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

CONCERNING

N,N'-BIS-(2-HYDROXYETHYL)-2-NITRO-P-PHENYLENEDIAMINE

COLIPA n° B34

adopted by the SCCNFP on 23 April 2004
by means of the written procedure

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 N,N'-bis-(2-hydroxyethylamino)-2-nitro-p-phenylenediamine safe for use in cosmetic products as a hair dye ingredient?

* 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

N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (INCI)

2.1.2. Chemical names

* 2,2'-[(2-nitro-1,4-phenylene)diimino]bisethanol (EU inventory)
* 1,4-Bis(2-hydroxyethylamino)-2-nitrobenzene
* N1,N4-bis(β-hydroxyethyl)-2-nitro-1,4-phenylenediamine

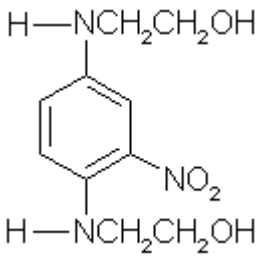
2.1.3. Trade names and abbreviations

COLIPA n° : B 34
Trade name : WS I 75
Other names : HC Violet BS

2.1.4. CAS / EINECS / Colour Index number

CAS : 84041-77-0
EINECS : 281-856-4
Colour Index : /

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_{10}H_{15}N_3O_4$
Mol weight : 241.25

2.1.7. Purity, composition and substance codes

Substance code : /
Batches used : /

Purity : /
Relative chromatographic purity
(HPLC - UV/VIS peak area method) : 99.3% at 248 nm

Impurities : 1-(β-hydroxyethyl)-amino-2-nitro-4-aminobenzene (0.422%, HPLC peak area)
1-amino-2-nitro-4-(β-hydroxyethyl)-aminobenzene (0.323%, HPLC peak area)

2.1.8. Physical properties

Appearance : Dark blue-violet powder
Melting point : 101-102°C
Boiling point : /
Density : /
Rel. vap. dens. : /
Vapour Press. : /
Log P_{ow} : /

2.1.9. Solubility

Water : 10 g/l at 20°C
DMSO : /

2.1.10 Stability

Stability at room temperature in dark bottles : > 5 years
Stability of aqueous solution stored in dark : > 90 days

General comments on analytical and physico-chemical characterisation

* No data (UV-Vis, IR, NMR, MS) is provided as to the characterisation of the test material.
* Purity of the test material as well as the organic impurities in the test material are recorded only as HPLC peak area (detection at 248 nm). Absolute content of the hair dye N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in the test material is not reported. Similarly the absolute content of the identified impurities has not been reported.
* A small peak present in the HPLC chromatogram has not been identified. Furthermore, no evidence is provided to assume that the test material may not contain other organic impurities, which do not absorb at 248 nm.
* Residual solvents, loss in drying, and metals in the test material have not been reported.
* Log P_{ow} and density of the test material have not been reported.
* The hair dye N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is a secondary amine, and thus, it is prone to nitrosation. No information is provided on the nitrosamine content in the test material.
* Batch number and purity of the test material in several of the tests are not reported.
* No information is provided on the stability of the dye in prototype hair dyeing formulations.

2.2. Function and uses

N,N'-bis(2-hydroxyethylamino)-2-nitro-p-phenylenediamine is intended for use in direct hair dye formulations at a maximum concentration of 1.5% and in oxidative hair dyes at a maximum final concentration of 1.0 % after mixing with hydrogen peroxide solution.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	/
Species/strain	:	BOR: WISW Wistar rats
Group size	:	5 males + 5 females
Test substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine, 50 % suspension in distilled water
Batch no	:	/
Purity	:	/
Dose	:	5 g/kg bw, once by gavage
GLP	:	/

In a dose range finding study 2 females per dose group were treated with 1, 2.5 and 5 g/kg bw of the test substance and no mortalities were observed. In the main study, 5 g/kg bw of the test substance was administered to five males and females. Skin and mucosa showed discolouration, no clinical signs were observed and the weight gain was normal.

