OPINION OF THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS INTENDED FOR CONSUMERS

CONCERNING

2,6-DIHYDROXYETHYLAMINOTOLUENE

COLIPA n° A138

adopted by the SCCNFP during the 25th plenary meeting
of 20 October 2003

1. Terms of Reference

1.1. Context of the question

The adaptation to technical progress of the Annexes to Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products.

1.2. Request to the SCCNFP

The SCCNFP is requested to answer the following questions:

* Is 1-Methyl-2,6-bis-(2-hydroxyethylamino) benzene safe for use in cosmetic products?

* Does the SCCNFP propose any restrictions or conditions for its use in cosmetic products?

1.3. Statement on the toxicological evaluation

The SCCNFP is the scientific advisory body to the European Commission in matters of consumer protection with respect to cosmetics and non-food products intended for consumers.

The Commission's general policy regarding research on animals supports the development of alternative methods to replace or to reduce animal testing when possible. In this context, the SCCNFP has a specific working group on alternatives to animal testing which, in co-operation with other Commission services such as ECVAM (European Centre for Validation of Alternative Methods), evaluates these methods.
The extent to which these validated methods are applicable to cosmetic products and its ingredients is a matter of the SCCNFP.

SCCNFP opinions include evaluations of experiments using laboratory animals; such tests are conducted in accordance with all legal provisions and preferably under chemical law regulations. Only in cases where no alternative method is available will such tests be evaluated and the resulting data accepted, in order to meet the fundamental requirements of the protection of consumer health.

2. Toxicological Evaluation and Characterisation

2.1. General

2.1.1. Primary name

2,6-Dihydroxyethylaminotoluene (INCI)

2.1.2. Chemical names

Chemical name : 1-Methyl-2,6-bis-(2-hydroxyethylamino)-benzene
CAS name : Ethanol, 2,2'-[(2-methyl-1,3-phenylene)diimino]bis-
Synonyms : 2,6-Di-(2-hydroxyethylamino)toluene;
2,6-bis-(β-hydroxyethylamino)toluene;
2,6-bis[(2-hydroxyethyl)amino]toluene

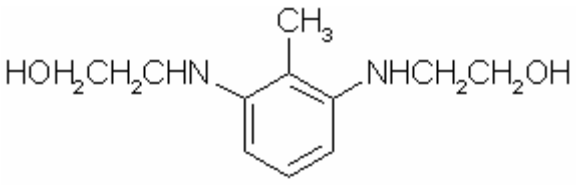
2.1.3. Trade names and abbreviations

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

2.1.4. CAS No. / EINECS No.

CAS No. : 149330-25-6
EINECS No. : /

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. formula : $C_{11}H_{18}N_2O_2$
Mol. weight : 210.28

2.1.7. Purity, composition, and substance codes

Purity (batch not identified)
titre as determined by HPLC : ≥ 99.5%
water content : not assessed or not reported
ash content : not assessed or not reported

Potential impurities and reaction intermediates	:	not assessed or not reported
Solvent residues	:	not assessed or not reported
Other		
NaCl	:	0.2%
Unidentified material	:	0.1%

2.1.8. Physical properties

Appearance	:	Odourless, light brown-greyish powder
Melting point	:	118-121 °C
Boiling point	:	/
Density	:	/
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{OW}	:	/

HPLC procedure and features provided. TLC and UV-Vis and MS spectral characteristics also available for identification purposes.

2.1.9. Solubility

Water	:	27 g/l (20°C)
Ethanol	:	100 g/l (20°C)
Receptor fluid	:	/

2.1.10 Stability

Stable for years when pure; stable for hours to months in an aqueous medium at room temperature, as specifically assessed

General comments on analytical and physico-chemical characterisation

* 1-Methyl-2,6-bis-(2-hydroxyethylamino)-benzene purity was reported in Section 2 "General Data" of Submission I (2001) with no clear identification of the batch (see next).
* No official measure of purity consistency is available from the analysis of more than one batch. However, only Batches No. 2 and 1/91 seem to have been used in toxicological testing, the purity of both complying with the lower limit value (≥ 99.5%) reported.
* The high and consistent level of chemical purity assessed by HPLC, combined with the tiny amounts (0.2%) of NaCl detected, should allow other substances (unidentified) to be present up to 0.3% only.
* 1-Methyl-2,6-bis-(2-hydroxyethylamino)-benzene is a secondary alkanolamine, and thus it is prone to nitrosation. No data are provided on the nitrosamine content of the dye and its formulations.
* The physico-chemical profile of the substance is insufficiently characterized (e.g., density and Log P_{OW} not reported). However, essential parameters are given.

