macroscopic observations were recorded and selected organs were weighed.

Results
All animals given 200 or 400 mg/kg/day and two given 100 mg/kg/day died or were sacrificed in a moribund condition.
Test article-related clinical observations for surviving animals given 100/25 mg/kg/day included thin appearance, mild tremors, few faeces, and rough hair coat for males and females and ocular fasciculation and swaying gait for females only. There were no remarkable clinical observations for control animals or animals given 50/5 mg/kg/day.
There was a decrease in mean body weights and an increase in kidney weight in animals in the 100/25 mg/kg group. No adverse effects were observed in the 50/5 mg/kg bw/day group, and therefore the NOAEL in this study was 5 mg/kg bw/day.

Ref: 18

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

Guideline:	OECD 408
Species/strain:	Rat, Crl:CD(SD)IGS BR
Group size:	15 animals per dose and sex
Test substance:	A50, GTS03849
Batch:	A203G157
Purity:	100.4%
Doses:	0, 1, 4 or 20 mg/kg bw/day oral gavage
Vehicle:	0.2% w/v erythorbic acid in reverse osmosis water
Dosing period:	91 days
GLP:	in compliance

Groups of rats (15 males and 15 females) were exposed to the test substance at dose levels of 1, 4 or 20 mg/kg bw/day by oral gavage once daily for 91 days. An additional group of 5 animals/sex of the control and high dose group was exposed for 91 days and was observed during a 4 week recovery period.
The animals were observed twice daily for mortality. Detailed clinical and neurobehavioral observations were performed weekly. Motor activity data and ophthalmic examinations were done in week 13. Body weights and food consumption data were measured weekly. Vaginal cytology data were collected once daily for 21 consecutive days, beginning after Week 10. Blood and urine samples for haematology, coagulation, clinical chemistry, urinalysis, and urine chemistry were collected at each scheduled sacrifice. At each necropsy, macroscopic observations were recorded, selected organs weighed, and selected tissues collected and preserved. Sperm was collected and analysed for motility, morphology and total counts.

Results
Up to the highest dose level, no adverse effects were noted after administration of the test substance for 91 days, and therefore the NOAEL in this study was 20 mg/kg bw/day.

Comment
According to OECD 408, the highest dose level should induce toxicity.

Ref.: 19

3.3.5.3. Chronic (> 12 months) toxicity

See point 3.3.7. Carcinogenicity

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, and TA1537, *Escherichia coli* WP2*uvr*A(pKM101)

Replicates:	In the initial trial, duplicates were investigated per test concentration. In the confirmatory trial, triplicates were investigated per test concentration.
Assay conditions:	Pre-incubation assay with and without S9 mix from rat livers (Aroclor™ induced)
Test substance:	GTS03849
Batch:	A203G157
Purity:	100.4% (as stated in study report)
Concentrations:	2.50 – 5000 µg/plate with and without metabolic activation
GLP:	in compliance

The ability of the test substance to induce reverse mutations in the presence and absence of Aroclor-induced rat liver S9 was tested at the histidine locus in several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2*uvr*A(pKM101). The assay was conducted with concurrent vehicle and positive controls using two according to OECD guidelines. The doses tested in the initial mutagenicity assay, using the preincubation method, were 2.50 - 5000 µg per plate in both the presence and absence of S9 mix. The results of the initial mutagenicity assay were confirmed in an independent experiment, using the preincubation method, at 200-5000 µg per with and without S9 mix.

Results
In the initial mutagenicity assay, positive increases in the mean number of revertants per plate were observed with tester strains TA100 (2.2-fold) and WP2*uvr*A(pKM101) (2.2-fold) in the absence of S9 mix. No increases in the mean number of revertants per plate were observed with any of the other tester strains. In the confirmatory mutagenicity assay, a 2.1-fold increase in the mean number of revertants per plate was observed with tester strain WP2*uvr*A(pKM101) in the absence of S9 mix. No increases in the mean number of revertants per plate were observed with any of the other tester strains.

Conclusion
Under the conditions of this study, the test substance increased revertant count in *E. coli* strain WP2*uvr*A(pKM101) without S9 activation and is therefore evaluated positive in this assay. In *Salmonella* strain TA100, increases that met the criteria for a positive response without S9 activation were seen in one trial, but the response was not duplicated in subsequent trials.

