Scientific Committee on Consumer Safety

SCCS

OPINION ON

N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (B34)

The SCCS adopted the final version of this Opinion by written procedure
on 16 September 2016

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SCCS

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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All Declarations of Working Group members are available on the following webpage:
http://ec.europa.eu/health/scientific_committees/experts/declarations/sccs_en.htm

This opinion has been subject to a commenting period of 8 weeks after its initial publication (from 14 April until 9 June 2016). Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meetings. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged.
For this Opinion, main revisions have been made on sections 3.3.9.1 Toxicokinetics in laboratory animals, 3.3.13 safety evaluation, as well as the references' list.

Keywords: SCCS, scientific opinion, N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (B34), Regulation 1223/2009, CAS No. 84041-77-0, EC No. 281-856-4

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1. BACKGROUND

The hair dye N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylamine (B34) CAS No 84041-77-0, EC No 281-856-4 is intended to be used as hair dye in cosmetic hair colouring products with or without the addition of an oxidizing agent (e.g. hydrogen peroxide). The final on-head concentration of B34 is up to a maximum of 1.0 % or 1.5 % respectively in the presence or absence of hydrogen peroxide.

Submission I and II on hair dye N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylamine (B34) were transmitted by COLIPA[1] in May 1994 and May 2005 respectively.

The latest safety evaluation on hair dye N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylamine (B34) was adopted by the Scientific Committee on Consumer Safety (SCCS) in September 2012 in reply to Submission III of December 2010. The SCCS concluded that:

"*a conclusion on the gene mutation potential of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine cannot be drawn without further testing.*

On the basis of the submitted data, the SCCS considers N,N'-bis(2-hydroxyethylamino)-2-nitro-p-phenylenediamine not safe for use as an oxidative hair dye with a concentration on head of maximum 1.0 % or as a non-oxidative hair dye with a concentration up to 1.5 %."
(SCCS/1463/12)

In December 2014, Cosmetics Europe submitted additional data on mutagenicity in order to review the issues raised by the SCCS in the Opinion of September 2012.

2. TERMS OF REFERENCE

1. In light of the new data provided, does the SCCS consider N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylamine (B34) safe when used as an oxidative hair dye with an on-head concentration of maximum 1.0 % and as a non-oxidative hair dye with a concentration up to 1.5 %?

2. Does the SCCS have any further scientific concerns with regard to the use of N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylamine (B34) in other cosmetic products?

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1. Primary name and/or INCI name

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

3.1.1.2. Chemical names

Ethanol, 2,2'-[(2-nitro-1,4-phenylene)diimino]bis-(9CI)
1,4-Bis-(2-hydroxyethylamino)-2-nitrobenzene
2,2'-[(2-Nitro-1,4-phenylene)diimino]bisethanol
2-({4-[(2-Hydroxyethyl)amino]-2-nitrophenyl}amino)ethanol

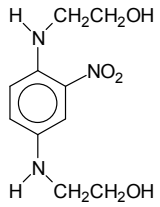
3.1.1.3. Trade names and abbreviations

HC Violet BS
Marcus Violet HFI
WS I 75
COLIPA B 034

3.1.1.4. CAS / EC number

CAS: 84041-77-0
EC: 281-856-4

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{10}H_{15}N_3O_4$

3.1.2 Physical form

Dark blue-violet powder

3.1.3 Molecular weight

Molecular weight: 241.24 g/mol

3.1.4 Purity, composition and substance codes

Batch DALA 013106 (= SAT 040271 = SAT 040411)
Chemical characterisation by NMR and IR spectroscopy

Purity

NMR assay:	98.6% (w/w)
HPLC assay:	98.8% (area)
Solvent content (water):	< 0.26% (w/w)
Sulphated ash:	0.3% (w/w)

Impurities

2-[(4-Amino-2-nitrophenyl)amino]ethanol (HC Red 3, CAS 2871-01-4)	500 ppm (Ref. 2) <1% (Ref. 1)
N-Nitrosodiethanolamine	< 50 ppb
2-Nitrobenzene-1,4-diamine	< 15 ppm
2-[(4-Amino-3-nitrophenyl)amino]ethanol	< 50 ppm
4-Amino-3-nitrophenol	< 50 ppm
2-[(4-Amino-3-nitrophenyl)(2-hydroxyethyl) amino]ethanol hydrochloride	< 40 ppm
4-Fluoro-3-nitroaniline	< 70 ppm
2-({4-[Bis(2-hydroxyethyl)amino]- 2-nitrophenyl}amino)ethanol	> 100 ppm

Comment
N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is a secondary amine and prone to nitrosation. The nitrosamine content in the dye should be <50 ppb. It should not be used in the presence of nitrosating agents.

The study report of the determination of nitrosamine in N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine described only the principle of the analytical method used and the result. No analytical details and/or analytical data were provided.

Ref.: 6, submission III

Declarations by the applicant

1. Currently used material

Purity

NMR assay:	*> 98.0% (w/w)*
HPLC assay:	*> 98.0% (area) Solvent content (water): < 1.0% (w/w)*

Impurities

2-[(4-Amino-2-nitrophenyl)amino]ethanol:	*< 0.5% (w/w)*
N-Nitrosodiethanolamine:	*< 50 ppb*

2. *Other batches*

The applicant declared that: "*The batch of COLIPA B34 used in the acute oral toxicity test (of 1984) is not fully analytically described. However, information is available from the laboratories that have synthesized this batch concerning the identity and purity of the material produced at that time. From this information it can be concluded that the former not-fully described batch is representative and its specification is quite similar to the fully characterized batch (DALA 013106)*".

3.1.5 Impurities / accompanying contaminants

See point 3.1.4. Purity, composition and substance codes

3.1.6 Solubility

Water: 7.5 g/l at room temperature

Ref.: 7, submission III

Ethanol: 3 – 30 g/l at room temperature
DMSO: > 100 g/l at room temperature

3.1.7 Partition coefficient (Log Pow)

Log P_{ow}: -0.44 (calculated)
0.285 (EU A.8)

Ref.: 8, submission III

3.1.8 Additional physical and chemical specifications

Melting point:	100 – 105 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	/

3.1.9 Homogeneity and Stability

Test solutions (30-120 mg/ml in 0.5% aqueous carboxymethylcellulose) used for developmental toxicity were homogeneous and stable (CV <7%) during the study period.
The stability under use conditions of N,N'-Bis(2-hydroxyethyl)-2-nitro-p-phenylenediamine in an oxidative hair coloration product containing 3% hydrogen peroxide at pH 9 over 60 minutes was tested by means of UV spectrophotometry. Therefore a solution of 1.0 % N,N'-Bis(2-hydroxyethyl)-2-nitro-p-phenylenediamine, batch DALA 013106 in a basic gel formulation (FARO7G00045\01) was adjusted to a pH value of 10.0 using ammonia. This solution was mixed with a 6.0% aqueous H_2O_2 solution 1:1 (v/v) resulting in a pH of 9.0. This mixture was tested over a period of 60 min by measuring the absorption of the test compound at the absorption maxima of 516 nm and 248 nm. The recovery of N,N'-Bis(2-hydroxyethyl)-2-nitro-p-phenylenediamine over 60 min in the described mixture containing 3% hydrogen peroxide at pH 9 is within 98%. Thus, the test compound is stable under oxidative application conditions.

General comments to physico-chemical characterisation

- N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is a secondary amine. It should not be used in the presence of nitrosating agents.
- The study report of the determination of nitrosamine in N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine described only the principle of the analytical method used and the result. No analytical details and/or analytical data were provided.
- The applicant provided a simple UV method to evaluate stability in an oxidative hair coloration product. Stability testing using a simple comparison of UV spectra is not acceptable. A more selective analytical method such as HPLC with PDA detection should be used to evaluate stability for up to 60 min.

3.2 Function and uses

N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is used as a direct hair dye for hair colouring products. It is also used in oxidative hair dye formulations with and without mixing with an oxidising agent (e.g. hydrogen peroxide).

The final concentration on head of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine can be up to 1.5% when it is used without an oxidising agent and up to 1.0% after mixing with an oxidising agent.