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. Subchronic oral toxicity

Guideline	:	OECD 408 (1981)
Species/strain	:	SPF-bred Wistar rats
Group size	:	25 males + 25 females in the control and high dose group, 20 males + 20 females in the low and medium dose group
Test substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine as aqueous suspension
Batch number	:	/
Purity	:	/
Dose levels	:	0, 5, 50 and 500 mg/kg bw/day via stomach tube
Exposure period	:	13 weeks, followed by a 4 weeks recovery period
GLP	:	in compliance

The test substance was administered for 13 weeks, once daily, to the animals by use of a stomach tube. 10 animals (5 of each sex) of the high dose and the control group remained for further 4 weeks untreated for recovery. Clinical signs were observed daily, body weights, food and water consumption were recorded in weekly intervals. Ophthalmological examinations, hearing tests and reflex-examinations were carried out at pretest and week 13 using 10 males and 10 females of each dose group. Haematology was performed at pretest, after 6 and 13 weeks and at the end of the recovery phase using 10 males and 10 females of each group. Urinalysis was performed at pretest, after 6 and 13 weeks with samples of 5 males and females. At the end of the study organ weights were recorded and histopathology was performed with 10 males and 10 females of the high dose group and controls.

Results
Violet staining of urine, fur, paw and tails was observed in all substance-treated animals. Ophthalmoscopy, hearing tests and reflex testing did not reveal differences between the groups. No differences in body weight development, food and water consumption were observed. No substance related changes in haematological parameters were found. Statistical significant changes in blood glucose levels, seen only at week 6 in females in all dose groups were not dose-related and are due to low actual control values at this time. SGOT (now AST) and CPK values were reduced at week 6 in the middle and the high dose group and at week 13 in the high dose group only, calcium levels were increased in males and females at week 6 in the middle and the high dose group.
Liver and kidney weights of males were increased in the high dose group. With the exception of inflammatory changes found in several organs in treated and control groups no relevant morphological changes were revealed. After the recovery period no differences in haematology, clinical chemistry and organ weights were seen.
The NOAEL is 50 mg/kg bw/day, the NOEL 5 mg/kg bw/day.

Ref.: 6

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 & corrosivity

2.4.1. Irritation (skin)

Guideline	:	/
Species/strain	:	Albino rabbits, strain not reported
Group size	:	6
Test substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine, 1% in distilled water
Batch No.	:	/
Dose	:	0.5 ml of a 1% suspension in distilled water applied under tape for 24 hrs
GLP	:	/

The acute dermal irritation of the test substance was investigated in healthy adult albino rabbits. Each animal served as its own control. The test was performed on shaved and scarified skin. An aliquot of 0.5 ml of the test substance was applied to the test areas under occlusion. After 24 hours the patches were removed. Animals were examined for signs of erythema and oedema formation. The skin reactions were observed 24, 48 and 72 hours after termination of the exposure and the effects were scored according to the scheme of Draize.

Results
Under the conditions of the study, the test substance was neither irritating nor corrosive when applied to the rabbit skin under occlusive conditions.

Ref.: 3

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405
Species/strain	:	Albino rabbits, New Zealand
Group size	:	9 rabbits, sex not specified
Test substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine 1% in aqueous suspension or solution
Batch number	:	/
Purity	:	/
Dose	:	0.1ml 5% w/w aqueous dilution (pH 4.5)
GLP	:	/

A volume of 0.1ml of the test substance was instilled into the conjunctival sac of the left eyes of the test animals. The right eyes served as controls. The test substance remained in contact with the eyes until rinsing with a 1% fluorescein solution 24 hours after instillation. The eyes were examined 1, 2, 8 hours and 1, 2, 3, 4, 5, 6 and 7 days after instillation of the test material.
Results
The test material did not cause any observable effect on the corneas or irises at any time. Under the conditions of the test, the test material was considered to be non-irritating to the eye.