2.2. Function and uses

1-Methyl-2,6-bis(2-hydroxyethylamino)-benzene will be used in oxidative hair dye formulations at a maximum concentration of 1%, after adding the oxidative agent.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

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 No.	:	#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 body weight 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

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

No data

2.3.5. Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline	:	OECD n° 408
Species/strain	:	Wistar rats
Group size	:	20 males and 20 females
Test substance	:	A 138 in 0.5 % aqueous Na-carboxymethylcellulose solution
Batch No.	:	Pt 1/91 (purity 99.5 %), 0.2 % NaCl
Dose	:	0, 100, 316 and 1000 mg/kg bw/day
Exposure period	:	90 days
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 body weight 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 an 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 and day 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, that were treated as their corresponding groups for 92 days and then maintained without treatment for additional 28 days.

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 practically in all 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).

The NOAEL was reported to be 100 mg/kg bw/day. 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).

Ref. : 5

2.3.8. Sub-chronic dermal toxicity

No data

2.3.9. Sub-chronic inhalation toxicity

No data

2.3.10. Chronic toxicity

No data

2.4. Irritation and corrosivity

2.4.1. Irritation (skin)

Guideline	:	OECD n° 404 / EC B.4
Species/strain	:	New Zealand white rabbits
Group size	:	3
Test substance	:	A138 1 % (w/v) a.i. in water
Batch No.	:	#2 (purity : 99.5%)
Dose	:	0.5 ml (patch test)
GLP	:	in compliance

Three New Zealand white rabbits were used. Their body weights ranged between 2.28 - 2.54 kg. The method used followed OECD Guideline n° 404 (1981), referenced as Method B4 in Commission Directive 84/449/EEC.

The test compound was made up to a 1 % (w/v) dilution with distilled water and mixed thoroughly on a rotamixer. An amount of 0.5 ml of the test compound preparation was introduced under a 2.5 cm x 2.5 cm gauze patch and placed on clipped skin. The patch was fixed and the rabbits were wrapped in an elasticated corset (TUBIGRIP). At 4 h after application, the corset and the patches were removed and the residual test material was removed with distilled water. Readings of skin reactions were made at 1, 24, 48 and 72 h after removal of the patches and scored following the scale of Draize.

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

Ref. : 2

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD n° 405 / EC B.5
Species/strain	:	New Zealand White Rabbits
Group size	:	3
Test substance	:	1 % (w/v) a.i. in water
Batch No.	:	#2 (purity : 99.5%)
Dose	:	0.1 ml
GLP	:	in compliance

Three New Zealand white rabbits were used. Their body weights 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 scale of Draize.

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

Ref. : 3

2.5. Sensitisation

Guideline	:	OECD n° 406
Species/strain	:	Guinea Pigs
Group size	:	15 male and 15 female (10/10 + 5/5)
Test substance	:	WS I-111
Batch No.	:	#2 (purity : 99.5%)
Concentration	:	0.5 % (w/v) a.i. olive oil / 1 % day 8
GLP	:	in compliance

30 Dunkin Hartley guinea pigs (15 males and 15 females) were used. 10 males and 10 females were treated, 5 males and 5 females served as controls.
The test was performed according to the maximisation method of Magnusson and Kligman, OECD Guideline n° 406 and the Principles of GLP (OECD, 12.5.1981).

During a ten-day sensitisation period (day 1 to 10), the test substance was administered by intradermal injection (day 1, 0.1 ml at a concentration of 0.5% in paraffin oil) and by cutaneous application (day 8, 1 %). The control group received the vehicle (paraffin oil) only. The intradermal administrations of the test substance and vehicle (control) were performed in the presence of Freund's complete adjuvant in order to maximise any potential cutaneous sensitisation reactions. After a 12-day rest period, for challenge, another cutaneous application (0.5 ml at a concentration of 1 % in the vehicle) was performed (day 22; 24 h occlusion). The cutaneous reactions were then evaluated at 24 and 48 h after removal of the dressing.