3.3 Toxicological evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Taken from SCCNFP/0781/04

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:	/
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.: 3, submission II

3.3.1.2 Acute dermal toxicity

No data submitted

3.3.1.3 Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404 (2002)
Species/strain:	albino rabbit, New Zealand White, (SPF-Quality)
Group size:	3 males
Test substance:	B034

Batch: DALA 013106 (SAT 040271)
Purity: 99.5% (HPLC)
Vehicle: water (Milli-U)
Dose volume: 0.5 g B034 moistened with water (Milli-U)
GLP: in compliance
Study period: 29 June – 10 July 2004

Each animal served as its own control. Approximately 24 hours prior to the treatment, the dorsal fur was shaved to expose an area of about 150 cm².
An aliquot of 0.5 g of the moistened test substance was exposed to the intact shaved back skin of each animal. The patch was removed four hours after semi-occlusive contact.
Animals were examined for signs of erythema, eschar and oedema formation. The skin reactions were assessed after 1 hour, 24, 48 and 72 hours.

Results
Although there was purple staining of this skin, this was reported as not affecting the observations. No reaction was seen at any time point.

Conclusion
B034 was not an irritant to rabbit skin under the conditions of the experiment.

Ref.: 4, submission II

Comment
Purple staining of the skin was noted in all animals at all-time points. The study authors stated that this staining did nor mask any erythema.

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405 (2002)
Species/strain: albino rabbit, New Zealand White, (SPF-Quality)
Group size: 3 males
Test substance: B034
Batch: DALA 013106 (SAT 040271)
Purity: 99.5% (HPLC)
Vehicle: /
Dose level: 52 mg
Dosing volume: 0.1 ml
GLP: in compliance
Study period: 6 July – 2 August 2004

52 mg (equivalent of 0.1 ml) of B034 was instilled into the conjunctival sac of one eye of the test animals. The substance remained in permanent contact with the eyes until rinsing with warm tap water, 24 hours after instillation. The other eyes served as controls.
The eye irritation reactions were scored approx. 1 hour, 24, 48 and 72 hours and 7 days after instillation of the test solution.
Results
Instillation of undiluted B034 into one eye of each of three rabbits resulted in irritation of the conjunctivae, which consisted of redness and chemosis. The irritation had completely resolved in one animal within 72 hours and in the other two animals within 7 days.
No scoring after 1 hour of the iris and lower part of the eyelids was possible, because of staining by the test substance.
No iridial irritation or corneal opacity was observed, and treatment of the eyes with 2% fluorescein 24 hours after test substance instillation revealed no corneal epithelial damage.

Conclusion
Under the conditions of this experiment, B034 was an irritant to rabbit eyes.

Ref.: 5, submission II

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species/strain:	mice, CBA/CaOlaHsd (nulliparous and non-pregnant)
Group size:	16 females (4 females per group)
Test substance:	B034
Batch:	DALA 013106 (SAT 040271)
Purity:	99.5% (HPLC)
Vehicle:	acetone:olive oil, 4:1 (v/v)
Concentration:	0, 2.5, 5, 10% (w/v)
Positive control:	α-hexylcinnamaldehyde (March 2004); acetone:olive oil, 4:1 (v/v)
GLP:	in compliance
Study period:	28 April – 12 May 2004

A homogenous solution of the test item in a mixture of acetone:olive oil (4:1 v/v) was made shortly before each dosing. The highest non-irritating and technically applicable test item concentration was found in a pre-test with two mice. Based on these test results, 2.5%, 5% and 10% solutions were chosen for the main study.
Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with the different test item concentrations. The application volume, 25 µl, was spread over the entire dorsal surface of each ear lobe once daily for three consecutive days. The control group was treated with the vehicle exclusively. Five days after the first topical application, all mice were administered with radio-labelled thymidine (^{3}HTdR) by intravenous injection via the tail vein.
Approximately five hours after ^{3}HTdR application, the draining lymph nodes were excised and pooled for each experimental group. After preparation of the lymph nodes, disaggregation and overnight precipitation of macromolecules, these precipitations were re-suspended and transferred to scintillation vials.
The level of ^{3}HTdR incorporation was then measured by scintillation counting. The proliferative response of lymph node cells is expressed as the ratio of ^{3}HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index).
The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of ^{3}H-methyl thymidine.

Results
Slight skin irritation was noted on the ear dorsum of the treated mice at a concentration of 10% (w/v).
The Stimulation Index (S.I.) was below 3 in all dose groups. No dose response relation was noted.
The EC3 value was not calculated because no test concentrations produced a S.I. of 3 or higher.
The EC3 value for the positive control was 11.7% w/v.

Based on the criteria of the test system, B034 was found to be a non-sensitiser when tested up to the highest applicable concentration of 10% (w/v) in acetone:olive oil (4:1) in mice.

Conclusion
A higher concentration of B034 than 10% (w/v) in acetone:olive oil could not be used because of irritation. Therefore, B034 at more than 10% is assumed to have irritant potential. B34 was not determined to be a skin sensitiser under these particular experimental conditions.

Ref.: 6, submission II

3.3.4 Dermal / percutaneous absorption

Guideline:	OECD 428 (2000)
Tissue:	dermatomed pig skin, 0.75 mm
Group size:	8 replicates per experiment, 4 from each of the 2 donors
Skin integrity:	transcutaneous electrical resistance, TER > 7kΩ
Diffusion cell:	static penetration cells (Franz-cells), 1.0 cm²
Test substance:	B034
Batch:	DALA 013106 (SAT 040271)
Purity:	99.5% (area%, HPLC)
Test item:	experiment A: B034 in direct dye cream (= 1.5 mg B034) experiment B: B034 in oxidative dye cream with developer and hydrogen peroxide (= 1.0 mg B034) experiment C: B034 in oxidative dye cream with developer and without hydrogen peroxide (= 1.0 mg B034) experiment D: B034 in aqueous solution (= 0.7 mg B034)
Application:	100 mg/cm²
Receptor fluid:	autoclaved Dulbecco's phosphate buffered saline
Solubility receptor fluid:	(7.4g/L in water)
Stability receptor fluid:	(stable in water) Method of Analysis: HPLC
GLP:	in compliance
Study period:	16 August – 17 September 2004

The dermal absorption/percutaneous penetration of the test substance was investigated for the open application of about 100 mg final formulation per cm² pig skin. Therefore, the resulting dose of the test substance was approx. 1.5 mg/cm² skin with the direct dye cream and 1.0 mg/cm² skin with the oxidative dye cream with and without hydrogen peroxide. Skin discs of 1.0 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with a 0.01% Tween 80 solution and deionised water.

Each of the formulations and the solution were analysed in two experiments with four replicates per experiment for adsorbed, absorbed and penetrated amount of the test substance. The receptor fluid used was Dulbecco´s phosphate buffered saline. Samples of the receptor fluid were drawn before the application of the test substance formulation and 0.5, 1, 2, 4, 6, 24, 29 and 48 hours after application. The removed volume was replaced by fresh receptor fluid.

Results summary
The absorption (bioavailability) from the three experiments with B034 in formulations are:

	Bioavailable (µg/cm²) ± ***SD***	Range (µg/cm²)	Bioavailable (%) ± ***SD***	Dose 100mg
B034 in direct dye cream	***1.454*** *± 0.758*	0.650 - 2.956	***0.107*** *± 0.060*	1.5%
B034 in oxidative dye cream with developer and with hydrogen peroxide	***0.939*** *± 0.407*	0.470 - 1.743	***0.093*** *± 0.041*	1.0%
B034 in oxidative dye cream with developer and without hydrogen peroxide	***1.466*** *± 0.599*	0.825 - 2.397	***0.149*** *± 0.059*	1.0%

Under the conditions of the experiments the amounts of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine considered absorbed were:

A) from 1.5% N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in a direct dye cream: 1.454 ± 0.758 µg/cm²;

b) from 1.0% N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in an oxidative dye cream with developer and with hydrogen peroxide: 0.939 ± 0.407 µg/cm²;

C) from 1.0% N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in an oxidative dye cream with developer and without hydrogen peroxide: 1.466 ± 0.599 µg/cm².