Ref.: 2

2.5. Sensitisation

Guinea pigs, modification of Magnusson-Kligman test

Guideline	:	/
Species/strain	:	Pirbright, Hoe: DHPK
Group size	:	20 test animals, 10 positive controls and 10 negative controls
Test substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine, 1% preparation
Batch No.	:	/
Concentration	:	1% aq. for intradermal and 1% pet. for topical induction, and 1%, 0.5% and 0.1% aq. for challenge
GLP	:	/

The test compound is not described in detail and was supplied as a 1% preparation, described as a violet powder.
Intradermal injections with FCA and tests substance were given pair-wise in the shaved shoulder region. 10% sodium lauryl sulphate in petrolatum was rubbed in the skin the day after. 6-8 hours later topical induction patches were applied under semi-occlusion. On the third day the last pair of intradermal injections was given in the shoulder area with 1% N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in FCA/water emulsion.
Challenge on the flank of the guinea pigs was performed 2 weeks later. Appropriate controls were included.

Results
No reaction was seen in test and negative animals, while DNCB sensitized all positive control animals.

Comment
The sensitization test is reported to be performed according to the method described by Magnusson and Kligman. However, pre-tests to establish the proper moderately irritating concentrations for intradermal and topical induction, and the non-irritant concentration for challenge were not performed. Further, intradermal induction and topical induction were performed almost simultaneously, and not a week apart as described in the original protocol. The sensitization test report is not acceptable because of deleterious modifications from the original protocol. It cannot be excluded that the test substance has sensitization potential.

Ref.: 5

Human volunteers
The dossier includes a one page description of a Shelanski and Shelanski repeated insult patch test (Ludwig modification) performed in 20 volunteers with WSI-75 in Igofluor with the purpose to reveal irritant and sensitizing properties of the compound. After a 3 weeks induction period challenge was performed. No reaction was seen. However, the report does not include any detailed information about test substance, test concentration and test performance. The short report is inadequate.

Ref.: 4

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Crl:CD(SD) BR rats

Group size	:	24 mated females per group
Test substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine, suspended in 0.5 % carboxymethylcellulose
Batch number	:	/
Purity	:	/
Dose levels	:	0, 10, 100 and 1000 mg/kg bw/day by oral gavage
Treatment period	:	day 6-15 of gestation
GLP	:	in compliance

The test substance was given to 24 female rats once daily by gavage on days 6-15 of gestation. Clinical observations were recorded daily. Bodyweights were recorded on day 0, 6, 15 and 20 day of gestation while food consumption was measured over the respective periods. Necropsy was performed on day 20 of gestation. The common reproduction parameters were recorded (corpora lutea, uterus weight, live and dead foetuses, foetal weight, implantations, resorptions, external abnormalities). Alternate foetuses of each litter were preserved and analysed for skeletal or visceral anomalies.

Results
Dose-related purple stained urine, fur and tail was observed in all substance-treated groups. In the high dose group maternal food consumption and body weight gain was significantly reduced while the slightly lower body weight gain compared to controls in the 100 mg/kg bw/day group was not statistically significant. Reproduction parameters remained unaffected in all dose groups. The incidence of external, visceral, major and minor skeletal abnormalities was not changed by substance treatment. N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine elicited maternal toxicity at 1000 mg/kg, the NOAEL in this study is 100 mg/kg bw/day. The NOAEL of embryotoxicity and teratogenicity is 1000 mg/kg bw/day.