Ref.: 21

***In vitro* chromosome aberration test**

Guideline:	OECD 473 (1997)
Species/strain:	Chinese hamster ovary cells (CHO-WBL)
Replicates:	Duplicate cultures per concentration
Test substance:	GTS03849
Batch:	A203G157
Purity:	100.4% (as stated in study report)
Concentrations:	1.88 – 60.0 µg/ml without (4 hour), 1.88 – 60.0 µg/ml without (~20 hour), and 50.0 - 700 µg/ml with metabolic activation
GLP:	in compliance

The objective of this *in vitro* assay was to evaluate the potential of the test substance and its metabolites to induce structural and numerical chromosomal aberrations in cultured Chinese

hamster ovary (CHO) cells with and without an exogenous metabolic activation system. The highest concentration tested in the assay was 5000 µg/ml, a dose above the solubility limit. Concentration selection was based on preliminary toxicity assessment. Vehicle control and positive control were included according to OECD guidelines.

In the initial toxicity assay, concentrations 0.167, 0.500, 1.67, 5.00, 16.7, 50.0, 167, 500, 1670, and 5000 µg/ml were tested in single cultures of CHO cells. Treatment period was for 4 hours with and without metabolic activation or ~20 hours without metabolic activation, and cultures were harvested ~20 hours from the initiation of treatment. Doses were selected for the confirmatory assay based on the initial assay. In the confirmatory assay, the treatment period was for 4 hours with and without metabolic activation or ~20 hours without metabolic activation, and cultures were harvested ~20 hours from the initiation of treatment. Concentrations of 1.88, 3.75, 7.50, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 55.0, and 60.0 µg/ml were tested for 4 hours and ~20 hours without metabolic activation and concentrations of 50.0, 100, 200, 250, 300, 350, 375, 400, 425, 450, 475, 500, 550, 600, and 700 µg/ml were tested for 4 hours with metabolic activation.

Results
Significant increases in cells with chromosomal aberrations were observed in cultures treated with 200 and 250 µg/ml of the test substance 4 h in the presence of metabolic activation. Significant increases in cells with endoreduplication were also observed in all the cultures analyzed with metabolic activation. No positive effects were seen without metabolic activation. The increase in total cells with numerical aberrations was within historical control values so this is not considered biologically relevant.

Conclusion
The test substance induced chromosomal aberrations in the presence of metabolic activation in Chinese hamster ovary (CHO) cells. Treatment with metabolic activation was also associated with an increase in endoreduplications.

Ref.: 22

In Vitro Mouse Lymphoma (L5178Y *tk*+/-) Mutation Assay

Guideline:	OECD 476 (1997)
Species/strain:	Mouse lymphoma cell line L5178Y *tk*$^{+/-}$
Group size:	Single cultures per concentration, 5 to 8 concentrations analyzed with and without S9 mix; second independent experiment, 8 concentrations analyzed without activation (verification)
Test substance:	GTS03849
Batch:	A-203G157
Purity:	100.4% (as stated in study report)
Concentrations:	1.00 – 10.00 µg/ml without (second experiment 2.50 – 4.50 µg/ml) and 20.0 – 100 µg/ml with metabolic activation.
Vehicle:	Water
GLP:	In compliance

The potential of the test substance to induce forward mutations at the thymidine kinase (*tk*) locus was tested in cultured L5178Y *tk*$^{+/-}$ mouse lymphoma cells with and without metabolic activation. Selection of concentrations was based on preliminary toxicity tests. Negative and

positive controls were included according to the OECD guidelines. Based on the results of the toxicity test, four independent experiments were carried out without metabolic activation; the initial mutagenicity trial was evaluated using five concentrations ranging from 1.00 to 7.50 µg/ml, the repeat of the initial mutagenicity trial was evaluated using eight concentrations ranging from 3.00 to 10.00 µg/ml, the final initial mutagenicity trial was evaluated using eight concentrations ranging from 4.00 to 9.00 µg/ml and the confirmatory mutagenicity trial was evaluated using eight concentrations ranging from 2.50 to 4.50 µg/ml. An independent experiment with metabolic activation was carried out using eight concentrations ranging from 20.0 to 100 µg/ml. Single cultures were investigated for each concentration and test group. Mutant frequency and cell survival (measured as relative total growth) were determined. In addition to the numbers of mutant colonies, the size of the colonies was determined and the ratio of small versus large colonies was calculated.

