Scientific Committee on Consumer Safety

SCCS

OPINION ON

2,6-Dihydroxyethylaminotoluene

COLIPA n° A138

The SCCS adopted this opinion at its 10th plenary meeting

on 25 June 2015

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

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

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

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1. BACKGROUND

The hair dye 2,6-Dihydroxyethylaminotoluene (Colipa No A138) CAS No. 149330-25-6 is intended to be used as a direct dye and as an ingredient in oxidative hair dye formulations up to a final on-head concentration of 1%.

Submission I and II on hair dye 2,6-Dihydroxyethylaminotoluene (A 138) were transmitted by COLIPA in May 1995 and January 2006 respectively.

Following Submission II, in the safety evaluation of December 2011 the SCCS (SCCS/1425/11) concluded that:

"*the positive results found in the in vitro gene mutation assay in bacteria were not confirmed nor ruled out in an appropriate in vivo test on the same genetic endpoint. Consequently, a final conclusion on the genotoxicpotential of 2,6-dihydroxyethylaminotoluene cannot be drawn*."

In September 2014, Cosmetics Europe submitted additional data on genotoxicity in order to review the issues raised by the SCCS in the Opinion of December 2011.

2. TERMS OF REFERENCE

(1) In light of the new data provided, does the SCCS consider 2,6-Dihydroxyethylaminotoluene (A138) safe when used as a direct dye and as an ingredient in oxidative hair dye formulations up to a final on-head concentration of 1%?

(2) Does the SCCS have any further scientific concerns with regard to the use of 2,6-Dihydroxyethylaminotoluene (A138) in cosmetic products?

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

2,6-Dihydroxyethylaminotoluene (INCI)

3.1.1.2. Chemical names

1-Methyl-2,6-bis-(2-hydroxyethylamino)-benzene
Ethanol, 2,2'-[(2-methyl-1,3-phenylene)diimino]bis-
2,6-Di-(2-hydroxyethylamino)toluene;
2,6-bis-(β-hydroxyethylamino)toluene;
2,6-bis[(2-hydroxyethyl)amino]toluene
2,6-di(2-hydroxyethylamino)-toluol

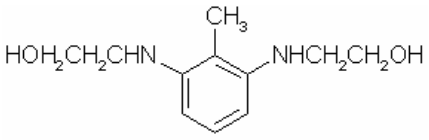
3.1.1.3. Trade names and abbreviations

HC Violet AS, HC Purple BS, WS I-111
COLIPA n° A138

3.1.1.4. CAS / EC number

CAS: 149330-25-6
EC: 443-210-1

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{11}H_{18}N_2O_2$ (free base)

3.1.2. Physical form

Odourless, light brown-greyish powder

3.1.3. Molecular weight

Molecular weight: 210.28 g/mol

3.1.4. Purity, composition and substance codes

Batch PVS 11/02 = SAT 040796

Identity:	verified by NMR-spectroscopy, IR- and UV-spectrometry
Purity by NMR assay:	101% (w/w)
Purity by HPLC assay:	99.7% (area)
Sulphated ash	1.1% (w/w)
Impurities:	
2,6-Diaminotoluene	< 50 ppm (detection limit)
Sulphate ion	0.1% (w/w)
Solvent content (water):	0.4% (w/w)
Heavy Metal Content:	
Pb	< 20 ppm
Sb and Ni	< 10 ppm
As and Cd	< 5 ppm
Hg	< 1 ppm

Batch Pt. 1/91 and Batch 2

Identity:	verified by HPLC chromatography, UV-/VIS-spectroscopy and thin layer chromatography
Purity by HPLC:	> 99.8 area%
Impurities:	unspecified 0.07area% (HPLC)

Comment
Batches No. 2 and 1/91 seem to have been used in toxicological testing and the HPLC purity of both batches is similar to that of batch PVS 11/02 (HPLC peak area ≥ 99.7%).

3.1.5. Impurities / accompanying contaminants

See 3.1.4
NDELA content in HC violet AS; Syntharo, Lot #2010101: <25 ppb.

3.1.6. Solubility

Water:	39.9 g/l	at 20°C, pH 8.2, measured by EC Method A.6
Ethanol:	10 – 100 g/l	at room temperature
DMSO:	> 100 g/l	at room temperature

3.1.7. Partition coefficient (Log Pow)

Log P_{ow}: 0.037 (pH 7, 23) measured by EC method A.8

3.1.8. Additional physical and chemical specifications

Melting point:	115 – 121 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/

Refractive index: /
UV_Vis spectrum (200-800 nm): λmax at 221nm and 293 nm

3.1.9. Homogeneity and Stability

The homogeneity and stability of 2,6-dihydroxyethylaminotoluene in test suspensions is described as follows:

0.1%, 1% and 10% suspensions of 2,6-dihydroxyethylaminotoluene were prepared in 0.5% aqueous CMC and stored at room temperature under access of daylight. Analyses were performed immediately after and after 2h, 4h and 24 h storage. Deviations of 5% of the initially determined concentration were tolerated.

2,6-Dihydroxyethylaminotoluene was assumed to be homogeneously distributed in the preparation, when values of the 3 samples were within ± 10% of the mean.

Ref.: 14, 16

General Comments to physico-chemical characterisation

- 2,6-Dihydroxyethylaminotoluene is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in 2,6-dihydroxyethylaminotoluene is not reported. The NDELA content cannot be related to possible nitrosamine that can be formed by 2,6-dihydroxyethylaminotoluene.
- Stability of 2,6-dihydroxyethylaminotoluene in typical hair dye formulations is not reported.

3.2. Function and uses

2,6-Dihydroxyethylaminotoluene is used as a precursor for hair colours. It reacts with primary intermediates to form the final dye-stuff. The reaction can be accelerated by addition of an oxidising agent (e.g. hydrogen peroxide), but it can also be achieved by air oxidation.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCNFP/0697/03

Guideline:	OECD n° 401 / ECB 1 / Limit test
Species/strain:	Sprague Dawley rats
Group size:	5 males and 5 females
Test substance:	A 138 (in water)
Batch:	#2
Dose:	2000 mg/kg bw
Observ. Period:	14 days
GLP:	in compliance

Five male (140-152 g) and five female (129-140 g) Sprague-Dawley rats were used for the test. The method used followed OECD Guideline n° 401 (1981), referenced as Method B1 in Commission Directive 84/449/EEC.
The rats were given a single oral dose of test material as a suspension in distilled water at a dose level of 2000 mg/kg bw. They were observed for 14 days after the day of dosing and were then killed for gross pathological examination.