Ref.: 13, submission II

Comment
The amount of test material applied on the skin (100 mg/cm^2) is too high compared to the recommended dose of 20 mg/cm². Measurements over 48 hours were too long.
An absorption of 1.76 µg/cm² (0.94 + 2 x 0.41 (mean + 2SD)) may be used for calculating the MOS for oxidative hair dyes.
For non-oxidative hair dyes, an absorption of 2.97 µg /cm² (1.45 + 2 x 0.76) may be used for the calculation of the MoS.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (14 days) oral / dermal / inhalation toxicity

No data submitted

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

Taken from SCCNFP/0781/04

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-week 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 untreated for a further 4 weeks 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 pre-test, 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, submission I

Comment
The dose-spacing between the mid and high dose was very large.

Guideline:	OECD 408 (1998)
Species/strain:	rat, Wistar Hannover (HsdBrlHan:Wist)
Group size:	120 (10 males and 10 females per group; control and high dose group: additional 5 males and 5 females for 4 weeks of recovery)
Test substance:	B034
Batch:	DALA 013106 (SAT 040411)
Purity:	99.5%
Vehicle:	0.5% aqueous carboxymethylcellulose
Dose levels:	0, 80, 240 and 720 mg/kg bw
Stability:	> 6 hours at room temperature
Dose volume:	10 ml/kg bw
Route:	oral gavage
Administration:	once daily for minimum 13 weeks
GLP:	in compliance
Study period:	9 August – 6 December 2004

The dose levels were selected based on a preliminary range finding study, in which three groups, each of 5 male and 5 female rats, received the test item by gavage at dosages of 300, 600 or 1200 mg/kg/day for 28 days.
Results
One high-dose female and one control female were found dead on day 77 and 78 of the study, respectively. The death of the control female was probably due to miss-dosing, while the death of the high-dose female was considered treatment-related.
Body weight gain and food consumption were comparable to the controls.
Staining (violet) of the fur, generally on the body surface or localised on the dorsal region and tail, was seen in all treated groups with increased incidence with the dose, from treatment week 1. On day 14, salivation was observed approximately 15 minutes post-dosing at the high dose (2 males, 1 female). On day 77, again 15 minutes post-dosing but continuing up to 1 h, some high dose females exhibited increased salivation, ocular discharge, lethargy, hunched posture, decreased activity and ataxia. No other treatment-

related signs were recorded during the treatment period. No treatment-related neurotoxic signs were noted.
At week 12, a dose-related decrease in motor activity was noted in females. This was statistically significant at the high dose (up to 29% of control). This persisted during the 4 week recovery period and showed only a slight improvement (up to 14% of control).
No treatment-related haematological effects were noted. In males, dose-related increases in triglycerides (up to 56% of control) and urea (up to 24% of control) were statistically significant at the high dose. No other toxicologically significant changes were observed over the treatment period. By the end of the recovery period, triglycerides remained slightly increased (14% above the control) while urea was slightly decreased (14% under the control) in high-dose males.
Slight dose-related increases in urinary specific gravity were observed in all treated groups, statistically significant in the mid and high dose males and in all treated females at the end of the treatment period. A complete recovery was observed after 4 weeks.
Dose-related increases in absolute and relative liver weights were seen in all treated males (up to 19% of controls), statistically significant in the mid- and/or high dose groups. Slight, dose-related increases in absolute and relative kidney weights were seen in all treated males (up to 11% of controls), with the relative weights being statistically significant in the high dose group. At the end of the recovery period, these were comparable with the controls. No other toxicologically significant changes were observed.

Post mortem
Post mortem examination of the 2 unscheduled deaths showed that a possible miss-dosing was the cause of the control female as there was multi-focal, moderate alveolar haemorrhage and oedema in the lungs, whereas the high-dose female showed dark purple staining of most organs/tissues in the skin, thorax and abdomen, which was probably treatment related.

A generalised violet coloration was described on the skin of the animals from all treated groups. At the end of the recovery period, the skin was still stained violet in the treated animals. This was considered to be due to excretion via urine of test substance or its metabolite(s).
Coloured, granular contents and/or violet colour of the glandular and non-glandular stomach of some high dose animals. Violet fluid was also reported in the urinary bladder of 1 mid-dose male and in many high-dose males and females. These changes were considered dose-related, but they were not regarded as lesions.
In the kidneys, the cortical tubules showed a dose-related increase of hyaline droplets in all males, but with a higher incidence and severity level at the high dose. In addition, it was noted that in the cortical tubules, in some control and all treated animals, there was a dose-related increase in the incidence of yellow-brown, intra-cytoplasmic pigmentation. These deposits were not evaluated as an adverse effect, since no toxicologically significant differences were observed in the accumulation of pigment deposits. By the end of the recovery period, the distribution of these deposits was multi-focal in the treated animals and focal in the controls.
No other organ was affected by treatment.

Conclusion
On the basis of these results, the mid-dose (240 mg/kg/day) may be considered the No Observed Adverse Effect Level (NOAEL) for N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine.

Ref.: 11, submission II

Comment
The NOAEL of 240 mg/kg bw/d could be used for the calculation of the Margin of Safety (MoS).

3.3.5.3 Chronic (> 12 months) toxicity

No data submitted

3.3.6 Mutagenicity / Genotoxicity

In submission I, II and III the genotoxicity of B34 was extensively investigated in the three endpoints of genotoxicity: gene mutations, structural chromosome aberrations and aneuploidy. B34 was positive in the gene mutation assays in bacteria (predominantly strain TA98) but did not induce gene mutation in mammalian cells at the *tk* or *hprt* locus of mouse lymphoma cells. In *in vitro* UDS tests treatment of hepatocytes with B34 gave contradictory results with negative result in more reliably performed experiment. Also in two *in vitro* chromosome aberration tests, both not performed according to current OECD guidelines and thus of limited value, contradictory results were found. In one test, B34 treatment resulted in an increase in the number of cells with chromosome aberrations, whereas the second test was negative. In *in vitro* micronucleus tests performed according to OECD guidelines, measuring the same genotoxic endpoint, B34 induced an increase in the number of cells with micronuclei. B34 was found to be clastogenic by micronucleus assay *in vitro* on V79 cells, both in the absence and presence of metabolic activation. This clastogenicity was, however, not confirmed in an *in vivo* micronucleus tests in mice.

However, the positive findings found in the *in vitro* gene mutation assays in bacteria were not confirmed nor ruled out in an appropriate *in vivo* test on the same genotoxic endpoint, thus a mutagenic potency could not be excluded.

In submission IV, an additional mammalian *g*ene mutation test at the hypoxanthine-guanine-phosphoribosyl transferase *(HPRT)* locus (ref. 6) was performed on mouse lymphoma cells L5178Y. Additionally, B34 was investigated on DNA damage *in vitro* with 3D-Comet assay using a human reconstructed 3D-skin tissue model and under conditions relevant to the dermal exposure (ref 7).

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Taken from submission III

Taken from SCCNFP/0781/04, modified

Reverse Mutation Testing Using Bacteria

Guideline:	OECD 471 (1983)
Species/Strain:	*Salmonella typhimurium* TA98 and TA98 NR
Replicates:	triplicates in 2 experiments
Test substance:	B 034
Batch:	WSI – 75 h/52
Purity:	99.3%
Vehicle:	DMSO
Concentration:	100, 500, 1000, 2500, 5000, 7500 and 10000 µg/plate
Treatment:	direct plate incorporation with 48 h incubation without and with S9-mix
GLP:	in compliance
Study period:	9 June 1993 – 29 October 1993

B 034 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-test by the sponsor. Since no relevant toxic effects were observed, 10000 µg/plate was chosen as the maximal concentration. The experiment was performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
A concentration-dependent increase in the number of revertants was observed in both *Salmonella* strains both with and without S9-mix. As comparable effects were found for both *Salmonella* strains, the mutagenicity of B 034 may not be dependent on the enzymatic reduction of the amino-group but may be due to a direct reaction of B 034 with DNA.