Results

In the initial mutation assay without metabolic activation with a 4-hour treatment period, five treatments ranging from 1.00 to 7.50 µg/ml were analyzed for mutant induction and no toxicity to moderately high toxicity was induced (98.7% to 34.2% relative total growth). The background mutant frequency for the vehicle control in the initial nonactivation assay was 87.9 x 10^{-6}, therefore a mutant frequency greater than or equal to 177.9 x 10^{-6} was required for a treatment to be evaluated as exhibiting a positive response. Mutant frequencies for the analyzed treatments ranged from 49.1 to 123.3 x 10^{-6}. None of the analyzed treatments induced a mutant frequency that met criteria for a positive response. However, since sufficient toxicity was not achieved, the assay was repeated. In the repeat of the initial mutation assay without metabolic activation with a 4-h treatment period, eight treatments ranging from 3.00 to 10.00 µg/ml were analyzed for mutant induction and no toxicity to high toxicity was induced (94.4% to 17.6% relative total growth). The background mutant frequency for the vehicle control in the repeat of the initial nonactivation assay was 62.1 x 10^{-6}, therefore a mutant frequency greater than or equal to 152.1 x 10^{-6} was required for a treatment to be evaluated as exhibiting a positive response. Mutant frequencies for the analyzed treatments ranged from 60.8 to 102.2 x 10^{-6}. None of the analyzed treatments induced a mutant frequency that met criteria for a positive response. However, the assay was repeated because the concentration verification results fell outside the acceptable range. In the final initial mutation assay without metabolic activation with a 4-h treatment period, eight treatments ranging from 4.00 to 9.00 µg/ml were analyzed for mutant induction, and weak to high toxicity was induced (68.6% to 11.5% relative total growth). The background mutant frequency for the vehicle control in the final initial nonactivation assay was 39.8 x 10^{-6}, therefore a mutant frequency greater than or equal to 129.8 x 10^{-6} was required for a treatment to be evaluated as exhibiting a positive response. Mutant frequencies for the analyzed treatments ranged from 42.2 to 72.2 x 10^{-6}. None of the analyzed treatments induced a mutant frequency that met criteria for a positive response. In the confirmatory nonactivation mutation assay with a 24-h treatment period, eight treatments ranging from 2.25 to 4.50 µg/ml were analyzed for mutant induction, and weak to high toxicity was induced (76.3% to 10.7% relative total growth). The background mutant frequency for the vehicle control in the confirmatory nonactivation assay was 84.6 x 10^{-6}, therefore a mutant frequency greater than or equal to 174.6 x 10^{-6} was required for a treatment to be evaluated as exhibiting a positive response. Mutant frequencies for the treatments analyzed ranged from 81.8 to 122.6 x 10^{-6}. None of the analyzed treatments induced a mutant frequency that met criteria for a positive response. In the initial activation mutation assay with a 4-h treatment period, eight concentrations ranging from 20.0 to 100 µg/ml were analyzed for mutant induction, and weak to high toxicity was observed (75.8% to 9.7% relative total growth). The background mutant frequency for the vehicle control was 55.5 x 10^{-6}, therefore a mutant frequency greater than or equal to 145.5 x 10^{-6} was required for a treatment to

be evaluated as exhibiting a positive response. Mutant frequencies for the treatments analyzed ranged from 56.4 to 93.4 x 10^{-6}. None of the analyzed treatments induced an increase in the mutant frequency that met the criteria for a positive response. A confirmatory assay was not required. Mutant colonies from all the cultures showed the expected bimodal distribution.

Conclusion
The test substance was evaluated as negative with and without metabolic activation for inducing forward mutations at the *tk* locus in L5178Y mouse lymphoma cells, under the conditions of this assay

Ref.: 23

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474 (1997)
Species/strain:	Mice, CD-1®(ICR)BR
Group size:	5 males per dose group and per sacrifice time (including vehicle and positive control); satellite group of 15 males for toxicokinetics (high dose only)
Test substance:	GTS03849
Batch:	A203G157
Purity:	100.4 %
Dose level:	15.625, 31.25, 62.5 mg/kg bw administrated as single dose
Route:	Oral gavage
Vehicle:	Reverse osmosis water
Sacrifice times:	24 and 48 hours (vehicle and high dose only)
GLP:	In compliance

The test substance was examined for micronucleus induction in mouse polychromatic erythrocytes *in vivo*. In the dose range-finding study, the test substance was formulated in reverse osmosis water and administered once by oral gavage to three males and three females per dose group. The animals were dosed at 62.5, 125, 250, 500, or 1000 mg/kg and observed for up to 2 days after dosing for toxic signs and/or mortality. Mortality was observed in 2/3 males and 3/3 females in the 125 mg/kg dose group and in 3/3 males and 3/3 females in the 250, 500, and 1000 mg/kg dose groups. A clinical sign of slight hypoactivity was observed in all animals in the 62.5 mg/kg dose group at the 3- to 3.5-hour and 4-hour observation intervals. Based on the results of the dose range-finding assay, the test agent was administered once in reverse osmosis water at doses of 15.625, 31.25 and 62.5 mg/kg to groups of five male mice per bone marrow sampling time. Five animals from all the groups were sacrificed 24 hours after dosing and from the vehicle control and high dose group at 48 hours after dosing. Plasma samples were collected from groups of three animals in the high dose group at 1, 2, 4, 6, 8, 24 and 48 hours after dosing for detection of the test article. For all groups at 24 hours and vehicle and high dose group only at 48 hours, bone marrow was extracted and at least 2000 PCEs per animal were subsequently microscopically analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 1000 total erythrocytes for each animal.