Results
There were no mortalities and all animals showed the expected gain in bodyweight during the study. Hunched posture and lethargy were noted in all animals on the day of dosing. No abnormalities were noted at necropsy. The acute median lethal dose (LD50) of the test compound was found to be greater than 2000 mg/kg bw in rats.

Ref.: 1 (submission I)

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Taken from SCCS/1425/11 (=submission II)

Guideline:	OECD 404 (1992)
Species/strain:	rabbit, white New Zealand (SPF Crl:NZW)
Group size:	3 males
Test substance:	2,6-di(2-hydroxyethylamino)-toluol
Batch:	Pt. 1/91
Purity:	99.8 area%
Vehicle:	water

Dose level: single dose of 500 mg
GLP: in compliance
Study period: 14 – 28 March 1995

The acute dermal irritation/corrosion of 2,6-di(2-hydroxyethylamino)-toluol was tested in three albino rabbits. The substance was applied in a single dose of 500 mg to a shaved dorsal area of trunk and covered with a gauze patch and aluminium foil held in contact with the skin by an occlusive dressing. Exposure duration was 4 hours. Thereafter residual substance was removed with water. Animals were examined for mortality, clinical signs and signs of irritation response 60 minutes, 24, 48 and 72 hours after patch removal.

Results
The treated skin area of all animals showed no signs of erythema or oedema formation.

Conclusion
2,6-di(2-hydroxyethylamino)-toluol was not irritant to rabbit skin under the conditions of the experiment.

Ref.: 5 (submission II)

3.3.2.2. Mucous membrane irritation

Taken from SCCNFP/0697/03

Guideline: OECD n° 405
Species/strain: New Zealand White Rabbits
Group size: 3
Test substance: 1 % (w/v) a.i. in water
Batch: #2
Purity: 99.5%
Dose: 0.1 ml
GLP: in compliance

Three New Zealand white rabbits were used. Their bodyweights ranged from 2.73 to 3.06 kg. The test was performed according to OECD Guideline n° 405 (1987), referenced as Method B5 in Commission Directive 84/449/EEC.
The test material was prepared as a 1 % (w/v) dilution in distilled water. An amount of 0.1 ml of the test material preparation was instilled into the conjunctival sac of the right eye, the left eye remained untreated and was used for control purposes. Assessment of ocular damage/irritation was made at about 1, 24, 48 and 72 h following treatment according to the Draize scale.

Results
The test substance was not irritating at 1% in water.

Ref.: 3 (submission I)

Taken from SCCS/1425/11 (=submission II)

Guideline: OECD 405 (1987)
Species/strain: rabbit, white New Zealand (SPF Crl:NZW)
Group size: 3 males
Test substance: 2,6-di(2-hydroxyethylamino)-toluol
Batch: Pt. 1/91
Purity: 99.8 area%
Vehicle: /
Dose level: 100 mg (neat)
GLP: in compliance

Study period: March 1995

The acute eye irritation/corrosion of 2,6-di(2-hydroxyethylamino)-toluol was tested in three albino rabbits. The substance was applied in a single dose of 100 mg to one of the eyes of each animal. The untreated eye was used as control. The eyes were washed out 24 hours after instillation of the test substance.
The animals were examined for clinical signs and the eyes were examined for lesions of conjunctivae, cornea and iris 60 minutes, 24, 48, 72 and 96 hours after application of the test substance.

Results
One hour after instillation a slight redness and a swelling of the conjunctivae with lids about half closed were observed. 24 hours after application a diffuse, crimson colour but only light swelling were seen. All animals were free of irritation signs 96 hours after application.

Ref.: 6 (submission II)

Comment
Under the conditions of the test, 2,6-di(2-hydroxyethylamino)-toluol was irritant to the rabbit eye.

3.3.3. Skin sensitisation

Taken from SCCS/1425/11 (=submission II)

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species/strain:	mouse, CBA strain, inbred, SPF-quality
Group size:	20 females (4 groups of 5 animals), nulliparous and non-pregnant
Test substance:	2,6-Di-(2-hydroxyethylamino)toluene
Batch:	PVS 11/02
Purity:	99.7 area% (HPLC)
Vehicle:	ethanol:water (7:3 v/v)
Concentration:	0, 10, 25 and 50%
Positive control:	alpha-hexylcinnamic aldehyde (in acetone:olive oil 4:1)
GLP:	in compliance
Study period:	7 – 21 June 2005

Three groups of five female mice each were treated with 2,6-di-(2-hydroxyethylamino)toluene at concentrations of 10, 25 and 50 % in ethanol:water by topical application to the dorsum of each ear lobe (25 µL) on three consecutive days. A control group of four mice was treated with the vehicle (ethanol:water (7:3 v/v)) only. Five days after the first topical application the mice were injected intravenously into a tail vein with ^{3}H-methyl thymidine.
Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a ß-scintillation counter.

Results
No symptoms of local toxicity at the ears of treated mice and no systemic findings were observed during the study period. The Stimulation Index (S.I.) was below 3 in all dose groups. No dose response relation was noted.

Compound	Concentration %	Stimulation Index
2,6-Di-(2-hydroxyethylamino)toluene	10	1.2
	25	1.0
	50	0.9
alpha-hexylcinnamic aldehyde	5	1.5
	10	2.9
	25	6.1

Conclusion
2,6-Di-(2-hydroxyethylamino)toluene is not a sensitiser.

Ref.: 7 (Submission II)

3.3.4. Dermal / percutaneous absorption

Taken from SCCS/1425/11 (=submission II)

Guideline:	OECD 428
Species/strain:	pigs both sexes
Group size:	8 dermatomed skin preparations from two young pigs
Skin thickness:	680-720 µm
Skin integrity:	TER >7kΩ
Test substance:	Experiment A: [^{14}C]-labelled "A 138" included in a hair dye formulation TM 0038-1a was mixed with a developer, not containing hydrogen peroxide. Experiment B: [^{14}C]-labelled "A 138" included in a hair dye formulation TM 0038-1a was mixed with another developer, containing hydrogen peroxide.
Batch:	PVS 11/02
Purity:	99.7% by HPLC
Receptor fluid:	Dulbecco's phosphate buffered saline
Test item:	A 138
Dose:	20 mg/cm² (corresponding to 0.21 mg/cm²)
Application area:	1 cm²
Method of Analysis:	Liquid scintillation
GLP:	in compliance
Study period:	2005

The test substance was studied as an ingredient of representative formulations:

Experiment A: A 138 included in a hair dye formulation TM 0038-1a was mixed with a developer, not containing hydrogen peroxide.
Experiment B: A 138 included in a hair dye formulation TM 0038-1a was mixed with a developer, containing hydrogen peroxide.
Eight integrity-checked dermatomed skin preparations of two young pigs of both sexes were used in each experiment. Skins were inserted in static penetration cells (Franz-cells) with an application area of 1.0 cm². The non-occlusive exposure under temperature controlled conditions lasted 30 minutes before rinsing.
The test substance formulation was applied topically to the horny layer of the skin in nominal quantities of 20 mg/cm², which corresponded to nominally 0.21 mg of the test substance per cm^2 for the experiments A and B.