Conclusion
Under the experimental conditions, used B 034 was mutagenic in this gene mutation test in bacteria.

Ref.: 11, submission I

Comment
The study followed the OECD guideline except that not all recommended strains were used.

Reverse Mutation Testing Using Bacteria

Guideline:	OECD 471 (1983)
Species/Strain:	*Salmonella typhimurium* TA98 NR
Replicates:	triplicates in 3 independent experiments
Test substance:	B 034
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	experiment 1: 50, 100, 500, 1000, 2500, 5000 µg/plate experiments 2-3: 100, 500, 1000, 2500, 5000, 7500, 10000 µg/plate
Treatment:	direct plate incorporation with 48 h incubation without and with S9-mix
GLP:	in compliance
Study period:	test was performed between September and December 1993

B 034 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. 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 B 034 showed no toxic effects up to the highest concentration tested. Without metabolic activation, a concentration-dependent increase in the number of revertants was observed when concentrations up to 10000 µg/plate were used. With S9-mix, only singular increases in the number of revertants were observed without concentration-dependency.

Conclusion
Under the experimental conditions used, B 034 was mutagenic in this gene mutation tests in bacteria.

Ref.: 10, submission I

Comment
The study followed the OECD guideline except that not all recommended strains were used. Purity and batch number are not reported. The value of this test is limited.

Reverse Mutation Testing Using Bacteria

Guideline:	OECD 471 (1983)
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538
Replicates:	triplicates in 2 independent experiments
Test substance:	B 034 or HC Violet BS
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	experiment 1: 50, 100, 500, 1000, 2500 and 5000 µg/plate experiment 2: 100, 500, 1000, 2500, 5000, 7500 and 10000 µg/plate
Treatment:	direct plate incorporation with 48 h incubation without and with S9-mix
GLP:	in compliance
Study period:	11 September 1990 – 10 December 1990

B 034 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 level of toxicity in a pre-experiment with strains TA98 and TA100 both without and with S9-mix. The experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
The plates incubated with B 034 showed normal background growth up to 5000 µg/plate with and without S9-mix. Without metabolic activation a concentration-dependent increase in the number of revertants was observed in TA98. With metabolic activation, the increase is smaller and without concentration-dependency. Biologically relevant increases in the number of revertant colonies were not observed in the other strains tested both with and without S9-mix.

Conclusion
Under the experimental conditions used B 034 was mutagenic in this gene mutation tests in bacteria.

Ref.: 9, submission I

Comment
Purity and batch number are not reported. The value of this test is limited.

Reverse Mutation Testing Using Bacteria

Guideline:	/
Species/Strain:	*Salmonella typhimurium* TA1535, TA1537, TA98 and TA100
Replicates:	triplicates in 2 independent experiments
Test substance:	B 34
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	0, 8, 40, 200, 1000 and 5000 µg/plate
Treatment:	direct plate incorporation with 48 h incubation with and without S9-mix
GLP:	/
Study period:	the experiment was reported in March 1985

B 34 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in a preliminary toxicity rangefinder with TA98 both with and without S9-mix. The experiment was

performed with the direct plate incorporation method. Negative and positive controls were included.

Results
The plates incubated with B 34 showed normal background growth up to 5000 µg/plate with and without S9-mix. In the absence of S9-mix a concentration-dependent increase in the number of revertants was found in TA98 whereas in the presences of S9-mix an increase was found in both TA98 and TA1537.
A biologically relevant increase in the number of revertant colonies was not observed in the other strains tested.

Conclusion
Under the experimental conditions used, B 34 was mutagenic in this gene mutation test in bacteria.

Ref.: 8, submission I

Comment
The test was not performed according to OECD guideline 471 and was not in compliance with GLP. Purity and batch number were not reported. The value of this test is therefore limited.

Reverse Mutation Testing Using Bacteria

Guideline:	/
Species/Strain:	*Salmonella typhimurium* (TA98; TA100) and *E. coli* (wP2uvrA$^-$p)
Replicates:	triplicate cultures
Test substance:	WSI-75
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	3.2, 16, 80, 400, 2000 and 10000 µg/plate
Treatment:	direct plate incorporation with 48 h incubation without and with S9-mix
GLP:	/
Study period:	experiment was performed in June 1983

WSI-75 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. The experiment was performed with the direct plate incorporation method. All manipulations were performed under an orange photographic safelight. Negative and positive controls were included.

Results
At the top concentrations, WSI-75 was toxic to the bacteria and precipitated out of the top concentrations. A statistically significant and concentration-dependent increase in the number of revertants was found for TA98 and TA100 both with and without S9-mix. In the presence of S9-mix, but not in its absence, a concentration-dependent but statistically insignificant increase in the number of revertants was found in *E. coli*.

Conclusion
Under the experimental conditions used, WSI-75 was mutagenic in this gene mutation test in bacteria.

Ref.: 7, submission I

Comment
The test was not performed according to OECD guideline 471 and was not in compliance with GLP. Purity and batch number were not reported. The value of this test is therefore limited.

Submission II, 2005

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537
Replicates:	triplicates
Test substance:	B 034
Batch:	DALA 013106
Purity:	99.5% (area%, HPLC)
Vehicle:	DMSO
Concentration:	33, 100, 333, 1000, 2500 and 5000 µg/plate, with and without S9-mix
Treatment:	direct plate incorporation with 48 h incubation with and without S9-mix
GLP:	in compliance
Study period:	19 July – 3 August 2004

B 034 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 level of toxicity in a pre-experiment with 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 spontaneous 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 experiment I. Since no relevant toxic effects were observed, 5000 µg/plate was chosen as the maximal concentration. Both the pre and main experiment were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
The plates incubated with B 034 showed normal background growth up to 5000 µg/plate with and without S9-mix. A minor toxic effect observed as a reduction in the number of revertants was observed at 5000 µg/plate in TA102 without S9-mix.
A biologically relevant increase in the number of revertant colonies was observed in strain TA98 both with and without S9-mix. In the other strains tested, an increase in the number of revertants was not found.

Conclusion
Under the experimental conditions used, B 034 was mutagenic in this gene mutation test in bacteria.

Ref.: 7, submission II

Comment
Since a positive result was obtained, a second confirmatory experiment was not performed.

Taken from submission III (SCCNFP/0781/04, modified)

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

Guideline:	/
Species/Strain:	Mouse Lymphoma L5178Y cells (*hprt* locus
Replicates:	duplicate cultures in 1 experiment
Test substance:	B34
Batch:	/
Purity:	/

Vehicle:	DMSO
Concentrations:	313, 625, 1250, 2500 and 5000 µg/ml without S9-mix 250, 500, 1000, 2000 and 4000 µg/ml with S9-mix
Treatment:	treatment both without and with S9-mix; expression period 7 days and a selection period of 14 days.
GLP:	/
Study date:	8 January 1985 – 3 May 1985

B34 was assayed for mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. Liver-S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a toxicity range finder with 9 concentrations up to 5000 µg/ml without metabolic activation measuring survival. In the main test, treatment was followed by an expression period of 72 h, to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured as survival relative to the survival of the solvent control cultures. Negative and positive controls were included.

Results
In the toxicity rangefinder without metabolic activation, the top concentration of 5000 µg/ml reduced survival to 5% which is a suitable top concentration. With metabolic activation, no survivals were seen at this concentration. As no evidence of toxicity was observed at the next lower concentration, 4000 µg/ml was chosen as top concentration in the presence of metabolic activation.
In the main experiment, no concentration-related toxicity was observed in the absence of S9-mix up to 2500 µg/ml; the maximum concentration (5000 µg/ml) induced 75% mortality; in the presence of S9-mix, a concentration reduction survival was observed starting from 1000 µg/ml (70%) to 4000 µg/ml (20%).
A biologically relevant increase in mutant frequency was not observed at any concentration tested both with and without S9-mix as compared to the concurrent controls.

Conclusion
Under the experimental conditions used, B34 was not genotoxic (mutagenic) in this gene mutation assay in mouse lymphoma cells.