Results

In the definitive assay, the test article induced a clinical sign of slight hypoactivity in the treated animals at 62.5 mg/kg. It did not induce statistically significant increases in micronucleated PCEs at any test article dose examined (15.625, 31.25, and 62.5 mg/kg). In addition, the test substance was not cytotoxic to the bone marrow (i.e., no statistically significant decreases in the % PCEs at any dose of the test article. Although there were no indications of bone marrow toxicity in the present study, the oral bioavailability of the test substance was evident based on the clinical signs seen at the 62.5 mg/kg dose in the definitive study and by the deaths and clinical signs seen at 125 mg/kg and above in the dose range-finding study. Moreover, in a 14-day oral gavage study in rats, a change in blood parameters was observed at a dose of 100/25 mg/kg/day, building upon the weight of evidence for systemic exposure of the test article in the *in vivo* micronucleus study.

Conclusion
The test substance was not genotoxic in the *in vivo* micronucleus assay to mice after a single oral gavage conducted up to a maximum tolerated dose.

Ref.: 24

Rat liver *in vivo/in vitro* UDS assay

Guidelines:	OECD 486 (1998)
Species/Strain:	8 to 10 week old male Sprague-Dawley rats.
Replicates:	Three rats evaluated, three slides scored per rat per dose level, vehicle and positive controls at 2 to 4 hours and 12 to 16 hours of exposure.
Animals per dose:	3
Assay conditions:	Single oral gavage at 10 ml/kg bw dosing volume.
Test Substance:	GTS03849
Batch:	A203G157
Purity:	100.4%
Concentrations:	UDS assay: 100 and 200 mg/kg bw
GLP:	In compliance

The ability of the test substance to induce DNA damage was assessed in the *in vivo* unscheduled DNA synthesis (UDS) test in hepatocytes of rats. In all phases of the study, test and control articles were administered as a single dose at a constant volume of 10 ml/kg bw by oral gavage. Deionized distilled water was used as the vehicle. The high dose for the UDS assay was set at the maximum tolerated dose which was estimated to be 200 mg/kg bw in male Sprague-Dawley rats. Vehicle control and positive control groups were included according to the OECD guidelines.

Results
The 100 mg/kg bw 12 to 16 hour-treated animals were observed to be normal immediately following dosing but one animal appeared to have a crusty nose prior to harvest. The 200 mg/kg bw 12 to 16 hour treated animals were observed to be normal immediately following dosing and were observed to have crusty eyes and crusty noses prior to harvest. One 200 mg/kg bw 12 to 16 hour-treated animal was found dead at time of harvest and was not considered for use in the UDS assay. The group mean net nuclear grain (NG) counts for animals treated with the test agent were not increased when compared with the vehicle control. For the 2 to 4 hour time point, the group mean NG counts for the test article-treated animals were -0.2 and 0.2 with 1% and 3% of cells in repair (cells with ≥ 5NG) for the 100 and 200 mg/kg bw, respectively. The group

mean NG count for the vehicle control group was -0.1 with 2% of cells in repair. For the 12 to 16 hour time point, the group mean NG counts for the test article-treated animals were -0.1 and 0.0 with 1% and 1% of cells in repair (cells with ≥ 5 NG) for the 100 and 200 mg/kg bw, respectively. The group mean NG count for the vehicle control group was -0.3 with 1% of cells in repair.

Conclusion
The test substance did not induce a significant increase in the mean number of net nuclear grain counts (i.e., an increase of at least 5 counts over the vehicle control group) in hepatocytes isolated either 2 to 4 hours or 12 to 16 hours after dose administration. It was concluded to be negative in the *in vivo* unscheduled DNA synthesis (UDS) test in rats.

Ref.: 25

3.3.7. Carcinogenicity

N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate alone

Induction of γ-glutamyl transpeptidase-positive foci in rat liver

Guideline:	/
Species/strain:	Male F344/DuCrj rats
Group size:	25 Animals, negative control group 50 animals
Test substance:	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch:	Lot no 3 (Lowenstein Dyes Cosmetics, Inc, USA)
Purity:	92.9%
Dose level:	110, 330, and 1000 ppm N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate in the diet
Route:	Oral
Exposure:	6 weeks
GLP:	not in compliance

The effect of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate on liver carcinogenesis was investigated in male F344 rats initially treated with N-nitrosodiethylamine (DEN). Two weeks after a single dose of DEN (200 mg/kg, intraperitoneally) (6 weeks old rats at the commencement of the experiment), the rats were given N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate at dietary levels of 110, 330 and 1000 ppm for 6 weeks. At week 3 following the DEN treatment, all animals were subjected to 2/3 partial hepatectomy. Positive control: 600 ppm 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) in the diet for 6 weeks. All survivors were sacrificed under anaesthesia for examination at week 8.