48 hours after the application, the *stratum corneum* was removed by repeated stripping with adhesive tapes to obtain the adsorbed test substance. The remaining skin was taken to determine the absorbed test substance. The penetration was calculated from the mass of the test substance in the receptor fluid, consisting of phosphate buffered saline. The overall amount of bioavailable test substance is defined as the sum of absorbed and penetrated quantities.

Results
The mean values of bioavailability were 1.97 ± 0.57 µg/cm² and 2.33 ± 0.70 µg/cm² for the formulation without and with hydrogen peroxide respectively.

Ref.: 17 (submission II)

Comment
There were only two donors. The mean + 2SD (3.73 µg/cm²) should be used for the MoS calculation under oxidative conditions.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral toxicity

No data submitted

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

Taken from SCCS/1425/11 (=submission II)

Dose Range Finding in Rats

The test substance was administered orally per gavage to 5 groups of 5 male and 5 female Wistar rats each once a day for 14 consecutive days. An equally sized negative control group was treated with distilled water. The test substance was administered freshly dissolved in distilled water (doses of 100 mg/kg and less) respectively suspended in a 0.5% aqueous CMC (carboxymethylcellulose; doses of 316 mg/kg bw and more) solution at a dose volume of 10 ml per kg body weight. Doses of 0 (control), 10, 32, 100, 316 and 1000 mg test substance per kg body weight and day were used.

Investigations performed: Observations in life, bodyweight, feed consumption and terminal necropsy.

Results
Mortality
All animals survived until the scheduled termination of the study.

Body weight
There were no significant differences of any dosed group to the control group.

Feed consumption
There were no significant differences of any dosed group to the control group.

Observations in life
Bedding material of all animals dosed with 1000 mg/kg bw/day was stained bright brown, resembling the colour of the test substance. This is most likely due to urinary excretion of the test substance and/or its metabolites. These stains are not regarded as toxic changes.

Some of the animals of the highest dosed group (1000 mg/kg bw/day) suffered from transient apathy, starting about 30 min after test substance administration and lasting for about another 30 min. This is regarded to be a toxic change.

Necropsy findings
A few single findings are altogether interpreted as incidental without test substance relationship.

Dose suggestions for a 90-Day Study:
The dose of 316 mg/kg bw represents the No-adverse-effect-level for both sexes, where no significant differences to the control group were found. Test compound-related alterations in the highest dosed group (1000 mg/kg bw) were of only low severity. Therefore, a dose of 1000 mg per kg bw/d may be used as the high dose in the main study whereas 100 and ca. 300 mg per kg bw/d may serve as low- and mid-dose, respectively.

Ref.: 13 (Submission II)

Taken from SCCNFP/0697/03

Guideline:	OECD n° 408
Species/strain:	Wistar rats
Group size:	15/sex + 20/sex for control and high dose group
Test substance:	A 138 in 0.5% aqueous Na-carboxymethylcellulose solution
Batch:	Pt 1/91
Purity:	>99.8 area% (HPLC)
Dose:	0, 100, 316 and 1000 mg/kg bw/d
Exposure period:	90 days
Route:	oral, gavage
GLP:	in compliance

80 male and 80 female Wistar rats, Crl:(WI) BR, SPF, were used. The age at first administration was approximately 7 - 10 weeks and the mean bodyweight of the animals at the beginning of the study ranged between 197 - 219 g for males and 159 - 168 g for females.
The study was conducted according to OECD-Guideline 408. The test substance (purity >99.5%) was suspended in a 0.5% aqueous Na-carboxymethylcellulose solution and preparations were made freshly every day immediately before use. Doses of 100, 316 and 1000 mg test substance/kg bw/d were applied to groups of 15 male and 15 female rats for 90 or 91 consecutive days in males and 91 or 92 consecutive days in females. An equally sized control group received the vehicle only. In all groups the dose volume was 10 ml/kg bw. Recovery was investigated in two groups of 10 males and 10 females each; one high-dose recovery group and one control recovery group, both of which were treated like the corresponding groups for 92 days and then maintained without treatment for an additional 28 days. Observations in life, ophthalmoscopy, bodyweight, feed consumption, haematology and clinical chemistry, urinalysis, gross pathology, organ weight determination and histopathology were performed.

Results
One male animal (low-dose group) died accidentally during blood sampling on day 29. All other animals survived until the scheduled sacrifice. A dose-dependent light to dark staining of skin, fur, urine and bedding material was observed.
In all high-dosed animals, transient apathy was reported within the first two weeks of treatment within about half an hour after gavage. Salivation was noted for a short time after application of the test substance in one animal of the mid-dose group and in practically all of the high-dosed animals.

In a few animals, abnormal head posture and stereotype was observed occasionally. Serum bilirubin was significantly elevated in males of the high-dose group. Serum creatinine was significantly lower in mid- and high-dosed females.
In the urine of mid- and high-dosed groups, bilirubin and urobilinogen were found in abnormally high concentrations. High-dosed recovery group males showed a significantly increased relative liver weight. The urine pH was significantly lowered in male rats of the high-dose group. In male animals of the high-dose group, renal tubular epithelial basophilia was observed. A trend towards a dose-related increase in relative kidney weight was seen in males, but comparison of the relative kidney weights in the low-dose group and the corresponding control group showed no differences (% of bw control : 0.77 - 0.91%; low-dose group: 0.79 - 0.91%). An increased absolute and relative kidney weight was observed in high-dosed recovery group males only.
High-dosed females had a significantly increased relative weight of kidneys and high-dosed recovery females had an increased absolute adrenal weight.
Study results have been described by means of descriptive statistics. Due to multiple testing, some probably unspecific effects have been reported as "significantly different from actual corresponding controls" (i.e., elevated mean cell volume of females on day 84, caused by an unusual low mean of the control group).