Ref.: 13, submission I

Comment
The test was not performed according to the OECD guideline and not in compliance with GLP. Purity and batch number were not reported. The value of this test is therefore limited.

Submission II, 2005

***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:	B 034	
Batch:	DALA 013106	
Purity:	99.5 area%	
Vehicle:	DMSO	
Concentrations:	Experiment I:	150, 300, 600, 1200 and 2400 µg/ml with and without S9-mix
	Experiment II:	9.4, 18.8, 37.5, 75, 150 and 600 µg/ml without 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: 5 July – 16 August 2004

B 034 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 (≈ 2400 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. Colony sizing was performed to discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect). Negative and positive controls were in accordance with the OECD guideline.

Results
The appropriate level of toxicity (10-20% survival after the highest concentration) was reached in both experiments both with and without S9-mix.
In neither experiment, was a reproducible, biologically-relevant increase in the number of mutant colonies observed independent of the presence or absence of S9-mix. Isolated minor increases of the mutant frequency exceeding the historical control data occurred in one culture of experiment II, but were considered biologically irrelevant since these effects were not observed in the parallel culture.

Conclusion
Under the experimental conditions used, B 034 was not mutagenic in this mouse lymphoma assay using the *tk* locus as reporter gene.

Ref. 8, submission II

Taken from SCCNFP/0781/04, modified

***In Vitro* Mammalian Chromosome Aberration Test**

Guideline: /
Species/Strain: CHO cells
Replicates: duplicate cultures per concentration
Test substance: B34
Batch: /
Purity: /
Vehicle: DMSO
Concentrations: 625, 1250, 2500 and 5000 µg/ml without S9-mix
1250, 2500 and 5000 µg/ml with S9-mix
Treatment 2 h with and without S9-mix; harvest time 24 h after start of treatment
GLP: /
Study date: 4 January 1985 – 6 March 1985

B34 has been investigated for the induction of chromosomal aberrations in CHO cells both in the absence and presence of metabolic activation. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a toxicity range-finder measuring mitotic index with concentrations up to 5000 µg/ml. Cytotoxicity was determined 24 h after start of treatment.
In the main test, cells were treated for 2 h (with and without S9-mix) and harvested 24 h after the start of treatment. Approximately 2 h before harvest, each culture was treated with colchicine (1 µg/ml) to block cells at metaphase of mitosis. Chromosome (metaphase)

preparations were stained with 4% Gurr's Giemsa R66 and examined microscopically for chromosomal aberrations. Negative and positive controls were included.

Results
A preliminary toxicity study demonstrated that a concentration of 5000 µg/ml in the presence of S9-mix induced a 66% reduction of mitotic index. No toxicity was observed in the absence of S9-mix at the same concentration.
In the main test, the concentration of 5000 µg/ml both in the presence and in the absence of metabolic activation in both cultures induced a statistically significant increase in the number of cells with chromosome aberrations.

Conclusion
Under the experimental conditions used, B34 was genotoxic (clastogenic) in the chromosome aberration test in CHO cells

Ref.: 16, submission I

Comment
The test was not performed according to the OECD guideline and was not in compliance with GLP. Purity and batch number were not reported. The value of this test is therefore limited.

In Vitro Mammalian Chromosome Aberration Test

Guideline:	OECD 473 (1983)
Species/Strain:	Human peripheral lymphocytes from two healthy donors
Replicates:	duplicate cultures per concentration
Test substance:	B 034
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	25, 50, 100 and 200 µg/ml without S9-mix 100, 200 400 and 600µg/ml with S9-mix
Treatment	24 h treatment without S9-mix or 2 h treatment with S9-mix; harvest time 24 h after the start of treatment
GLP:	in compliance
Study date:	22 August 1990 – 13 May 1991

B 034 has been investigated for the induction of chromosomal aberrations in human lymphocytes of 2 different donors both in the absence and presence of metabolic activation. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a preliminary cytotoxicity assay with and without metabolic activation measuring the mitotic index with 9 concentrations up to 5000 µg ml. Cytotoxicity was determined 24 h after start of treatment.
In the main test, cells were treated for 2 h (with S9-mix) or 24 h (without S9-mix) and harvested 24 h after the start of treatment. Approximately 2 h before harvest, each culture was treated with colcemid (0.25 µg/ml culture medium) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with 5-10% Giemsa and examined microscopically for chromosomal aberrations. Negative and positive controls were in accordance with the OECD guideline.

Results
In the cytotoxicity assay without S9-mix, there was a reduction of the mitotic index of about 50% at 100 and 200 µg/ml. With S9-mix a reduction of 50% was observed between 400 and 600 µg/ml. Therefore 200 and 600 µg/ml were chosen as top concentrations.
There was no a biologically relevant increase in cells with chromosome aberrations found in either the absence and presence of S9-mix, Exclusively, at 200 µg/ml in the presence of

metabolic activation endoreduplication was observed. As this was a single observation it is not considered biologically relevant.

Conclusion
Under the experimental conditions used, B 034 was not genotoxic (clastogenic) in the chromosome aberration test in human lymphocytes.

Ref.: 17, submission I

Comment
Purity and batch number were not reported. The value of this test is limited.

Submission II, 2005

***In vitro* Micronucleus Test**

Guideline:	draft OECD 487 and OECD 473
Species/strain:	Chinese hamster V79 cells
Replicates:	two parallel cultures per group
Test item:	B 034
Batch:	DALA 013106
Purity:	99.5% (area%, HPLC)
Vehicle:	DMSO
Concentrations:	1200, 1800 and 2400 µg/ml without and with S9-mix

Treatment 4 h treatment; harvest time 24 hours after the beginning of treatment, with or without S9-mix

GLP:	in compliance
Study period:	21 September – 29 October 2004

B 034 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 was performed in order to determine the toxicity of B 034, the solubility during exposure and changes in osmolarity and pH value at experimental conditions. The highest concentration should produce clear toxicity with reduced cell growth. Considering the toxicity data of the pre-test and the occurrence of precipitation of B 034, 2400 µg/ml (≈ 10 mM, the prescribed maximum concentration) was chosen as top concentration in the main experiment. The treatment period in the main test was 4 h (with and without S9-mix). Harvest time was 24 h after the beginning of culture. For assessment of cytotoxicity, a XTT test was also carried out in the main test in parallel to the micronucleus test. Negative and positive controls were in accordance with the draft guideline.

Results
After 4 h treatment with B 034, clear toxic effects, indicated by reduced cell numbers below 40% of control values, were observed at the highest concentration scored (2400 µg/ml). In contrast, regardless of whether it was with or without S9-mix, the XTT activity was reduced after 4 h treatment up to the highest concentration applied.
Both with and without S9-mix, a statistically significant and concentration-dependent increase in the number of cells with micronuclei was observed.

Conclusion
Under the experimental conditions used, B 034 induced an increase in micronucleated cells and, consequently, is genotoxic (clastogenic and/or aneugenic) in V79 cells.

Ref.: 9, submission II

Comment
Since B 034 was genotoxic after 4 h treatment, no second experiment was performed, in accordance with international guidelines.

Taken from SCCNFP/0781/04, modified

***In vitro* unscheduled DNA synthesis (UDS) test**

Guideline:	/
Species/Strain:	HeLa S3 cells
Test substance:	B 34
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	0.0064, 0.032, 0.16, 0.8, 4, 20, 100 and 500 µg/ml both with and without S9-mix
Treatment	2.5 h treatment and cells were harvested immediately
GLP:	/
Study period:	18 January 1985 – 22 February 1985

B 34 was investigated for the induction of unscheduled DNA synthesis (UDS) in HeLa cells both without and with S9-mix. Liver- S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Cells were treated for 2.5 h with various concentrations of B 34 and [^{3}H] thymidine (5 µCi/ml) and harvested immediately after treatment. DNA was extracted and hydrolysed by heating. After removing the proteins (present as precipitate), the supernantant was counted in a liquid scintillation counter. The DNA concentration was measured using the Burton colorimetric assay, with 18 h for colour development and measurements at 600 and 700 nm. Absolute DNA concentrations were determined against a standard graph plotted from solutions of hydrolysed calf thymus DNA of known concentrations. The UDS values (dpm/µg DNA) were calculated from the determined DNA concentration and the results of scintillation counting (dpm/ml). Positive and negative control compounds were included.