Ref.: 21

2.7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous penetration / dermal absorption of a hair dye formulation in rats

Guideline	:	/
Animal strain	:	Sprague Dawley rats (Him:OFA) 5 males and 5 females in each of 2 experiments
Method	:	^{14}C ring labelled N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in a hair dye formulation (without developer), radiochemical purity > 98 %, applied to the back skin for 30 minutes and then rinsed off.
Test substance	:	1% radio labelled N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in a hair dye formulation
Batch no	:	/
Dose levels	:	see below
GLP	:	/

The percutaneous absorption of radio labelled ^{14}C-N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was studied in rats after 30 minutes application of a formulation containing [^{14}C]- labelled N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine 1%, water 84.75%, solvents 5%, detergents and emulsifiers 7.5%, ammonia 0.25% and other vehicle constituents 2.5%. The formulation was spread on shaved dorsal skin until wetted. The mean mass of test

substance applied was 1.06-1.08 mg/cm^2. The dye was removed by shampooing and rinsing and the rinsings collected. The test area was covered with gauze to prevent licking. The detection limit for radioactivity from the various samples from the cutaneous application experiments was ≤ 0.01% of applied ^{14}C.

Experiment A : Faeces and urine were collected daily for analysis. After 72 hours the animals were sacrificed and the treated skin as well as the carcass were analysed for remaining radioactivity.

Experiment B : Blood was drawn at 35 minutes, 1, 2, 4, 8 and 24 hours, the animals were sacrificed and 13 organs and carcass analysed for remaining radioactivity.
Mass balance was calculated.

Further, two experiments were performed with peroral dosing.

Results
One animal died before end of study. The mean percutaneous absorption of the test substance was 0.22% of applied ^{14}C equivalent to 2.37 µg/cm^2 test substance. N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was excreted mainly via urine (67%) and to a lesser extent via faeces (33%). The excretion was fast with 81% eliminated within the first 24 hours. The mass balance in experiment A gave a recovery of 99.4% of ^{14}C doses from the various samples.
The blood level after cutaneous application was highest at the first sampling time at 35 minutes (mean 0.000129% ± 0.000061%) and with a half life of 0.7 hours. After 24 hours the ^{14}C content in the organs was below or near detection limit. The highest concentration of ^{14}C was found in ovaries, thyroids, blood, liver and kidney. There was no measured retention in any tissue except in the treated skin after 24 and 72 hours.

A oral dose study in rats found the highest organ concentrations after 24 hours in kidneys, liver, adrenals and thyroid. Although the concentrations after cutaneous application are close to the detection limit and conclusions therefore restricted, it seems that the distribution pattern is similar after cutaneous and oral application of test substance.

Ref.: 22

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Reverse Mutation Testing Using Bacteria

Guideline	:	OECD 471 (1983)
Species/Strain	:	*Salmonella typhimurium* (TA98, TA98 NR)
Test item	:	HC Violet B5
Batch No.	:	/
Purity	:	/
Replicate	:	2 experiments
Doses	:	100, 500, 1000, 2500, 5000, 7500, 10000 µg/plate
Metabolic Act.	:	Phenobarbital / Naphthoflavone-treated rat liver homogenate
Positive controls	:	2NF (-S9); 2AA (+S9)
GLP	:	in compliance

Results
A strong mutagenic activity, dose-dependent, was observed in both strains.

Conclusions
This test item is mutagenic on bacterial cells in the presence and in the absence of Nitro-Reductase enzymes.

Ref.: 11

Reverse Mutation Testing Using Bacteria

Guideline	:	OECD 471 (1983)
Species/Strain	:	*Salmonella typhimurium* (TA98 NR: his D 3052 rfa, uvrB$^-$, R-factor)
Test item	:	HC Violet B5
Batch No.	:	/
Purity	:	/
Replicate	:	3 experiments
Doses	:	50, 100, 500, 1000, 2500, 5000 µg/plate
Metabolic Act.	:	Phenobarbital / Naphthoflavone-treated rat liver homogenate
Positive controls	:	2NF (-S9); 2AA (+S9)
GLP	:	in compliance

Results
A weak mutagenic effect was observed in the presence of metabolic activation.

Conclusions
The test item is mutagenic on bacterial cells.