No adverse effects on survival and body weight were seen even at the highest dietary level of the test substance. The treatment did not affect the relative liver weight. Marked growth retardation and increased liver weight were found in rats given 3'Me-DAB. N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate did not significantly increase the level of γ-glutamyl transpeptidase-positive foci observed after DEN initiation. Increased levels were found after treatment with the positive control 3'-Me-DAB.

Ref.: 26

Oral administration, rat

Guideline:	/
Species/strain:	F344/DuCrj rats
Group size:	50 Animals per sex and dose
Test substance:	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch:	Lot no 6 (Hoyu Co Ltd, Nagoya, Japan)
Purity:	92.9%, 98.7% after drying
Dose level:	0 (control) 300, 1000, and 3000 ppm in the diet
Route:	Oral
Exposure:	104 weeks
GLP:	not in compliance

Fischer 344 rats, groups of 50 males and 50 females (6 weeks old), were exposed to 0 (control) 300, 1000 or 3000 ppm N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate for 104 weeks. Daily average intake 300 ppm, males 12 mg/kg/d; females 14 mg/kg/d, 1000 ppm, males 40 mg/kg/d; females 48 mg/kg/d, and 3000 ppm, males 121 mg/kg/d; females 146 mg/kg/d. All surviving animals were sacrificed after 104 weeks. The animals were observed daily for abnormalities. Individual body weights were recorded weekly for the first 14 weeks and every other week thereafter.

There were no significant positive association between the concentration of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate administered and mortality of either sex. Statistical significant lower body weights were observed in males feed 3000 ppm N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate from week 2 to termination. No body weight retardation was found in rats in the other groups. Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumours.

Complete histopathology was performed on all of the control and high dose animals, as well as on all animals with unscheduled deaths or sacrificed when moribund. Histopathology for the terminal sacrifice animals of the 300 and 1000 ppm groups included selected tissues (lung, liver, kidney, thyroid and any gross lesions). Slightly increased values for thyroid weight and the thyroid/body weight ratio in the male 3000 ppm group, compared with those of male controls, were significant ($p<0.05$). Thyroid weight but not thyroid/body weight ratio in the male 1000 ppm group was also significantly higher than in male controls. No statistically significant differences in neoplastic and non-neoplastic lesions were observed between treated and control rats, either in tumour incidence or in the number of tumour bearing animals with single or multiple tumours. It was concluded that under the condition of this bioassay, there were no convincing evidence that dietary administration of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate was carcinogenic in Fischer 344 rats.

The dose selection in this study was based on a 13-week study, also described in ref. 20.
In this 13-week study animals were dosed 0, 100, 300, 1000 and 3000 ppm via the diet. In the highest dose group, males showed decreased body weight gain. Treated male groups (300, 1000 and 3000 ppm dose groups) exhibited higher thyroid to body weight ratios, whereas all treated female groups exhibited lower thyroid to body weight ratios. No histopathological abnormalities in the thyroids or other organs in males and females of the 3000 ppm dose group.

Ref.: 20

Topical administration, mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 animals per sex
Test substance:	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate. One semipermanent hair dye formulation (P22) containing 0.5 % N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate.
Batch:	/
Purity:	/
Dose level:	0.05 ml of a solution containing 0.5% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Route:	Topical, 1 application weekly
Exposure:	23 months
GLP:	not in compliance

The experiment involved altogether 12 different dye formulations and 3 negative control groups.

Dye applied topically to a 1 cm^2 area on a clipped (24 hours prior to application) site in the interscapular region. Mice received a dose of 0.05 ml topically without occlusion once weekly from 8 – 10 weeks of age for 23 months. The animals were observed daily for mortality and signs of toxicity, and were weighed monthly. A continuous weekly record was maintained for any skin lesions noted. After 9 months of treatment, 10 males and 10 females per group were necropsied and the study was terminated after 23 months. Skin and internal organs were evaluated histologically.

There were no overt sign of systemic toxicity in any of the dye-treated groups. Four male and 4 female survived 23 months compared to 3 males and 8 females in the control group. There were no significant differences in absolute or relative liver or kidney weights in groups of 10 male and 10 female mice necropsied after 7 and 9 months. Average body weights were comparable in all groups throughout the study. There were no statistically significant differences in the distribution of tumours among treated and control groups.

It is concluded that no evidence of carcinogenic activity was seen.

Ref.: 31

Topical administration, rats

Guideline:	
Species/strain:	Male and female weanling Sprague Dawley rats, 60 per sex per group
Group size:	60 animals per sex and dose
Test substance:	One semipermanent hair dye formulation, P22, containing 0.5% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch:	/
Purity:	/
Dose level:	0.5 ml of the test substance
Route:	Topical. 1 application twice weekly
Exposure:	114 weeks
GLP:	not in compliance

The experiment involved altogether 10 different dye formulations and 3 negative control groups.