Results
In the absence of S9-mix, a biologically relevant induction of unscheduled DNA synthesis was not observed. In presence of S9-mix, treatment with B 34 resulted in a statistically significant and concentration-related increase in DNA repair and thus unscheduled DNA synthesis.

Conclusions
Under the experimental conditions used, B 34 did induce unscheduled DNA synthesis and, consequently, is genotoxic in this UDS test.

Ref.: 14, submission I

Comment
The test was not performed according to the OECD guideline and was not in compliance with GLP. Purity and batch number were not reported. The value of this test is therefore limited.

***In vitro* unscheduled DNA synthesis (UDS) test**

Guideline:	OECD 482 (1986)
Species/Strain:	freshly isolated rat hepatocytes (Wistar/Wu rats)
Replicate:	triplicate cultures in 2 independent experiments
Test substance:	B 034

Batch:	WSI-75h/52
Purity:	99.3% (HPLC)
Vehicle:	DMSO
Concentration:	2.67, 8.0, 26.67, 80.0 and 266.67 µg/ml
Treatment	18 h treatment and cells were harvested immediately
GLP:	in compliance
Study period:	29 April 1993 – 21 June 1993

B 034 was investigated for the induction of unscheduled DNA synthesis (UDS) in freshly isolated hepatocytes from Wistar/Wu rats. The viability of the actual performed perfusion was determined by the trypan blue dye exclusion method. In addition the number of isolated cells was determined. To evaluate toxicity of B 034, a pre-experiment on cytotoxicity was performed with 10 concentrations under the same conditions as in the main experiment. Toxicity was evidenced by altered cell morphology, reduced number of adherent cells and uptake of the vital dye neutral red.
After an attachment period of approximately ninety minutes, the hepatocytes were exposed B 034 for 18 h in the presence of ^{3}H-thymidine (5 µCi/ml, specific activity 20 Ci/mmol). UDS was measured by autoradiography. The number of silver grains above the nucleus and the number of grains above a nuclear-sized cytoplastic area adjacent to the nucleus were counted. UDS is reported as the net nuclear grain count (nuclear grain count – average cytoplasm grain count). Unscheduled DNA synthesis was determined in 50 randomly selected hepatocytes/slide. Negative and positive controls were in accordance with the guideline.

Results
In the pre-experiment, cytotoxicity was observed at B 034 concentrations of 533.33 µg/ml and above. Concentrations higher than 266.67 µg/ml precipitated in the culture medium.
In neither independent experiment was a biologically relevant and reproducible concentration-dependent increase in the number of nuclear and net grain counts observed up to the highest concentration evaluated.
A slightly positive net grain value was found at the concentration of 266.67 µg/ml in experiment II. Since this positive value was neither due to increased nuclear grain counts nor higher then 5, it was considered of no biological relevance.

Conclusion
Under the experimental conditions reported, B 034 did not induce DNA-damage leading to unscheduled DNA synthesis in hepatocytes and, consequently, is not genotoxic in this *in vitro* UDS test.

Ref.: 15, submission I

Comment
It should be noticed that the positive observed concentration was partly precipitated, and that the concentrations in this experiment were lower than in the previous experiment on HeLa.

Submission III, 2010

***In vitro* unscheduled DNA synthesis (UDS) test**

Guideline:	OECD 482 (1986)
Cells:	primary hepatocytes from 6-10 weeks old Wistar rats
Replicate:	3-6 cultures per concentration in 2 independent experiments
Test substance:	COLIPA B 034
Batch:	DALA 013106
Purity:	98.8 area % (HPLC), 98.6 wt % (^{1}H-NMR)

Solvent:	culture medium	
Concentrations:	Experiment 1:	50, 100, 250, 500, 1000 and 2500 µg/ml
	Experiment 2:	250, 500, 600, 700, 800, 900, 1000, 1250, 1500 and 2500 µg/ml
Treatment	Experiment 1:	18 h and 40 minutes exposure, autoradiography after 7 days
	Experiment 2:	19 h exposure, autoradiography after 7 days
GLP:	in compliance	
Study period:	June 2010 – December 2010	

COLIPA B 034 was investigated in an *in vitro* unscheduled DNA synthesis (UDS) test in hepatocytes of rats. Hepatocytes were collected from 6-10 week old Wistar rats by liver perfusion with collagenase. The viability of the actual performed perfusion was determined by the trypan blue dye exclusion method. In addition, the number of isolated cells was determined. To evaluate toxicity of COLIPA B 034, a pre-experiment on cytotoxicity was performed with 10 concentrations under the same conditions as in the main experiment. Toxicity was evidenced by altered cell morphology, reduced number of adherent cells and uptake of the vital dye neutral red.
After an attachment period of approximately ninety minutes, the hepatocytes were exposed to COLIPA B 034 for 40 min and 18 h (experiment 1) or 19 h (experiment 2) in the presence of ^{3}H-thymidine (5 µCi/ml, specific activity 20 Ci/mmol). The number of silver grains above the nucleus and the number of grains above a nuclear-sized cytoplastic area adjacent to the nucleus were counted. UDS is reported as the net nuclear grain count (nuclear grain count – average cytoplasm grain count). Additionally, the percentage of cells in repair (cells with ≥5 net nuclear grains) is reported. Unscheduled DNA synthesis was determined in 50 randomly selected hepatocytes/slide. Appropriate reference positive controls were included.

Results
In the pre-experiment on cytotoxicity, strong cytotoxicity was observed with COLIPA B 034 concentrations of 1000 µg/ml and above. At concentrations up to 500 µg/ml, the recommended cytotoxicity was found. The reduction in viability of the cells at these concentrations was less than 20%. Precipitation was seen at 5000 µg/ml. Although strong cytotoxicity was present at 2000 µg/ml, this concentration was still chosen as the highest concentration.
In the main experiment, no precipitation of COLIPA B 034 was found. At the highest concentration, strong cytotoxicity was observed but only slight cytotoxicity at the next lower concentration. No biologically relevant increase in mean net nuclear grain count as compared to the untreated control was found in hepatocytes at any concentration tested.
A 24 % increase in the percentage of cells in repair was found in experiment 1. However, as a mean nuclear grain count of +5 was not found and no such increase was found in experiment 2, this increase in cells in repair is not considered biologically relevant.

Conclusion
Under the experimental conditions reported, COLIPA B 034 did not induce DNA-damage leading to unscheduled DNA synthesis in hepatocytes and, consequently, is not genotoxic in this *in vitro* UDS test.

Ref.: 5, submission III

Submission IV, 2014
***In vitro* Mammalian Cell Gene Mutation Test (*HPRT*-locus)**

Guideline:	OECD 476 (1997)
Cells:	L5178Y cells
Replicates:	duplicate cultures in two independent experiments
Test substance:	COLIPA B 034

Batch:	DALA013106, SAT 120007	
Purity:	99.5 % (HPLC);	
Solvent:	deionised water	
Concentrations:	experiment I:	154.1, 308.1, 616.3, 1232.5, 1848.8 and 2465 µg/ml with and without S9-mix
	experiment II	154.1, 308.1, 616.3, 1232.5, 1848.8 and 2465 µg/ml with S9-mix and 4 h treatment 38.6, 77, 154.1, 308.1, 462.2, 616.3 and 1232.5 µg/ml without S9-mix and 24 h treatment
Treatment:	experiment I:	4 h treatment both with and without S9-mix; Expression period 48h and a selection period of 10-15 days
	experiment II:	4 h treatment both with S9-mix; expression period 48h and a selection period of 10-15 days 24 h treatment without S9-mix; expression period 48h and a selection period of 10-15 days
GLP:	in compliance	
Study period:	March 13, 2013	

B34 was assayed for gene mutations at the *HPRT* 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 (≈ 2465 µg/ml) measuring relative suspension growth. In the main test, cells were treated for 4 h (with and without S9-mix, Experiment I and II) or 24 h (without S9-mix experiment II) followed by an expression period of 48h to fix the DNA damage into a stable *HPRT* mutation. Liver S9-fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system.
In the second experiment, the cells were treated for 24 hrs in the absence and for 4 hrs in the presence of metabolic activation. 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. Negative and positive controls were in accordance with the OECD guideline.