Ref.: 10

Reverse Mutation Testing Using Bacteria

Guideline	:	OECD 471 (1983)
Species/Strain	:	*Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, TA1538)
Test item	:	HC Violet B5
Batch	:	/
Purity	:	/
Replicate	:	2 experiments
Doses	:	50, 100, 500, 1000, 2500, 5000 µg/plate
Metabolic Act.	:	Phenobarbital / Naphthoflavone -treated rat liver homogenate
Positive controls	:	2NF (TA98, TA100 and TA1538); SA (TA1535); 9AA (TA1537); 2AA (+S9)
GLP	:	in compliance

Results
A very slight mutagenic effect was observed on TA98 in the absence of metabolic activation.

Conclusions
The test item is slightly mutagenic on bacterial cells.

Ref.: 9

Reverse Mutation Testing Using Bacteria

Guideline	:	/
Species/Strain	:	*Salmonella typhimurium* (TA1535, TA1537, TA98; TA100)
Test item	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (blue/black powder)
Batch	:	/
Purity	:	/
Replicate	:	2 experiments
Doses	:	0, 8, 40, 200, 1000, 5000 µg/plate
Metabolic Act.	:	Aroclor induced rat liver homogenate (+S9)
Positive controls	:	2NF (TA98); SA (TA100 and TA1535); 9AA (TA1537); 2AA (+S9)
GLP	:	/

Results
The test item was mutagenic on TA98 (± S9) and on TA1537 (+S9)

Conclusions
The test item is mutagenic on bacterial cells.

Ref.: 8

Reverse Mutation Testing Using Bacteria

Guideline	:	/
Species/Strain	:	*Salmonella typhimurium* (TA98; TA100); *E. coli* (wP2uvrA$^-$p)
Test item	:	WS 1-75
Batch	:	/
Purity	:	/
Replicate	:	1 experiment
Doses	:	from 3.2 to 10000 µg/plate
Metabolic Act.	:	Aroclor induced rat liver homogenate
Positive control	:	2NF (TA98); Sodium Azide (TA100); MNNG (E. coli) and 2AA
GLP	:	/

Results
The test item has been found mutagenic on TA 98 and TA 100 (+ S9) and in *E. coli* (- S9).

Conclusion
The test item is mutagenic on bacterial cells.

Ref.: 7

Reverse Mutation Testing Using Bacteria

Guideline	:	/
Species/Strain	:	*E. coli* wP2; CH871
Test item	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine
Batch	:	/
Purity	:	/

Replicate : 4 experiments
Metabolic Act. : /
GLP : /

Results
The study is inadequate, because no numerical data were reported as results, but only + and - symbols.

Ref.: 12

In Vitro **Mammalian Cell Gene Mutation Test**

Guideline : /
Species/Strain : Mouse Lymphoma L5178Y cells (forward mutation at Thymidine Kinase ($TK^{+/-}$) locus
Test item : N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (dark blue powder)
Batch : /
Purity : /
Replicate : 1 experiment; duplicate cultures
Doses : 5000, 2500, 1250, 625, 313 µg/ml (-S9) 4000, 2000, 1000, 500, 250 µg/ml (+S9) 2 hours of treatment
Metabolic Act. : Aroclor-1254 induced rat liver homogenate (S9)
Positive control : 4NQO (-S9); B(a)P (+S9)
GLP : /

Results
Toxicity: (2 h treatment): 5000 µg/ml (-S9) induced 95% of lethality, whereas a lethality of 100% was induced in the presence of S9. The other doses did not show any lethality.

Mutagenicity
No dose related toxicity was observed in the absence of S9 up to 2500 µg/ml; the maximum dose (5000 µg/ml) induced 75% mortality; in the presence of S9 a dose reduction survival was observed starting from 1000 µg/ml (70%) to 4000 µg/ml (20%).
There was no counting of small and large colonies; there was no indication of induction of an increase of mutants compared to the control in both conditions. The positive controls induced significant increase in mutation frequency.