Groups of 60 male and 60 female were obtained from the first mating (F_{1a}) of a multi-generation reproduction study in rats treated with one semi-permanent hair dye formulation containing 0.5% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate. The F_0 parents had received topical application of the hair dye formulation from the time of their weaning to the weaning of their offspring. The dye formulations were administered topically to the shaved (24 hours prior to application) neck and back area twice weekly. An initial dosage level of 0.2 ml/rat was increased incrementally by 0.1 ml per week until 0.5 ml was achieved. There were three independent control groups each containing 60 males and 60 females, which received no treatment.

The rats were observed daily for overt signs of toxicity and for mortality. Detailed observations were recorded weekly. Individual body weights were recorded weekly for the first 14 weeks and monthly thereafter. Group food consumption was recorded weekly. Haematological, biochemical and urinalysis studies were done on 5 males and 5 females per group at 3, 12, 18, and 24 months of study. After 12 months of treatment, 5 males and 5 females from each group were sacrificed and necropsied. Histopathological evaluations were performed on 18 tissues (plus tumour masses) including treated skin.

Changes in body weight and food consumption have been similar for control and treated rats. Survival just prior to terminal sacrifice (at week 117 119) the survival was 16 males and 12 females for the exposed group. Survival was 15 males and 14 – 18 females for the control groups. After 114 weeks, group mean body weight in the treated group was 746 g in males and 468 g in females. Control group values ranged from 682 to 759 g in males and 477 to 513 g in females.

In the treated group at 18 months, the mean haematocrit values were significantly reduced in males and females versus two of the three control groups and one of the three control groups respectively. At 24 months, the mean urea nitrogen for treated females was significantly higher than two of the three control groups. Gross observations considered to possibly be test article related were skin lesions including ulceration, scabbing, abscesses, thickening at the application site and increased incidences of enlarged and/or firm livers in the treated group. Most of the treated group animals showed colouration of the stratum corneum of the skin and the hair shafts. The changes were considered to be a dying effect of the test compounds and not pathologically significant. No significant variation in incidences of tumour bearing animals between treated and control animals when compared with each of the control groups by sex

Ref.: 32

Comment
Three studies with N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate have been identified. One long-term rat (F344/DuCr) study with oral administration of the substance was negative.

In one experiment was a formulation containing 0.5% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate tested by topical application on Swiss-Webster mice and in another experiment by topical application on Sprague Dawley rats. Both experiments were negative. However, as known carcinogens have been tested by the same experimental procedures without any tumour formation, it is not possible to draw any conclusions from these experiments with regard to potential carcinogenic effects.

N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate and hydrogen peroxide

Topical administration, mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 animals per sex and dose
Test substance:	One permanent hair dye formulation, P21 containing 1% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate prior to mixing with equal volume 6% hydrogen peroxide. The mixture was used within 15 minutes after mixing
Batch:	/
Purity:	/
Dose level:	0.05 ml of a solution containing N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate and hydrogen peroxide
Route:	Topical, 1 application weekly
Exposure:	23 months
GLP:	not in compliance

Dye applied topically to a 1 cm^2 area on a clipped (24 hours prior to application) site in the interscapular region. Mice received a dose of 0.05 ml topically without occlusion once weekly from 8 – 10 weeks of age for 23 months. The animals were observed daily for mortality and signs of toxicity, and were weighed monthly. A continuous weekly record was maintained for any skin lesions noted. After 9 months of treatment, 10 males and 10 females per group were necropsied and the study was terminated after 23 months. Skin and internal organs were evaluated histologically.

There were no overt sign of systemic toxicity in any of the dye-treated groups. Two males and 6 females survived to 23 months in the group receiving the oxidative formulation containing N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate. At 23 months, there were 3 males and 8 females surviving in the control group. There were no significant differences in absolute or relative liver or kidney weights in groups of 10 male and 10 female mice necropsied after 7 and 9 months. There were no statistically significant differences in the distribution of tumours among treated and control groups.

Ref.: 31

Topical administration, rats

Guideline:	/
Species/strain:	Male and female weanling Sprague Dawley rats, 60 per sex per group
Group size:	60 animals per sex and dose
Test substance:	One permanent hair dye formulation, P21, 1% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate prior to mixing with equal volume 6% hydrogen peroxide. The mixture was used within 15 minutes after mixing
Batch:	/
Purity:	/
Dose level:	0.5 ml of the test substance
Route:	Topical. 1 application twice weekly
Exposure:	114 weeks
GLP:	not in compliance

Groups of 60 male and 60 female were obtained from the first mating (F_{1a}) of a multi-generation reproduction study in rats treated with three different hair dye formulations containing up to 0.5% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate. The F_0 parents had received topical application of the hair dye formulation from the time of their weaning to the weaning of their offspring. The dye formulations were administered topically to the shaved (24 hours prior to application) neck and back area twice weekly. An initial dosage level of 0.2 ml/rat was increased incrementally by 0.1 ml per week until 0.5 ml was achieved. There were three independent control groups each containing 60 males and 60 females, which received no treatment.