Target organs of the substance toxicity were the liver (based on serum bilirubin, urine urobilinogen and bilirubin, organ weight changes) and the kidney (based on serum creatinine, organ weights and histopathological changes, i.e., tubular epithelial basophilia). The NOAEL was considered to be 100 mg/kg bw/d.

Ref.: 5 (Submission I)

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Gene Mutation test in bacteria

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537
Replicates:	triplicates in a single experiment
Test substance:	A 138 [2-((3-((2-hydroxyethyl)-2-methylphenyl)amino)ethanol)}
Batch:	PVS 11/02
Purity:	99.7 area%
Solvent:	DMSO
Concentrations:	0, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with at least 48 h incubation, without and with S9-mix
GLP:	in compliance
Study period:	23 November 2004 – 20 December 2004

A 138 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment for toxicity and mutation induction in strains TA98 and TA100 both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of

revertant colonies and/or clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in the strains used, the pre-experiment is reported as part of the main experiment. The experiment was performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
The plates incubated with A 138 showed normal background growth and no toxic effects, evident as a reduction in the number of revertants, up to 5000 µg/plate without and with S9-mix.
In the presence of metabolic activation, a concentration-dependent increase in the number of revertants was observed for strain TA98. In the absence of metabolic activation, no biologically relevant increase was found.
In the other strains used a biologically relevant increase in the number of revertants was not found in the absence nor in the presence of S9-mix

Conclusion
Under the experimental conditions used, A138 was mutagenic in this gene mutation tests in bacteria.

Ref.: 8 (Submission II)

***In vitro* Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1997)
Species/strain:	L5178Y $tk^{+/-}$ mouse lymphoma cells
Replicates:	two parallel cultures in 2 independent experiments
Test substance:	A 138
Batch:	PVS 11/02
Purity:	99.9 (area%)
Vehicle:	DMSO
Concentrations:	65.5, 131.3, 262.5, 525, 1050 and 2100 µg/ml without and with S9-mix
Treatment	Experiment I: 4 h treatment without and with S9-mix; expression period 72 h and selection period of 10-15 days
	Experiment II: 24 h treatment without S9-mix; expression period 48 h and selection period of 10-15 days
GLP:	in compliance
Study period:	9 November 2004 – 3 January 2005

A 138 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity with exposure up to the prescribed maximum concentration of 10 mM (≈ 2100 µg/ml) measuring relative suspension growth. In the main test, cells were treated for 4 h or 24 h (without S9 experiment II) followed by an expression period of 72 or 48 h (experiment II) to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect), colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
There was no relevant shift in osmolarity or in the pH value of the medium, even at the maximal concentration of A 138 in the pre-test. No precipitation was observed up to the maximal concentration of 2100 µg/ml.
The appropriate level of toxicity (10-20% survival after the highest dose) was not reached in any of the experiments both without and with S9-mix.

In both experiments, no reproducible, biologically relevant increase in the number of mutant colonies was observed, independent of the presence or absence of S9-mix.

Conclusion
Under the experimental conditions used, A 138 was not mutagenic in this mouse lymphoma assay using the *tk* locus as reporter gene.

Ref. 9 (Submission II)

SCCS comment
The recommended toxic range of approximately 10-20% survival compared to the concurrent negative control was not reached. However, the maximum required concentration of 10 mM (≈ 2100 µg/ml) was tested in this gene mutation test in mammalian cells.

Gene mutation test in mammalian cells (*hprt*-locus)

Guideline:	OECD 476 (1997)
Cells:	mouse lymphoma cell line L5178Y
Replicates:	duplicate cultures in two independent experiments
Test substance:	COLIPA A 138
Batch:	PVS 11/02
Purity:	99.7 area% (by HPLC)
Solvent:	deionised water
Concentrations:	0, 131.3, 262.5, 525, 1050 and 2100 µg/ml without and with S9-mix
Treatment:	experiment I: 4 h treatment both without and with S9-mix; Expression period 48 h and a selection period of 10-15 days.
	experiment II: 4 h treatment with S9-mix; expression period 48 h and a selection period of 10-15 days. 24 h treatment without S9-mix; expression period 48 h and a selection period of 10-15 days.
GLP:	in compliance
Study period:	14 November 2012 – 21 December 2012

COLIPA A 138 was assayed for gene mutations at the *hprt* locus of mouse lymphoma cells in both the absence and presence of S9 metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth with 8 concentrations without and with metabolic activation and exposure for 4 and 24 h up to the maximum prescribed concentration of 2100 µg/ml (~10 mM). In the main tests, cells were treated for 4 h or 24 h (experiment II, without S9-mix only) followed by an expression period of 48 h to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured in the main experiments as percentage total growth of the treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
There was no relevant shift of pH and osmolarity of the medium even at the maximum concentration tested. No precipitation or phase separation was noted with and without metabolic activation.
In the pre-test, relevant cytotoxicity only occurred at 262.5 and above following 24 h treatment without metabolic activation. At 4 h treatment, only a transient reduction of the relative suspension growth was seen at 131.3 and 262.5 µg/ml with metabolic activation which was considered not biologically relevant. Consequently, 2100 µg/ml (~10 mM) was chosen as the maximum concentration.

The appropriate level of toxicity (10-20% relative total growth after the highest concentration) was generally not reached; occasionally single cultures did show a relative survival of 10-20%.
In both experiments, no biologically relevant and concentration dependent increase in the mutant frequency was observed, either in the presence or in the absence of metabolic activation. Moreover, a linear regression analysis did not indicate a significant concentration dependent trend of the mutant frequency by a probability value of <0.05 in any of the experimental groups.

Conclusion
Under the experimental conditions used, COLIPA A 138 did not induce gene mutations in this gene mutation test in mammalian cells and, consequently, COLIPA A 138 is not mutagenic in mouse lymphoma cells.

Ref.: 1 (Submission III)

SCCS comment:
The required toxicity was not reached. However, COLIPA A 138 was tested up to the prescribed maximum concentration of ~10 mM (2100 µg/ml).