Results
The induction concentration used was far too low and therefore the study is considered inadequate.

Ref. : 4

2.6. Teratogenicity

Guideline	:	OECD n° 414
Species/strain	:	Wistar rats
Group size	:	24 females
Test substance	:	A138 gavage in CMC
Batch No.	:	Pt 1/91
Purity	:	> 99.5 %
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 body weight of females at day 0 of gestation ranged from 275 to 279 g. The test was performed according to OECD Guideline n° 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) and B (200 mg/kg). 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) was assumed due to a "statistically significant" decreased body weight 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. The overall NOAEL is 200 mg/kg bw.

Ref. : 14

2.7. Toxicokinetics (including Percutaneous Absorption)

5 male and 5 female Sprague-Dawley rats (Him: OFA, SPF) were used in each of the 5 experiments. The body weights were approximately 200 g.

The ^{14}C-labelled test substance was integrated in a hair dyeing formulation (0.18% ^{14}C-"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 hair dyeing formulation was mixed with oxigenta lotion (containing 6% hydrogen peroxide) before application.

Cutaneous application

Guideline	:	/
Species/Strain	:	Rats, male, female, Sprague-Dawley, SPF-quality
Test substance	:	^{14}C-labelled test substance incorporated in a hair dye formulation and in an aqueous solution
Batch No	:	2(WSI-111) (Purity: 99%)
Dose level	:	0.66 mg/cm² of the test substance (56.6 mg/cm² of formulation) 0.56 mg/cm² of the test substance (33.6 mg/cm² of solution)
Exposure time	:	30 min exposure and 24 h or 72 h follow up
GLP	:	not in compliance

5 Females and 5 males were used in each of the following studies:

Study A	:	0.5 h dermal exposure (formulation), sacrifice after 72 h
Study B	:	0.5 h dermal exposure (solution), sacrifice after 72 h
Study C	:	72 h oral exposure (solution)
Study D	:	0.5 h dermal exposure (formulation), sacrifice after 24 h
Study E	:	24 h oral exposure (solution)

The test substance was integrated in a hair dyeing formulation or used as a solution in water. The stability of the test substance (in solution and in formulation) was checked and considered satisfactory.
^{14}C-labelled of the test substance was applied to the clipped dorsal skin of rats (3 cm x 3 cm) for 30 min and was then washed off. Radioactivity of rinsings, application site, urine, faeces, blood, organs and carcass was estimated by liquid scintillation counting.

Results
Under the present experimental conditions, total recoveries of the test substance of 97.7 % (formulation) and 99.3 % (aqueous solution) have been 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.
The ^{14}C-labelled test substance was excreted to a larger extent via urine (88 % of the eliminated ^{14}C) and to lesser extent via faeces (12%). The excretion was fast: A mean of 99 % of the totally eliminated ^{14}C was excreted within the first 24 h.

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.

Oral dosing

Two further groups (groups C, E) received the test solution (about 26 mg/kg) 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
The compound 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 mean excretion was fast: 88-94% were eliminated during the first 24 h in groups A and B. 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. Relatively highest concentrations of radioactivity were determined in group A in thyroids, adrenals and femur and in group B in thyroids, carcass and skin. 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).
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.

Oral dosing : An oral absorption of at least 83.8% (i.e., 22 mg test substance/kg bw) was calculated. 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 mean excretion was fast: 99% of the total eliminated radioactivity was excreted within the first 24 h. 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 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 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.
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 via the kidneys/urine.

Ref. : 15

2.8. Mutagenicity/Genotoxicity

2.8.1. Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline : /

Species/strain	:	*S. typhimurium,* TA98, TA100, TA1535, TA1537, TA1538
Replicates	:	Triplicate plates, No independent repeat test
Test substance	:	WS I-111/2 in water
Batch no	:	no data
Purity	:	no data
Concentrations	:	With and Without metabolic activation 50, 100, 500, 1000, 2500, 5000 μg/plate (6 doses)
GLP	:	not in compliance

A 138 has been investigated for gene mutation in *Salmonella typhimurium* using the plate incorporation method both with and without S9 mix. Liver S9 fraction from rats pretreated with β-naphthoflavone and phenobarbitone was used as the exogenous metabolic activation system.