Results
The appropriate level of toxicity (10-20% survival after the highest concentration) was reached in both experiments without S9-mix but not in the presence of metabolic activation. At highest test concentration in the 1st experiment precipitation was visible in the presence of metabolic activation.
No substantial and reproducible concentration dependent increase in the mutant colony numbers was observed in either main experiment up to the maximum concentration with and without metabolic activation.
Isolated minor increases of the mutant frequency exceeding the historical control data occurred in one culture of experiment I but were considered biologically irrelevant since these effects were not observed in the parallel culture.

Conclusion
Under the experimental conditions used, B34 was not mutagenic in mouse lymphoma cells at the *HPRT* locus as reporter gene.

Ref.: 6, submission IV

SCCS comment
B34 was dissolved in deionised water and precipitation occurred in the presence of S9-mix.
A concentration-dependent trend of increasing mutant frequencies that were assessed as a linear regression (least squares) was significant in Experiment I without S9-mix.

3D-Comet assay using the Phenion® Full-thickness Skin Model

Guideline: /
Cells: *in vitro* 3D system, consisting of fibroblasts and keratinocytes,
Replicates: triplicate skin tissues in two independent experiments
Test substance: COLIPA B 034
Batch: HCVBS121201H, SAT 140048
Purity: 99.5 % (HPLC);
Solvent: 70% ethanol in water
Concentrations: 15 to 50 mg/ml, or 240 to 800 µg/cm^2
Treatment: 3, 24 and 48 hours
GLP: in compliance
Study period: November 11 2014

The Phenion® Full-thickness Skin Model, an *in vitro* 3D system, consisting of fibroblasts and keratinocytes, was used to examine the potential of B 034 to induce DNA lesions resulting in comet tails.

The genotoxic potential of B 034 was evaluated in two independent tests consisting of three skin tissues each. The chosen test system allows the topical application of the test compound similar to the intended normal application of such a cosmetic ingredient. Since DNA damage can be the result of cell death, it is evaluated at non-cytotoxic concentrations to avoid false positive results.
B34 was applied three times at concentrations in the range of 1.5 to 5% (corresponding to 15 to 50 mg/ml, or 240 to 800 µg/cm^2 respectively) per 3D skin tissue. 10% is considered to be the maximum concentration for this *in vitro* test but was found to be extremely toxic in this test system. The three consecutive applications were placed to ensure an exposure duration of these three aliquots of 3, 24 and 48 hours. In each test run, a negative (untreated), solvent (70% ethanol), and positive control (5 µg methyl methane sulfonate, MMS) were tested in parallel.
The comet tails were evaluated with the Comet IV software (Perceptive Instruments) resulting in per cent tail intensity of the comet tail compared to the head signal. Cytotoxicity was assessed by measuring both the intracellular concentration of adenosine triphosphate (ATP) and the activity of adenylate kinase (AK) released into the culture medium. Due to the strong cytotoxicity, a third test run with the DNA polymerase inhibitor Aphidicolin was not considered.

Results
Concentrations of B34 tested were found to be cytotoxic at concentrations exceeding or equal to 320 µg/cm² in the first two experiments. The tail intensity values of all concentration groups of B34 tested up to 3% as the maximum non-cytotoxic concentration were within the range of the negative and solvent controls in both cell types in both test runs.

Conclusion
B34 was considered as non-genotoxic in this study as it did not meet the pre-defined criteria for a positive response.

Ref.: 7, submission IV

3.3.6.2 Mutagenicity / Genotoxicity in vivo

Taken from Submission III

SCCNFP/0781/04, modified

***In Vivo* Mammalian Erythrocyte Micronucleus Test**

Guideline: /
Species/Strain: CFLP Mice
Group size: 5 male and 5 female mice per group dosed.
Test Substance: B 34
Batch: /
Purity: /
Vehicle: 10% DMSO in water
Dose level: 0 and 600 mg/kg bw
Treatment: intraperitoneal injection
Sacrifice Time: 24, 48 and 72h
GLP: /
Study period: experiment was reported in April 1985

B 34 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on acute toxicity in a dose-range finding study with doses up to 1000 mg/kg bw. In the main experiment, mice were exposed to single *i.p*. doses of 0 and 600 mg/kg bw. Bone marrow cells were collected 24, 48 or 72 h after dosing. Toxicity, and thus exposure of the target cells, was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Negative and positive controls were included.

Results
In a preliminary toxicity test, no signs of toxicity were seen at doses < 400 mg/kg bw. At 600 and 1000 mg/kg bw all mice were lethargic and had tremors one h after dosing. Periorbital staining was obvious in one female dosed at 600 mg/kg bw and in both females at 1000 mg/kg bw. At 17 h after dosing all male and female mice died at a dose of 1000 mg/kg.
In the micronucleus test, no toxicity, measured as PCE/TE ratio, was observed up to 24, 48 and 72h after treatment. Mice treated with B 34 did not show any statistically significant increase in the number of cells with micronuclei for either sex at any sacrifice time, compared to the control frequencies.

Conclusion
Under the experimental conditions used, B34 did not induce micronuclei in bone marrow cells of treated mice and, consequently, B34 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 18, Submission I

Comment
The study was not performed under GLP compliance and not according to the OECD guideline. Purity and batch number were not reported. Indications that bone marrow cells were exposed were lacking. Consequently, the value of this test is limited.

In Vivo Mammalian Erythrocyte Micronucleus Test

Guideline: OECD 474
Species/Strain: Cr1:NMRI BR Mice
Group size: 5 males and 5 female mice/group dosed
Test Substance: B 34
Batch: /
Purity: /
Vehicle: 0.5 % aqueous carboxymethylcellulose
Dose: 1200 mg/kg bw
Treatment: stomach intubation
Sacrifice Time: 24, 48 and 72h after start of treatment

GLP: in compliance
Study period: 26 April 1988 – September 1988

B 34 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on acute toxicity in a preliminary range finding study. In the main experiment, mice were exposed to oral doses of 0 and 1200 mg/kg bw. Bone marrow cells were collected 24, 48 or 72 h after dosing. Toxicity, and thus exposure of the target cells, was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Negative and positive controls were in accordance with the OECD draft guideline.

Results
All animals survived until the scheduled termination of the experiment and no adverse test substance-related effects were noted.
Treatment with B 034 did not result in decreased PCE/TE ratios compared to the untreated controls indicating that B 034 did not have cytotoxic properties in the bone marrow. No biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were found following treatment with B 034 at any time point.
Conclusion
Under the experimental conditions used, B 34 did not induce micronuclei in bone marrow cells of treated mice and, consequently, B 34 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 19, Submission I

Comment
The study was not performed under GLP compliance and not according to the OECD guidelines. Purity and batch number were not reported. Indications that bone marrow cells were exposed were lacking. Consequently, the value of this test is limited.