The rats were observed daily for overt signs of toxicity and for mortality. Detailed observations were recorded weekly. Individual body weights were recorded weekly for the first 14 weeks and monthly thereafter. Group food consumption was recorded weekly. Haematological, biochemical and urinalysis studies were done on 5 males and 5 females per group at 3, 12, 18, and 24 months of study. After 12 months of treatment, 5 males and 5 females from each group were sacrificed and necropsied. Histopathological evaluations were performed on 18 tissues (plus tumour masses) including treated skin.

Survival just prior to terminal sacrifice (week 117 – 119) the survival was 15 males and 18 females for the exposed groups. Survival was 17 – 20 males and 22 – 26 females for the control groups. After 114 weeks, group mean body weights in the treated groups were 662 g in males and 473 g in females. Control group values ranged from 682 to 759 g in males and 477 to 513 g in females.

Changes in body weight and food consumption values were similar for control and treated animals. The weight gain was, however, slightly lower in the treated animals. Evidence of slight anaemia (reductions in erythrocytes, haemoglobin, and/or haematocrit) associated with an increase in reticulocytes occurred in one male at 12 months. The incidence of hyperkeratosis and dermatitis was considered higher in the treated animals than in controls. Most of the treated animals showed colouration of the stratum corneum of the skin and the hair shafts. The changes were considered to be a dying effect of the test compounds and not pathologically significant. No significant variation in incidences of tumour bearing animals between the treated and control animals when compared with each of the control groups by sex.

Ref.: 32

Comment
Two studies on N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate together with hydrogen peroxide were identified. One study involved topical application of a formulation containing 1% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate after mixing with an equal volume with 6% hydrogen peroxide on Swiss-Webster mice and another study involved application of the same mixture on Sprague Dawley rats. Both experiments were negative. However, as known carcinogens have been tested by the same experimental procedures without any tumour formation, it is not possible to draw any conclusions from these experiments with regard to potential carcinogenic effects.

Conclusion on carcinogenicity
N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate did not induce tumours after oral administration in a long-term rat study. The substance has also been studied alone and after mixing with hydrogen peroxide in a hair dye formulation, by topical application to mice and rats. However, as known carcinogens have been tested by the same experimental procedures without any tumour formation, it is not possible to draw any conclusions from these experiments with regard to potential carcinogenic effects.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	OECD 414 (1984)
Species/strain:	Rat, Crl:CD® (SD)IGS BR VAF/Plus®
Group size:	25 pregnant female per dose
Test substance:	A50, GTS03849
Batch:	A203G157
Purity:	100.4%
Doses:	0, 5, 20 or 50 mg/kg bw/day oral gavage, 7days/week
Vehicle:	0.2% w/v erythorbic acid in reverse osmosis water.
Dosing period:	Gestation Days (GD) 6 -20
GLP:	in compliance

Groups of rats (25 females per group) were administered orally (gavage) once daily on days 6 through 20 of gestation at dose levels of 0, 5, 20 and 50 mg A50/kg bw/day.
Viabilities, clinical observations, body weights and feed consumption values were recorded. After sacrifice, the gravid uterus was excised, weighed and subsequently examined for the number and distribution of corpora lutea, implantation sites and uterine contents. A gross necropsy was performed. Foetuses were weighed and examined for gross external, soft tissue and skeletal alterations and sex.

Results
One dam in the 50 mg/kg/day dosage group was found dead on gestation day 18. It is not clear whether this death was exposure related.

Maternal body weight gains and food consumption were reduced in the 20 and 50 mg/kg/day dosage group during the entire dosage period. Also gravid uterine weights were reduced in these dose groups.

Administration of the test substance did not affect litter observations or cause gross external, soft tissue or skeletal foetal alterations. In this study, the maternal no-observable-adverse-effect-level (NOAEL) was 5 mg/kg/day based on reductions on body weight and feed consumption. The developmental NOAEL was 50 mg/kg/day, since no developmental toxicity was observed up to the highest dose tested.