Results
Dose range finding assay
No data

Final test
In the absence of activation : no dose related and biologically relevant increase in revertant numbers was observed, in any of the tester strains.
In the presence of rat activation : A dose related and biologically relevant increase in revertant numbers was observed, in the TA 98 and TA 1538 tester strains.

Conclusions
Based on the reversion rate, and under the conditions of the 2 assays performed, it could be concluded that the test agent, in the presence of reductive S9 mix, shows clear evidence of mutagenic activity in tester strain TA 98.
The test is unsuitable for genotoxicity and/or mutagenicity evaluation. (test substance, purity, batch not characterised; no dose range finding data, no repeat experiment).

Ref. : 6

In Vitro Mammalian Cell Gene Mutation Test

Guideline	:	/
Cells	:	Chinese Hamster V-79 cell line (mutation at the HPRT locus)
Replicates	:	2 independent tests with and without S9 mix but performed at different dates
Test substance	:	WSI-111 in water
Batch no	:	No data
Purity	:	No data
GLP	:	In compliance
Concentrations	:	Test # 1 and test # 2 With or Without metabolic activation : 10, 30, 100, 300, 1000 μg/ml (5 doses) With metabolic activation 10, 30, 100, 300, 1000 μg/ml (5 doses)

A 138 has been investigated for gene mutation at the HPRT locus in V79 Chinese hamster cell line in the presence or absence of activation system. The origin of the S9 activation system is not described.

Results
Not taken into consideration due to inadequacy of the protocol used.

Conclusions
The test is unsuitable for genotoxicity and/or mutagenicity evaluation. (test substance, purity, batch not characterised; no dose range finding data, initial and repeat experiments performed at different dates, no guarantee exists that the same dilutions factors of the test agent were used because they do not originate from the same stock solution. The number of cells seeded may not be identical).

Ref. : 7

***In Vitro* Mammalian Cell Gene Mutation Test**

Guideline	:	OECD 476
Cells	:	Chinese Hamster V-79 cell line (mutation at the HPRT locus)
Replicates	:	2 independent tests
Test substance	:	WSI-111 in DMSO
Batch no	:	2
Purity	:	99.8 % (HPLC)
Concentrations	:	Test # 1 & 2 Without metabolic activation : 100, 500, 1000, 3000, 5000 µg/ml (5 doses) With metabolic activation : 100, 500, 1000, 3000, 5000 µg/ml (5 doses)
GLP	:	In compliance

A 138 has been investigated for gene mutation at the HPRT locus in V79 Chinese hamster cell line in the presence or absence of activation system. No visible precipitate occurred. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guidelines.

Results
Toxicity
No sign of cytotoxicity were seen in both assays in the presence or absence of activation systems

Precipitate
No visible precipitation was observed.

Mutant frequency
Test # 1
No statistically or biologically significant increase in mutant frequency was observed over the concurrent solvent controls for any doses in all conditions.
Test # 2
No statistically or biologically significant increase in mutant frequency was observed over the concurrent solvent controls for any doses in all conditions.

Conclusions
The test is acceptable for evaluation. A 138 did not demonstrate mutagenic potential on the HPRT gene of V79 cells.

Ref. : 8

***In Vitro* Mammalian Chromosomal Aberration Test**

Guideline	:	88/320 EEC
Species/strain	:	Human lymphocytes
Replicates	:	Duplicate cultures, 2 independent experiments
Test substance	:	WSI-111 in water
Batch no	:	2
Purity	:	99.8 % (HPLC)
Concentrations	:	Test #1 With or without S9 mix 4 h treatment – 20 h harvest : 1250, 2500, 5000 μg/ml Test # 2 With or without S9 mix 4 h treatment – 30 h harvest : 1250, 2500, 5000 μg/ml
GLP	:	In compliance

A 138 has been investigated for induction of chromosomal aberrations in human lymphocytes withdrawn from one volunteer. The test concentrations were established from a preliminary toxicity study.
Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

Results
Toxicity
A dose dependent decrease in the mitotic index has been observed without S9 mix ; no significant alteration was observed in the presence of activation system.