Micronucleus Test in mammalian cells

Guideline:	draft OECD 487 and OECD 473 (1997)	
Cells:	V79 cells	
Replicates:	duplicate cultures in 3 independent experiments	
Test substance:	A 138	
Solvent:	DMSO	
Batch:	PVS 11/02	
Purity:	99.7 area%	
Concentrations:	experiment I:	0, 525, 1050 and 2100 µg/ml without and with S9-mix
	experiment IIA:	0, 525, 1050 and 2100 µg/ml without and with S9-mix
	experiment IIB:	0, 1050, 1400 and 2100 µg/ml without S9-mix
Treatment:	experiment I:	4 h treatment both without and with S9-mix; harvest time 24 h after the beginning of treatment
	experiment IIA:	20 h treatment without S9-mix; harvest time 24 h after the beginning of treatment 4 h treatment both with S9-mix; harvest time 48 h after the beginning of treatment
	experiment IIB:	20 h treatment both without S9-mix; harvest time 24 h after the beginning of treatment
GLP:	in compliance	
Study period:	15 February 2005 – 7 June 2005	

A 138 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in V79 cells. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. A pre-test on cell growth inhibition (XTT assay) with 4 h treatment and concentrations up to 2100 µg/ml, the maximum prescribed concentration (approximately 10 mM) was performed in order to determine the toxicity of A 138, the solubility during exposure and thus the test concentrations for the main micronucleus test. The highest concentration should produce clear toxicity with reduced cell growth. The treatment period in the main test was either 4 h without and with S9-mix or 20 h without S9-mix. The harvest time was 24 or 48 h (experiment IIA with S9 mix) after the beginning of culture. For assessment of cytotoxicity the relative cell count was estimated as compared to the respective solvent control. In

parallel to the micronucleus test, a XTT test was carried out as a second measure of cytotoxicity. Negative and positive controls were in accordance with the draft guideline.

Results
In the pre-test, precipitation of A 138 was observed at 2100 µg/ml in the absence of S9-mix and at 1050 µg/ml and above in the presence of S9-mix. In the pre-test using reduced XTT activity of about 40% of control or below as indicator for toxicity, no toxic effects were observed after 4 h treatment up to the highest concentration used. Therefore, 2100 µg/ml was chosen as the highest concentration.
In the main test, precipitation of A 138 was not observed. Only in experiment IIB after 20 h treatment slight toxic effects indicated by cell numbers of 54.1% of control were observed at the highest concentration of 2100 µg/ml. Cytotoxicity was not found in the other experiments.
In experiment I both without and with S9-mix a biologically relevant increase in cells with micronuclei was not found up to the highest concentration. In experiment IIA with a harvest time of 48 h in the presence of S9-mix only a single statistically significant increase in the number of cells with micronuclei was found. As this increase was within the range of the historical controls, it is considered not biologically relevant.
A statistically significant and concentration dependent increase in the number of cells with micronuclei was observed in experiment IIA without metabolic activation. As the positive result was only slightly outside the range of the historical control data, experiment IIB was performed with narrower dilution steps. In this confirmative experiment a statistically significant increase in the number of cells with micronuclei was seen at the highest concentration.

Conclusion
Under the experimental conditions used, A 138 induced an increase in micronucleated cells and, consequently, is clastogenic and/or aneugenic in V79 cells.

Ref.: 10 (Submission II)

Single cell gel electrophoresis assay (COMET assay) in reconstructed human skin

Guideline:	/
Tissue:	Phenion® Full-Thickness skin model
Replicates:	3 tissues/concentration in 3 independent runs
Test substance:	A138 [2,6-dihydroxyethylaminotoluene]
Batch:	WE#20100619
Purity:	99.9 area% (by HPLC), 98.6 weight% (by 1H-NMR)
Solvent:	70% ethanol in water
Concentrations:	25, 50, 75 and 100 mg/ml identical to 400, 800, 1200 and 1600 µg/cm^2
Treatment:	48 h treatment
GLP:	in compliance
Study period:	12 November 2013 – 30 April 2014

A138 has been investigated for induction of DNA damage in reconstructed human skin tissue using the comet assay. A reconstructed 3-dimensional skin tissue of non-transformed human keratinocytes and fibroblasts was used (Phenion® Full-Thickness skin model), allowing realistic exposure conditions by topical application, the evaluation of the skin as the first site of contact and the consideration of any possible species- and organ-specific metabolism. The tissues are equilibrated upon arrival in air-liquid-interphase medium without phenol red.
The test concentrations were based on the results of a concentration range-finding study measuring cytotoxicity, which was monitored by measuring the intracellular ATP concentration and the release of adenylate kinase. Only concentrations with limited or no cytotoxicity should be used in the main experiment.

In the main experiment 4 concentrations of A138 were used with 3 tissues per concentration. The tissues were exposed to A138 for 48 h; 24 and 45 h after the first application, a second and third aliquot of A138 was applied atop of the same tissue. After treatment the tissue was treated with thermosolin to allow degradation of the basal membrane between the dermis and the epidermis. Next the dermis was peeled off from the epidermis and each tissue was further developed separately on pre-coated glass slides.
Three slides were prepared from both the epidermis and dermis of each individual skin tissue. Electrophoresis was performed for 30 min at 1 V/cm. DNA was stained with the fluorescence dye SYBR Gold. For the evaluation of comets the % tail DNA (= tail intensity) was used as assessment parameter. 50 cells per slide, 2 slides per tissue compartment (epidermis and dermis) and 3 tissues per concentration were scored.
In case of a negative result after exposure to A138, an additional run is performed in which aphidicolin is added 4 h before the end of the run. Aphidicolin inhibits DNA polymerases α and δ transient DNA strand breaks, occurring during the DNA excision repair, will remain to increase the sensitivity of the test.
Cytotoxicity was monitored by measuring the intracellular ATP concentration and the release of adenylate kinase. Appropriate negative and positive controls were included.

Results
Based on the low cytotoxicity seen in the concentration range-finder, the maximum concentration considered for the comet assay was 10%, equivalent to 100 mg/ml or 1600 µg/cm^2.
In the first and second run, a biologically relevant increase in tail moment in keratinocytes or fibroblasts due to treatment with A138 was not observed. None of the four concentration groups exceeded the DNA tail intensity of the historical controls neither for keratinocytes not for fibroblasts.
To verify these negative results, a third run, introducing aphidicolin, was performed. In this experiment a biologically relevant increase in tail moment in keratinocytes or fibroblasts was not observed as well.

Conclusion
Under the experimental conditions used, A 138 was not genotoxic in this *in vitro* alkaline comet assay with reconstructed human skin tissue.