Conclusion
The test item did not induce gene mutation in mammalian cells *in vitro*.

Ref.: 14

In Vitro **Mammalian Chromosome Aberration Test**

Guideline : /
Species/Strain : CHO cells
Test item : N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (dark blue powder)

Batch	:	/
Purity	:	/
Replicate	:	two experiments
Doses	:	1250, 2500, 5000 µg/ml (2 hours of treatment)
Metabolic Act.	:	Aroclor-1254 induced rat liver homogenate
Positive control	:	MMS (-S9); CPA (+S9)
GLP	:	in compliance

Results
Toxicity: A preliminary study demonstrated that a dose of 5000 µg/ml in the presence of S9 induced a 66% reduction of Mitotic Index. No toxicity was observed in the absence of S9 at the same dose.

Clastogenicity
The dose of 5000 µg/ml (± S9) in both cultures induced a significant increase in the frequency of chromosome aberrations (± gaps).

Conclusions

The study indicates that the test substance induces chromosome aberrations at a dose of 5000 µg/ml in the presence and in the absence of metabolic activation.
The study is inadequate as no information is provided on the test item.

Ref.: 17

In Vitro Mammalian Chromosome Aberration Test

Guideline	:	OECD 473 (1983)
Species/Strain	:	Human peripheral blood from healthy people (two individuals: 1+S9; 1-S9)
Test item	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (dark/blue/violet powder)
Batch	:	/
Purity	:	/
Replicate	:	two cultures each donor
Doses	:	15, 100, 200, 400, 6000, µg/ml (+S9) 12, 25, 50, 100, 200 µg/ml (-S9) (2h; +S9; 24h: -S9)
Metabolic Act.	:	Aroclor-1254 induced rat liver homogenate
Positive controls	:	Bleomycin (+S9); B(a)P (-S9)
GLP	:	in compliance

Results
Toxicity: in a preliminary study it was shown that the Mitotic Index was reduced by 5% starting from 50 µg/ml; at 400 µg/ml there was no survival in the absence of S9; whereas in the presence of S9 the almost complete lethality was observed at a dose of 800 µg/ml.
Clastogenicity

No induction of clastogenicity was observed except on endoreduplication at the dose of 200 µg/ml in the presence of metabolic activation.

Conclusions

The test item is not clastogenic on human chromosome cells treated *in vitro*; the doses used in this experiment were almost 10 times lower than the previous study.

Ref.: 18

DNA Damage – Unscheduled DNA Synthesis – Mammalian Cells *In Vitro*

Guideline	:	/
Species/Strain	:	HeLa S3 cells
Test item	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (dark blue powder)
Batch	:	/
Purity	:	/
Replicate	:	3 replicate/dose
Doses	:	500, 100, 20, 4, 0.8; 0.16; 0.032; 0.0064 µg/ml (+S9) (1 hour of treatment)
Method	:	Extraction of DNA and evaluation by count in a liquid scintillation counter (LSC)
Metabolic Act.	:	Aroclor-1254 induced rat liver homogenate (S9)
Positive controls	:	4NQO (-S9); B(a)P (+S9)
GLP	:	in compliance

Results
In the absence of S9, the positive control (4NQ0) induced UDS (2560 dpm/µg DNA); untreated control: 103.5; $p<0.001$. All tested doses did not induce UDS. In the presence of S9 the positive control (B(a)P) induced UDS (1158.6 dpm/µg DNA); untreated control: 649.4; $p<0.025$.
All tested doses induced UDS with a $p<0.001$.
The study is inadequate for an evaluation, as no information on the nature of the test item is included.
The OECD Guideline 482 approved in 1986, indicates the need of 6 cultures in case of LSC method of evaluation.