Structural chromosome aberrations
Exp # 1 and Exp # 2 with or without S9 mix
No statistically or biologically significant increase in the number of aberrant cells was observed as compared to the corresponding solvent control for all doses at any treatment time.

Numerical chromosome aberrations
No significant increase of aneuploidy cells was noted.

Conclusions
A 138 is considered negative for its clastogenic and/or aneugenic potential in human lymphocytes in the presence or the absence of activation system under the conditions of the test.

Ref. : 9

Unscheduled DNA Synthesis in Mammalian Cells *in vitro*

Guideline	:	/
Species/strain	:	Rat hepatocytes

Replicates	:	2 independent tests
Test substance	:	WSI-111 in DMSO solution
Batch no	:	no data
Purity	:	no data
Concentrations	:	Test # 1, 10 concentrations : 1.95 - 1000 µg/ml Test # 2, 6 concentrations : 62.5 - 2000 µg/ml
GLP	:	not in compliance
Exposure time	:	3 h

A 138 in DMSO solution was tested for induction of UDS in freshly isolated hepatocytes in two independent experiments, at concentrations ranging from1.95 to 2000 µg/ml.

Results
Due to the several important technical lacks in the assay presented, results have been examined but were inadequate and uninformative, therefore, they are not described in this report.

Conclusions
This study is unsuitable for an accurate evaluation. (absence of physicochemical characterisation, no guidelines, not GLP, no information about the exposure times, only 20 cells scored instead of at least 50).

Ref. : 10

***In vitro* Sister Chromatid Exchange Assay in Mammalian Cells**

Guideline	:	OECD 479
Species/strain	:	Chinese hamster ovary cell lines (CHO)
Replicates	:	Duplicate cultures, no independent repeat experiment
Test substance	:	A 138 in DMSO
Batch no	:	Pt 1/91
Purity	:	99.8 % (HPLC)
Concentrations	:	Test #1 Without S9 mix 24 h treatment with BrdU – 300, 600, 1200, 2400 µg/ml 3h treatment then 24 incubation with BrdU – 300, 600, 1200, 2400 µg/ml With S9 mix 3h treatment then 24 incubation with BrdU – 300, 600, 1200, 2400 µg/ml
GLP	:	In compliance

A 138 has been investigated for induction of Sister Chromatid Exchange in Chinese hamster cells. The test concentrations were established from a preliminary toxicity study.
Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

Results

Test with or without S9 mix
Most of the cultures tested showed a statistically significant increase in the mean number of SCE/cell when compared to the corresponding negative control.

Conclusions
A 138 is considered positive for its recombinogenic potential in mammalian cells in the presence or the absence of activation system under the conditions of the test. While some shortcomings are present, such as the too few number of cells scored, positive results were obtained. However, the statistical significance has been set using the Student t test, which requires a normal distribution of data. Such distribution has not been tested. Not suitable for an appropriate evaluation.

Ref. : 11

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474
Species	:	CD-1 mice
Group sizes	:	5 males and 5 females
Material	:	WSI-111 in distilled water
Batch no	:	2
Purity	:	Not given in this study but indicated in the *in vitro* CA test (99.8 % (HPLC))
Dose levels	:	Maximum Tolerated Dose (MTD) A dose-range finding assay was conducted to determine the MTD and the appropriate route of administration. According to clinical signs and toxic reactions of the mice, the top dose has been chosen to be 5000 mg/kg bw to be given orally. One single oral dose (5000 mg/kg bw) of WSI-111 was administered by single intragastric gavage. Three sacrifice times were selected 24 h, 48 h, 72 h.
GLP	:	In compliance

WSI-111 has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. A preliminary range finding study in which toxic effects were observed determined dose levels. The test substance was administered by a single intragastric gavage and the groups of animals sacrificed 24, 48 and 72 hours after administration. Negative and positive controls were in accordance with the OECD guideline.

Number of cells scored : a total of at least 1000 erythrocytes were examined from each animal ; the incidence of micronucleated erythrocytes and the ratio of polychromatic erythrocytes to normochromatic erythrocytes were calculated.