Ref.: 2 (Submission III)

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Bone marrow micronucleus test in the mouse

Guideline:	OECD 474 (1997)
Species/strain:	mouse, NMRI
Group size:	5 males and 5 females per test group
Test substance:	A 138
Batch:	PVS 11/02
Purity:	99.7 area% (HPLC)
Vehicle:	30% DMSO/70% CMC (2%)
Dose level:	0, 312.5, 625 and 1250 mg/kg bw
Route:	intraperitoneal injection
Sacrifice times:	24 h and 48 (highest dose only) h after injection
GLP:	in compliance
Study period:	29 September 2005 – 22 November 2005

A 138 was investigated for the induction of micronuclei in bone marrow cells of mice. Test doses were based on the results of a pre-experiment for toxicity. Mice were treated ip with 100, 250, 1000, 125 and 1500 mg/kg bw under identical conditions as in the main test and

observed for acute toxic symptoms at intervals around 1, 2-4, 6, 24, 30 and 48 h post application.
In the micronucleus test mice were treated by ip injection with 0, 312.5, 625 and 1250 mg/kg bw. The mice of the highest dose group were examined for acute toxic symptoms at intervals of around 1, 6, 24 and 48 h after treatment. Bone marrow cells were collected 24 h and 48 h (highest dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/TE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment for toxicity, 2 of the 4 mice (1 females and 1 male/dose) treated with 1500 mg/kg bw died 24 h after treatment. Animals treated with 1250 mg/kg bw showed clinical signs like reduction spontaneous activity, ruffled fur, abdominal position, eyelid closure and apathy. These clinical signs decreased with lower doses applied. From 250 mg/kg bw and above, the urine of the treated mice had a distinct yellow orange colour.
In the micronucleus test identical clinical signs were observed after treatment with the highest dose. At 625 and 1250 mg/kg bw the urine of the mice had a yellow-orange colour.
In comparison to the concurrent negative controls, in the micronucleus test the PCE/TE ratio was not affected. However, the yellow-orange coloured urine points to systemic distribution of A 138 and thus bioavailability.
Compared to the concurrent vehicle controls, a dose dependent and statistically significant increase in the number of bone marrow cells with micronuclei was observed. The relevance of this increase could be confirmed by increasing the number of evaluated PCE's to 6000 per mouse. However, all values are within the range of the historical negative control values. As this leaves the possibility that the dose dependence of the results is not biologically relevant, the results are considered inconclusive.

Conclusion
Under the experimental conditions used, the results of the present experiment do not allow a conclusion on the genotoxic (clastogenic and/or aneugenic) potential of A 138.

Ref.: 11 (Submission II)

SCCS comment
The results of the present experiment are inconclusive since although a dose dependent increase in cells with micronuclei was found, the positive values were within the range of the historical control data. If results are inconclusive they should be clarified by further testing. The test was repeated as reference 12, submission II.

Bone marrow micronucleus test in the mouse

Guideline:	OECD 474 (1997)
Species/strain:	mouse, NMRI
Group size:	5 males and 5 females per test group
Test substance:	A 138
Batch:	PVS 11/02
Purity:	99.7 area% (HPLC)
Vehicle:	30% DMSO/70% CMC (2%)
Dose level:	0, 312.5, 625 and 1250 mg/kg bw
Route:	intraperitoneal injection
Sacrifice times:	24 h and 48 (highest dose only) h after injection
GLP:	in compliance
Study period:	14 December 2005 – 22 December 2005

A 138 was investigated for the induction of micronuclei in bone marrow cells of mice. As the study has previously been performed under the same conditions (reference 11, submission II), the doses were based on the results of a pre-experiment for toxicity performed in this study.
In the micronucleus test mice were treated by ip injection with 0, 312.5, 625 and 1250 mg/kg bw. The mice of the highest dose group were examined for acute toxic symptoms at intervals of around 1, 6, 24 and 48 h after treatment. Bone marrow cells were collected 24 h and 48 h (highest dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/TE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the micronucleus test, identical clinical signs, except for apathy, were observed after treatment with the highest dose compared to the results found in the pre-test for cytotoxicity described in reference 11, submission II. At 625 and 1250 mg/kg bw the urine of the mice had a yellow-orange colour.
In comparison to the concurrent negative controls, in the micronucleus test the PCE/TE ratio was not affected. However, the yellow-orange coloured urine points to systemic distribution of A 138 and thus bioavailability.
Compared to the concurrent vehicle controls, a biologically relevant increase in the number of bone marrow cells with micronuclei was not observed for the groups sacrificed 24 h after begin of treatment. All obtained values were within the range of the historical negative control values.
For the 48 h preparation group a statistically significant increase in the number of bone marrow cells with micronuclei was found compared to the untreated controls. This effect is strongly disputed since there are no indications for a delayed mutagenic effect of A 138. A delay can be expected when there is a high bone marrow cytotoxicity or the concentration of A138 in the target organs is at a later time point. In the present test the analysis of the PCE/TE ratio did not give any indications of induced bone marrow cytotoxicity.
Within 6 h the urine of the animals treated with A 138 already had a yellow orange colour, indicating bioavailability. Finally the individual and group value are within the range of the historical control data. Consequently, the results found in the 48 h preparation group are considered not biologically relevant.

Conclusion
Under the experimental conditions used A 138 is not genotoxic (clastogenic and/or aneugenic) in this micronucleus test in bone marrow cells of mice.

Ref.: 12 (Submission II)

SCCS comment
In the present experiment, a statistically significant increase in the number of bone marrow cells with micronuclei was found compared to the untreated controls for the 48 h preparation group. The authors demonstrate that this result can be considered as biologically not relevant. SCCS agrees with this explanation.

3.3.7. Carcinogenicity

Taken from SCCNFP/0697/03

Malignant transformation of C31-1-mouse M2-fibroblasts *in vitro*

The test substance was dissolved in dimethylsulfoxide (DMSO) and tested in a concentration range of 50-4000 µg/ml (without and with addition of an external metabolising system; S9, Aroclor-1254 induced). C3H-mouse M2-fibroblasts were used as indicator cells. The solvent served as negative control. Positive control substances were N-methyl-N'-nitro-N-nitrosoguanidine (0.5 µg/ml), methylcholanthrene (10 µg/ml) and 2-acetylaminofluorene (10 µg/ml).

Results
The test substance was tested up to concentrations inducing significant cytotoxicity; the compound was detoxified by microsomal metabolism. 2,6-Dihydroxyethylaminotoluene was inactive at inducing malignant transformation *in vitro*. The positive controls yielded the expected results indicating the proper functioning of the indicator cells and the external metabolising system. The cell transformation test used has not been validated.