Ref.: 15

DNA Damage – Unscheduled DNA Synthesis – Mammalian Cells *In Vitro*

Guideline	:	OECD 482 (1986)
Species/Strain	:	freshly isolated rat hepatocytes (Wistar/wu rats)
Test item	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine
Batch	:	WSI-75h/52
Purity	:	99.3% (HPLC)
Replicate	:	2 cell cultures; 2 experiments
Doses	:	2.67, 8.0, 26.67, 80.0, 266.67, 800 µg/ml
Method	:	Autoradiography
Positive controls	:	2AAF

GLP : in compliance

Results
Toxicity: A preliminary study with 10 doses (from 8.0 to 8000 µg/ml) indicated that a reduction of survival to 80% (53.33 µg/ml); for the remaining doses, a precipitation was observed.

UDS Reduction
1st experiment
The positive control (2AA) induced 116.40 net grains per nucleus (untreated= -2.48 grains/nucleus – grains/Cytoplasm).
All the five treated doses induced negative UDS, as the untreated control.

2nd experiment
The positive control (2AA) induced 43.86 net grains/nucleus (untreated control: -5.47). The dose of 266.67 µg/ml of the test item induced 1.09 net grains per nucleus, in the presence of a precipitate of the test item.

Conclusion
The test substance has been found to be unable to induce UDS in rat hepatocytes treated *in vitro*, because one observed positive dose was not reproducible. It should be noticed, however, that the positive observed dose was partly precipitated, and that the doses in this experiment were lower than in the previous experiment on HeLa.

Ref.: 16

2.8.2 Mutagenicity/Genotoxicity *in vivo*

In Vivo Mammalian Erythrocyte Micronucleus Test

Guideline	:	/
Species/Strain	:	CFLP Mice
Group size	:	5 M+5 F per group dosed.
Test Substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in 10 % DMSO/water
Batch	:	/
Purity	:	/
Treatment	:	intraperitoneal injection
Dose	:	600 mg/kg bw
Sacrifice Time	:	24, 48 and 72h
Replicate	:	two cultures each donor
Positive control	:	CPA (intraperitoneal; 24h after treatment)
GLP	:	/

Results
Toxicity: Males and females died at a dose of 1000 mg/kg
MN: No toxicity was observed 24, 48 and 72h. CPA produced 1.35% of MN, compared with 0.16% of the untreated control ($p<0.001$). The test item produced the following results:

GROUP	***24h***	***48h***	***72h***
CONTROL	0.16%	0.25%	0.06%

TREATMENT	0.11%	0.15%	0.16%

The males treated for 72 hours had a significant increase in the percentage of MN ($p>0.05$). By applying a chi-square test after 24h, the value of females reached the significant value of 0.05; after 48h the MN value of females was also significant and after 72h the MN value of males was significant at 0.05%.

Conclusions
The study is inadequate for the evaluation, due to the lack of toxicity in the bone marrow cells, thus indicating the presence of the test item in those cells.
Moreover, the statistical analysis may indicate a possible clastogenic/aneugenic effect.

Ref.: 19

In Vivo **Mammalian Erythrocyte Micronucleus Test**

Guideline	:	OECD 474
Species/Strain	:	Cr1:NMRI Mice
Group size	:	5 males/5 females/ group dosed
Test Substance	:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (black powder) in 0.5 % aqueous carboxymethylcellulose
Batch	:	/
Purity	:	/
Treatment	:	stomach intubation
Dose	:	1200 mg/kg
Sacrifice Time	:	24, 48 and 72h
Positive control	:	CPA (24h)
GLP	:	in compliance

Results
Toxicity: no experiment
MN: CPA induced MN and cytotoxicity. The test item did not induce neither MN, nor cytotoxicity.

Conclusions
The test item did not induce micronuclei in mice.