Results
NCE/PCE ratio
When pooling the male and female data, the mean ratio has been found to be significantly altered for the 48 h and 72 h sacrifice time. This indicates that the bone marrow has been reached and that the test agent induces cytotoxic effects on bone marrow cells.
Remark : there is a large inter-individual variability in the data even in the control group :
72 hours sample : ratio NCE/PCE in ctrl – 0.54, 0.44, 0.69, 1.32 and 0.50/5000 mg/kg;
48 h – 1.20, 0.68, 1.22, 0.84, 2.1

Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal or other damage leading to the micronucleus formation in polychromatic erythrocytes treated mice.

Conclusions
Under the conditions of the test it can be concluded that A 138 at doses at which some signs of clinical toxicity were recorded, does not induce a statistically significant increase in the frequency of micronucleated polychromatic erythrocytes.

Ref. : 12

2.9. Carcinogenicity

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. 1-Methyl-2,6-bis-(2-hydroxyethylamino)-benzene 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

2.10. Special investigations

No data

2.11. Safety evaluation

CALCULATION OF THE MARGIN OF SAFETY

(2,6-Dihydroxyethylaminotoluene)
(oxidative)

The maximum concentration of 2.0 % 2,6-Dihydroxyethylaminotoluene is mixed before use with H_2O_2. Thus the usage volume of 100 ml contains at maximum 1.0 %

Maximum absorption through the skin	**A (µg/cm²)**	**=**	**0.838 µg/cm²**
Typical body weight of human		**=**	**60 kg**
Skin Area Surface (scalp)	**SAS**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.587 mg**
Systemic exposure dose (SED)	**SAS x A x 0.001/ 60**	**=**	**0. 010 mg/kg**
No observed effect level (mg/kg) (rat, 13 weeks oral)	**NOAEL**	**=**	**100 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**10000**

2.12. Conclusions

2,6-Dihydroxyethylaminotoluene is a secondary alkanolamine, and thus it is prone to nitrosation. No data are provided on the nitrosamine content of the dye and its formulations. The physico-chemical profile of the substance is insufficiently characterized (e.g., density and log P_{OW} not reported), although essential parameters are given.

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, target organs were the liver and the kidneys.
The results of a teratogenicity study showed a NOAEL of 200 mg/kg bw. No teratological abnormalities were recorded.

2,6-Dihydroxyethylaminotoluene can be classified as non-irritating for both the skin and mucous membranes at 1% in water.
The induction concentration used was far too low and therefore the sensitisation study is considered inadequate.

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 via the kidneys/urine.

2,6-Dihydroxyethylaminotoluene has been tested once in procaryotic and twice in mammalian cells for gene mutation, in mammalian cells for chromosomal aberration and sister chromatid exchanges *in vitro*. An *in vitro* UDS on rat hepatocytes has also been performed. One *in vivo* bone marrow micronucleus test has been performed.

The *in vitro* test for gene mutation in prokaryotes is unsuitable for genotoxic/mutagenic evaluation.
The first *in vitro* test for gene mutation in mammalian cells is unsuitable for genotoxic/mutagenic evaluation.
The second *in vitro* test for gene mutation in mammalian cells showed that the test agent is non mutagenic.
The *in vitro* test for clastogenicity in human lymphocytes is negative.
The *in vitro* UDS on rat's hepatocytes is unsuitable for genotoxic evaluation.
The *in vivo* micronucleus test in mice gave negative results.

Derived from the data available, the substance is considered not mutagenic.

2.13. References

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15. Forschungszentrum Seibersdorf, Report OEFZS-A-2555 (May 1993), "Toxicokinetics of WSI-1 11 ".

3. Opinion of the SCCNFP

The data submitted were insufficient for a final evaluation. The SCCNFP is of the opinion that 2,6-Dihydroxyethyl aminotoluene should fulfil the demands of the SCCNFP "Strategy Paper" (doc. N° SCCNFP/0720/03 of 24.06.2003) as to mutagenicity of possible reaction products. Also it has to be shown that under in use conditions no nitrosation can occur.

Moreover, an appropriate sensitization study is required.

The compound as such can be regarded as safe.

4. Other considerations

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5. Minority opinions

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