Ref.: 13 (Submission I)

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Taken from SCCS/1425/11 (=submission II)

Dose range finding study in rats

At least six mated female rats were used per dose group. The test substance was suspended in 0.5 % aqueous Na-carboxymethylcellulose and administered by oral gavage at doses of 10, 32, 100, 316, and 1000 mg/kg bw/d from day 6 to 15 of gestation, the control group received water. Signs of toxicity, body-weight changes and food consumption were recorded. After dissection from the uterus the foetuses were weighed and externally examined.

Results
At 100 mg/kg bw/d statistically significant body-weight reduction was noted. No changes in the foetal parameters were noted. For the main study, the doses 40, 200 and 1000 mg/kg bw/d were chosen.

Ref.: 15 (Submission II)

Taken from SCCNFP/0697/03

Guideline:	OECD 414
Species/strain:	Wistar rats
Group size:	24 females
Test substance:	A138 gavage in CMC
Batch:	Pt 1/91
Purity:	> 99.8 area% (HPLC)
Dose levels:	0, 40, 200 and 1000 mg/kg bw
Treatment period:	day 6 to day 15
GLP:	in compliance

Virgin female and male Wistar rats (Crl: (WI) BR) were used. The mean bodyweight of females at day 0 of gestation ranged from 275 to 279 g. The test was performed according to OECD 414.

The test substance (purity >99.5%) was suspended in an aqueous 0.5% Na-carboxymethylcellulose solution.
Beginning on day 6 of gestation (day 0 = day of detecting vaginal plug or sperms), the test substance was applied by gavage once daily until day 15 to groups of 24 mated female rats at doses of 40, 200 or 1000 mg/kg bw (groups A, B and C). The control group K received the vehicle only. Signs of toxicity, body-weights and food consumptions of the dams were recorded.
On day 20, the dams were necropsied and examined for the number of *corpora lutea*, implantations, viable foetuses, early and late deaths. The viable foetuses were weighed, sexed and examined for gross malformations. Approximately one half of the foetuses was examined for skeletal anomalies and the other half for internal anomalies.

Results
In the control group, significantly more litters with foetuses with incompletely ossified or unossified hyoid were observed in comparison with all dosed groups. In one control foetus, an abnormal curvature of the spine and fused sternebrae were noted.
According to the authors, no test-substance related effects on dams or foetuses were diagnosed in groups A (40 mg/kg bw) and B (200 mg/kg bw). In all dosed groups the observed effects on foetuses were not considered as test-substance related, since they were infrequent and not dose-dependent.
A slight maternal toxicity caused by the test substance in the highest dose group C (1000 mg/kg bw) was assumed due to a "statistically significant" decreased bodyweight at the beginning of the dosing period. This finding is limited to one recorded measurement period only and inconsistent with the overall weight development. Thus, the relevance of this result is limited, no clear-cut dose for maternal toxicity was observed. A slight but dose-dependent increase in post implantation losses was noted (mean values: group K, 5.8; group A, 6.4; group B, 7.8; group C, 10.9) but not interpreted as abnormal.

Conclusion
The overall NOAEL is 200 mg/kg bw.

Ref.: 14 (Submission I)

3.3.9. Toxicokinetics

Taken from SCCS/1425/11 (=submission II)

Guideline:	/
Species/Strain:	Rats, male, female, Sprague-Dawley, SPF-quality
Test substance:	^{14}C-labelled test substance incorporated in a hair dye formulation (**A, D**) and in an aqueous solution (**B, C, E**)
Batch:	2(WSI-111) (Purity: 99%)
Study groups:	**A:** 0.5 h dermal exposure (formulation), sacrifice after 72 h **B:** 0.5 h dermal exposure (solution), sacrifice after 72 h **C:** 72 h oral exposure (solution) **D:** 0.5 h dermal exposure (formulation), sacrifice after 24 h **E:** 24 h oral exposure (solution)
Dose level:	**A** 0.66 mg/cm² of the test substance (56.9 mg/cm² of formulation) **B** 0.56 mg/cm² of the test substance (33.6 mg/cm² of solution) **D** 0.65 mg/cm² of the test substance (56.6 mg/cm² of formulation) **C** 26.3 mg/kg bw **E** 26.9 mg/kg bw
Exposure time:	30 min dermal exposure and 24 h or 72 h follow up
GLP:	not in compliance

5 male and 5 female Sprague-Dawley rats (Him: OFA, SPF) were used in each of the 5 experiments. The bodyweights were approximately 200 g. The ^{14}C-labelled test substance was integrated in a hair dyeing formulation (0.18% 14C-"WSI-111", 0.98% "WSI-111", 1% p-Phenylenediamine hydrochloride, 97.94% basic formulation) or used as a solution in water (**B**: 16.7 mg/ml, **C** and **E**: 5 mg/ml). The stability of the test substance (in solution and in formulation) was checked and considered satisfactory. The hair dyeing formulation was mixed with oxigenta lotion (containing 6% hydrogen peroxide) before application. The test substance was applied to the clipped dorsal skin of rats (3 cm x 3 cm) for 30 min and then washed off. The concentration on skin was 0.58% (formulation) and 0.5% (solution). Radioactivity of rinsings, application site, urine, faeces, blood, organs and carcass was estimated by liquid scintillation counting. Groups **C** and **E** received the test solution (about 26 mg/kg bw) orally by stomach tube. Group **C** was sacrificed after 72 h and urine, faeces, organs, and carcass without gastrointestinal tract were examined for radioactivity. Group **E** was killed after 24 h and radioactivity was determined in the blood.

Results, dermal application
Under the experimental conditions, total recoveries of the test substance of 97.7% (formulation) and 99.3% (aqueous solution) were obtained. The majority of the applied ^{14}C-labelled test substance was removed from the skin with the washing procedure (95.5% for the formulation and 96.1% for the aqueous solution). The amount of ^{14}C penetrated was calculated by adding the amounts eliminated from the body (i.e. urine 0-72 h plus faeces 0-72 h) and the amounts of ^{14}C still being present in the carcass.

When the formulation was used (group **A**), the application site contained a mean ^{14}C-activity of 2.1% of the dosed ^{14}C and 3% when the test substance solution was applied. In the animals of group **D**, the blood level of radioactivity was highest at 35 min p.a. and declined with a half-life of approximately 50 min. In groups **A** and **B**, the observed detection limit ranged from ca. 0.0005% dose/g for thyroids to 0.00002% dose/g for large organs (i.e., ca. 0.03-0.001 µg equivalents of the test substance/g). Mean radioactivity concentrations in blood and the 14 analysed organs were all below or at the detection limit in groups **A** and **B** at 72 h after dosing. The radioactivity was excreted mainly via urine (82 - 89% of eliminated ^{14}C) and to a lesser extent via faeces (11 - 18% of eliminated ^{14}C). The excretion was fast: A mean of 99% of the totally eliminated ^{14}C was excreted within the first 24 h in groups **A** and **B**. Relatively highest concentrations of radioactivity were determined in group **A** in thyroids, adrenals and femur and in group **B** in thyroids, carcass and skin.