Ref.: 28

Remark
Dose selection was based on a range finding study, in which at 40 mg/kg (highest dose tested) slight effects on maternal body weight were observed (ref: 27)

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

No data submitted

3.3.11. Human data

Repeated Insult Patch Test

Guideline:	/
Species/strain:	human female volunteers
Group size:	116
Test substance:	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate
Batch:	/
Purity:	/
Doses:	0.1ml of a formulation containing 3% N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate, 12% isopropanol, 20% Tween 20, 2% Natrasol, 0.05% sodium sulfite, , and made up to 100% with water
Dosing period:	72 hours
GLP:	not in compliance

N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate was tested to determine its ability to sensitize the skin of normal volunteer subjects using an occlusive repeated insult patch test.

Conclusion
A definite evidence of sensitization was found in one subject, and possibly in a second subject, to the test substance. In addition, one individual developed reactions to all three materials (two other substances were tested in parallel), which were interpreted as pre-sensitization, probably to the vehicle.

Comment

The SCCP considers that human maximisation studies of this type are unethical. It is realised that the study provided was performed in 1984.

Ref.: 16

3.3.12.	Special investigations

See point 3.3.7.

3.3.13.	Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(N,N-Bis(2-hydroxyethyl)-p-phenylenediamine sulfate)
(Oxidative/permanent)

Maximum absorption through the skin	**A (µg/cm²)**	**=**	**0.252 µg/cm²**
Skin Area surface	**SAS (cm²)**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.1764 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.003 mg/kg**
No observed adverse effect level (mg/kg) (rat, oral, maternal toxicity)	**NOAEL**	**=**	**5 mg/kg**

Margin of Safety	**NOAEL/SED**	**=**	**1667**

3.3.14.	Discussion

Physico-chemical specification
N,N-bis(2-hydroxyethyl)-p-phenylenediamine is a tertiary amine, and thus, it is prone to nitrosation. The substance should not be used in combination with nitrosating agents. Stability of *N,N*-Bis(2-hydroxyethyl)-p-phenylenediamine is marketed products is not reported. The physico- chemical characterisation and purity of the substance is not reported in several studies.

General toxicity
The oral LD_{50} in 2 (non-guideline) studies after oral administration ranged between 100 and 400 mg/kg bw. After i.p. administration the LD50 was between 16 and 31 mg/kg bw.
The NOAEL was set at 5 mg/kg bw/day (14 day range-finding study) and at 20 mg/kg bw/day (90 day study). The maternal NOAEL was 5 mg/kg/day; the developmental NOAEL was 50 mg/kg/day. The NOAEL from maternal toxicity will be used for the calculation of margin of safety.

Irritation / sensitisation
Most of the studies are old and not complying with GLP. Presentation of four of the studies is inadequate, among which 1 is unreadable. N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate was found to be irritant to mucous membrane. It induced skin sensitisation as shown by LLNA. The substance is a known skin sensitiser in humans.

Dermal absorption
The *in vitro* dermal absorption of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate from a hair dye formulation, containing 2.5% of the test substance, in combination with a placebo developer was 188 ± 0.242 μg/cm^2 (range 0.049-0.497 μg/cm^2). The *in vitro* dermal absorption of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate from a hair dye formulation, containing 2.5% of the test substance, in combination with a peroxide developer was 0.108± 0.064 μg/cm^2 (range 0.037-0.252 μg/cm^2). Maximum observed dermal absorption in the presence of developer mix, i.e. 0.252 μg/cm^2, is used for the calculation of margin of safety.

Mutagenicity
N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate induced mutations in *Escherichia coli* without exogenous metabolic activation. In *Salmonella typhimurium* TA100, an initial positive finding was not reproduced in another trial. *N*,*N*-bis(2-hydroxyethyl)-p-phenylenediamine sulfate did not either increase mutations at the *tk* locus in L5178Y mouse lymphoma cells. It induced chromosome aberrations and endoreduplications in Chinese hamster ovary (CHO) cells in the presence of metabolic activation. *In vivo*, N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate did not induce micronuclei in polychromatic erythrocytes of mice or DNA damage (measured by unscheduled DNA synthesis) in hepatocytes of rats. Thus, although *in vitro* assays in *E. coli* and CHO cells suggest that N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate has genotoxic potential, two *in vivo* assays indicated no genotoxic activity.

Carcinogenicity
N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate did not induce tumours after oral administration in a long-term rat study. The substance has also been studied alone and after mixing with hydrogen peroxide by topical application to mice and rats. However, as known carcinogens have been tested by the same experimental procedures without any tumour formation, it is not possible to draw any conclusions from these experiments with regard to potential carcinogenic effects.

4. CONCLUSION

Based on the information provided, the SCCP is of the opinion that the use of N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate itself as an oxidative hair dye substance at a maximum concentration of 2.5% in the finished cosmetic product (after mixing with hydrogen peroxide) does not pose a risk to the health of the consumer, apart from its sensitising potential.

N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate is a tertiary amine, and thus it is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 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

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