Ref.: 20

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

Not applicable

2.12. Conclusions

No data (UV-Vis, IR, NMR, MS) is provided as to the chemical characterisation of N,N'-bis-(2-hydroxyethyl-2-nitro-p-phenylenediamine in the test material(s). Purity of the hair dye as well as the impurities in the test material(s) have not been fully characterised. Log P_{ow} of the test material has not been reported. N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is a secondary amine, and thus, it is prone to nitrosation. No information is provided on the nitrosamine content in the test material.

In a subchronic oral toxicity study, the NOAEL was set at 50 mg/kg bw/day, the NOEL at 5 mg/kg bw/day. The test substance elicited maternal toxicity at 1000 mg/kg, the NOAEL in this study was set at 100 mg/kg bw/day. The NOAEL of embryotoxicity and teratogenicity was set at 1000 mg/kg bw/day.

A 1 % suspension of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was neither irritating nor corrosive when applied to the rabbit skin under occlusive conditions. It was considered to be non-irritating to the eye. However, it cannot be excluded that N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine has sensitization potential.

The mean percutaneous absorption of the test substance was 0.22% of applied ^{14}C equivalent to 2.37 μg/cm^2 test substance. However, the substance was tested at 1% in a hair dye formulation, where the in-use concentration in semi-permanent hair dyes is 1.5%. The percutaneous absorption of the dye has not been tested in an oxidative environment.

N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine has been tested in a set of assays, not always according to OECD Guidelines and following GLP, for gene mutation on *Salmonella typhimurium, E. coli* and on mammalian cells (mouse lymphoma L51784); for chromosome aberrations on CHO cells and human lymphocytes; for UDS on HeLa cells and rat hepatocytes *in vitro*; for the induction of MN in an *in vivo* mice micronucleus assay (2 experiments).
In a few studies the nature of the test item was indicated.
By evaluating only the adequate studies, it was demonstrated that N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine induces:

- gene mutations in bacteria

and that it does not induce:

- UDS in rat hepatocytes treated *in vitro*
- Chromosome aberrations on human lymphocytes
- gene mutations in mammalian cells *in vitro*
- micronucleus in mice *in vivo*.

2.13. References

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4. Dobzy A., N. Simon, Clinina Dermato-Venerologica Szeged, Hungaria, 15.6.1983.
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15. Microtest Research Limited, 8.10.1985, 'Study to determine the ability of B34 to introduce unscheduled DNA synthesis in HeLa cells.'
16. CCR project 414101 'Unscheduled DNA synthesis in primary hepatocytes of male rats in vitro with B34', 7.22.1993
17. Microtest Research Limited, 3.10.1984, 'Study to evaluate the chromosome damaging potential of B34 by its effects on cultured Chinese hamster ovary (CHO) cells using an in vitro cytogenetics assay.'
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19. Toxicol. Laboratories Limited. Ref. n° 181/8412, April 1984. 'Mouse micronucleus test.'
20. Österreichisches Forschungszentrum Seibersdorf, September 1988. 'Micronucleus test with B34.'
21. Toxicol. Laboratories Limited. Ref. n° SAK/13/87, December 1987. 'Colipa B34 rat teratology study.'
22. Österreichisches Forschungszentrum Seibersdorf, December 1987. 'Toxicokinetics of N1,N4-bis(β-Hydroxyethyl)-2-nitro-1,4-phenylenediamine.'

3. Opinion of the SCCNFP

The SCCNFP is of the opinion that the information submitted is inadequate to assess the safe use of the substance.

Before any further consideration, the following information is required :

* Chemical characterisation of N,N'-bis-(2-hydroxyethylamino)-2-nitro-p-phenylenediamine in the test material; determination of absolute concentration of the hair dye in various batches of test material; Log P_{ow} of the hair dye and the nitrosamine content in the test material and prototype formulations, data on stability in hair dye formulations.

* percutaneous absorption in an oxidative environment.

* data on the genotoxicity/mutagenicity following the relevant SCCNFP-opinions and in accordance with the Notes of Guidance.

4. Other considerations

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5. Minority opinions

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