The mean percutaneous penetration of the test substance was 0.078% of the administered ^{14}C for the formulation (0.515 µg/cm²) and 0.128% (0.838 µg/cm²) for the aqueous solution

Results, oral dosing
After application of 26.3 mg test substance/kg bw, 83.7% were recovered in urine and 0.068% in the carcass at 72 h p.a. (group **C**). The radioactivity was excreted mainly via urine (88% of eliminated ^{14}C) and to a lesser extent via faeces (12% of eliminated ^{14}C). The excretion was fast: 99% of the total eliminated radioactivity was excreted within the first 24 h. In group **C**, the detection limit ranged from approximately 0.002% dose/g for thyroids to 0.00006% dose/g for large organs (i.e., ca. 0.01 and 0.003 µg equivalents of test substance/g). As far as can be judged from the very low ^{14}C concentrations, the distribution into the organs was not too different from that observed after dermal application.
In the blood and in the 14 analysed organs, the ^{14}C-concentrations were below or near the detection limit. At 72 h p.a., relatively highest concentrations of radioactivity were found in thyroids, skin and kidneys, lowest in testes, brain and muscle. In the animals of group **E**, the blood levels of radioactivity were highest at 35 min. p.a. and declined with an initial half-life of approximately 40 min.
An oral absorption of at least 83.8% (i.e., 22 mg test substance/kg bw) was calculated.

Conclusion
Experiments carried out with radio-labelled test formulations in toxicokinetic investigations including cutaneous and oral (gavage) application showed a low dermal penetration rate – between 0.08 and 0.13% of the applied radioactivity – compared with an absorption rate of about 84% after oral application; in both cases, more than 99% of the radioactivity was excreted during the first 24 hours after application, about 88% via urine.

Ref.: 15 (Submission I)

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(……. conditions)
(…..% formulation, on head concentration …..%)

Absorption through the skin	**A**	**=**	**3.73 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.16 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.036 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**100 mg/kg bw/d**
Bioavailability 84%*		**=**	**84 mg/kg bw/d**

Margin of Safety	**adjusted NOAEL/SED**	**=**	**2300**

*.see reference 15, submission II

3.3.14. Discussion

Physico-chemical properties

2,6-Dihydroxyethylaminotoluene is used as a precursor for hair colours. It reacts with primary intermediates to form the final dye-stuff. The reaction can be accelerated by the addition of an oxidising agent (e.g. hydrogen peroxide), but it can also be achieved by air oxidation. The final concentration on head of 2,6-Dihydroxyethylaminotoluene can be up to 1.0%.

2,6-Dihydroxyethylaminotoluene is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in 2,6-dihydroxyethylaminotoluene is not reported. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb.

Stability of 2,6-dihydroxyethylaminotoluene in typical hair dye formulations is not reported.

Irritation, sensitisation

HC Violet AS is not irritant to rabbit skin and is an eye irritant. 2,6-Dihydroxyethylaminotoluene is not a sensitiser.

Dermal absorption

The mean value of dermal absorption of HC Violet AS was 2.33 ± 0.70 µg/cm^{2}under oxidative conditions. As only two donors were used, the mean + 2SD (3.73 µg/cm²) could be used for the MoS calculation.

Toxicokinetics

Experiments carried out with radio-labelled test formulations in toxicokinetic investigations including cutaneous and oral (gavage) application showed a low dermal penetration rate – between 0.08 and 0.13% of the applied radioactivity – compared with an absorption rate of about 84% after oral application; in both cases, more than 99% of the radioactivity was excreted during the first 24 hours after application, about 88% via urine.

General toxicity

2,6-Dihydroxyethylaminotoluene is of low acute toxicity; > 2 000 mg/kg bw in rats. The NOAEL, derived from a 90-day study in rats, is 100 mg/kg bw/d, target organs were the liver and the kidneys. The results of a teratogenicity study showed an overall NOAEL of 200 mg/kg bw/d. No teratological abnormalities were recorded. No reproductive toxicity study was provided.

Mutagenicity

Overall, the genotoxicity of 2,6-dihydroxyethylaminotoluene was investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy.

2,6-dihydroxyethylaminotoluene did induce gene mutations in bacteria. Two well performed gene mutation tests in mammalian cells (mouse lymphoma cells) were negative. The negative results in these gene mutation tests in mammalian cells were confirmed in a comet assay in reconstructed human skin. This *in vitro* model, which detects both substances that induce gene mutations and structural chromosome aberrations, resembles human skin and may be considered as a surrogate for an *in vivo* gene mutation test. The absence of DNA damage in this comet assay in reconstructed human skin may indicate that the probability that 2,6-dihydroxyethylaminotoluene has the potential to induce gene mutations is small. Consequently, 2,6-dihydroxyethylaminotoluene can be considered to have no gene mutation-inducing potential.

An *in vitro* micronucleus test demonstrated an increase in cells with micronuclei. The clastogenicity found *in vitro* could not be confirmed *in vivo*. 2,6-dihydroxyethylaminotoluene

did not induce micronuclei in an *in vivo* micronucleus test in erythrocytes of mice. A second test showed inconclusive results.
Consequently, 2,6-dihydroxyethylaminotoluene can be considered to have no genotoxic potential and additional tests are unnecessary.

Carcinogenicity
2,6-Dihydroxyethylaminotoluene did not induce transformation in a non-validated *in vitro* transformation test.

4. CONCLUSION

1. In light of the new data provided, does the SCCS consider 2,6-Dihydroxyethylaminotoluene (A138) safe when used as a direct dye and as an ingredient in oxidative hair dye formulations up to a final on-head concentration of 1%?

In the light of the new data provided, SCCS considers that the use of 2,6-dihydroxyethylaminotoluene as an ingredient in oxidative hair dye formulations at a maximum concentration of 1.0% on the head is safe.

2. Does the SCCS have any further scientific concerns with regard to the use of 2,6-Dihydroxyethylaminotoluene (A138) in cosmetic products?

2,6-Dihydroxyethylaminotoluene is a secondary amine, and thus, it is prone to nitrosation. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb.

5. MINORITY OPINION

Not applicable.

6. REFERENCES

Submission I, 1995

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