Submission II, 2005

***In vivo* Mammalian Erythrocytes Micronucleus Test**

Guideline: OECD 474 (1997)
Species/strain: mouse, NMRI BR (SPF)
Group size: 5 mice/sex/sampling time
Test substance: B 034
Batch: DALA 013106
Purity: 99.5%
Vehicle: corn oil
Dose level: 0, 187.5, 375, 750 mg/kg bw
Route: single intraperitoneal injection
Sacrifice times: 24 and 48 h (high dose and positive control only) after the treatment.
GLP: in compliance
Study period: 21 December 2004 – 31 January 2005

B 034 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on acute toxicity in a dose-range finding study, measured after 1, 1.5, 3.5 h and on day 2 and 3 after treatment. In the main experiment, mice were exposed to single *i.p*. doses of 0, 187.5, 375, 750 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (highest dose and positive control only) after dosing. Toxicity, and thus exposure of the target cells, was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Additional animals were dosed for blood sampling. Blood was sampled to be able to demonstrate the exposure of the bone marrow to B 034 in case exposure could not be demonstrated by the PCE/TE ratio, severe toxic

effects, discoloured urine or positive results. Negative and positive controls were in accordance with the OECD draft guideline.
Results
Treatment with B 034 did not result in decreased PCE/TE ratios compared to the untreated controls indicating that B 034 did not have cytotoxic properties in the bone marrow. During the first 1.5 h after treatment, all animals treated with 750 mg/kg bw were lethargic, showed ataxia and their hairless body parts were coloured purple. In the other groups the animals showed no reaction to treatment. Within 4 h after treatment, all animals had black-coloured urine. The animals from the 750 mg/kg bw groups were lethargic and most animals had a rough coat; one animal, had a hunched posture. Within 20 h all animals had recovered from treatment. Since bioavailability of the groups treated with B034 was demonstrated by the excretion of coloured urine, plasma levels in the blood samples were not measured.
No biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were found following treatment with B 034 at any time point or dose level tested.

Conclusion
Under the experimental conditions used, B 034 did not induce micronuclei in bone marrow cells of treated mice and, consequently, B 034 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 10, submission II

3.3.7 Carcinogenicity

No data submitted

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

No data submitted

3.3.8.2 Teratogenicity and other data on fertility and reproduction toxicity

Taken from submission III
Taken from SCCNFP/0781/04

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:	/
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
Study period:	1987

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 were 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.: 20, submission I

3.3.8.3 Developmental Toxicity

From submission III
Prenatal developmental study

Guideline:	OECD 414 (2001)
Species/strain:	rat, Wistar Hannover (HsdBrlHan:Wist)
Group size:	100 (25 females per dose group)
Test substance:	B034
Batch:	DALA 013106 (SAT 040411)
Purity:	99.5%
Vehicle:	0.5% aqueous carboxymethylcellulose
Dose levels:	0, 300, 600 and 1200 mg/kg bw
Dose volume:	10 ml/kg bw
Route:	oral gavage
Administration:	once daily from gestation day (GD) 5 through day 19
GLP statement:	in compliance
Study period:	9 – 28 August 2004

Dosages were based on the results of the previously performed toxicity studies. The mortality and the body weight gain were observed daily.
The dams were killed on GD 20. The number of alive and dead foetuses, their distribution and site in the uterus, early and late resorption, implantation and number of *corpora lutea* were determined. The weight of the foetuses, gravid uteri, uteri without foetuses, placentas and the sex of foetuses were recorded. Approximately one-half of the foetuses were selected at random and examined for visceral alterations. The remaining foetuses were examined for skeletal malformations, variations and retardation of the normal organogenesis after appropriate staining.

Results
No mortality occurred during the study.
One low-dose female had unilateral implantation and two high-dose females were not pregnant at autopsy.
Staining (violet) of the fur and of the cage tray were seen in all treated groups. This staining was considered to be related to the colour of the test substance and was probably eliminated in the urine. No other clinical signs were noted.

A slight but statistically significant reduction in body weight gain was noted at the mid-dose, compared with controls on GD 20. Food consumption was not affected by treatment. At autopsy, a generalised violet staining was noted in the skin and tail of all treated animals that was attributed to the colour of the test substance or its metabolites excreted in the urine. In addition, a treatment-related violet effect on the contents and walls of the stomach, jejunum and ileum of some treated animals granular was reported. This was considered substance-related and not regarded as a lesion. The remaining findings reported were considered to be incidental or spontaneous in origin.
A statistically significantly reduction in uterus weight was noted at the mid-dose compared with controls. This reduction was considered to be due to the reduced implantations noted in this group. Since the females were treated from GD 5, after corpora lutea production, this finding was not considered to be related to treatment.

Litter data and sex ratios
The statistically significantly reduced number of live young, reduced litter weight and disturbance in the sex ratio seen in the mid-dose group were considered to be due to the reduction in the number of implantations and to the increase in post-implantation loss noted in this group. The increase in total implantation loss was confined to three animals that had fewer corpora lutea with approximately 50% implantation losses. Since there was a lack of dose-relationship, these findings were considered incidental. A statistically significant increase in the percentage of male foetuses noted in the low-dose group was not considered to be of toxicological significance.
The number of small foetuses was comparable between groups. No relevance was attributed to the minor anomalies noted in one low dose foetus.
Visceral examination did not show any dose-related findings. No relevant foetal treatment-related skeletal changes were reported.

Conclusion
No maternal toxicity, foetal toxicity or teratogenicity was seen. On the basis of these results, the No Observed Adverse Effect Level (NOAEL) for N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine should be considered to be 1200 mg/kg/day.

Ref.: 12, submission II

3.3.9 Toxicokinetics

No new data submitted.

3.3.9.1 Toxicokinetics in laboratory animals

Taken from SCCNFP/0781/04

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:	/
Dose levels:	see below
GLP:	/

Study period: 1987

The percutaneous absorption of radio labelled ^{14}C-N,N'-bis-(2-hydroxyethyl)-2-nitro-pphenylenediamine 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/cm². 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/cm2 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.
An 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

3.3.9.2 Toxicokinetics in humans

No data submitted

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

No data submitted

3.3.10.2 Photomutagenicity / photoclastogenicity

No data submitted

3.3.11 Human data

No data submitted

3.3.12 Special investigations

3.3.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(Oxidative conditions)
(on head concentration 1%)

Absorption through the skin	**A**	**=**	**1.76 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.021 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.017 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**240 mg/kg bw/d**
Bioavailability 50%*		**=**	**120 mg/kg bw/d**
Margin of Safety	**adjusted NOAEL/SED**	**=**	**7100**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

(Non-oxidative conditions)
(on head concentration 1.5%)

Absorption through the skin	**A**	**=**	**2.97 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.723 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.029 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**240 mg/kg bw/d**
Bioavailability 50%*		**=**	**120 mg/kg bw/d**
Margin of Safety	**adjusted NOAEL/SED**	**=**	**4100**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14 Discussion

Physico-chemical properties
N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is used as a direct hair dye for hair colouring products. It is also used in oxidative hair dye formulations with and without an oxidising agent (e.g. hydrogen peroxide). The final on-head concentration of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine can be up to 1.5% when it is used without an oxidising agent and up to 1.0% after mixing with an oxidising agent.

N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is a secondary amine. It should not be used in the presence of nitrosating agents. The study report of the determination of

nitrosamine in N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine described only the principle of the analytical method used and the result. No analytical details and/or analytical data were provided. The applicant provided a simple UV method to evaluate stability in an oxidative hair coloration product. Stability testing using a simple comparison of UV spectra is not acceptable. A more selective analytical method such as HPLC with PDA detection should be used to evaluate stability for up to 60 min.

Toxicity

No mortalities occurred up to 5g/kg bw, so N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine showed no acute toxicity.

The No Observed Adverse Effect Level (NOAEL) for N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in the 90-day study was considered to be 240 mg/kg bw/day, based on the effects on the kidney at 720 mg/kg bw/d, which seemed to be the target organ.

Since no maternal toxicity, foetal toxicity or teratogenicity was seen, the No Observed Adverse Effect Level (NOAEL) for N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine for these endpoints was considered to be 1200 mg/kg bw/d.

Skin/eye irritation and sensitisation

Neat N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was not irritant when applied to rabbit skin. However, it was noted that 10% N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in acetone:olive oil was slightly irritant to the mouse ear in the LLNA test. It was irritant to rabbit eyes.

N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was not determined to be a skin sensitiser, under the experimental conditions.

Percutaneous absorption

The amount of test material applied on the skin (100mg/cm^2) is too high compared to the recommended dose of 20 mg/cm². Therefore, an absorption of 1.76 µg/cm² (0.94 + 2 x 0.41 (mean + 2SD)) may be used for the calculation of MoS for oxidative hair dyes.

For non-oxidative hair dyes, an absorption of 2.97 microgram/cm² (1.45 + 2 x 0.76) may be used for the calculation of the MoS.

Mutagenicity/genotoxicity

Overall, the genotoxicity programme on N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine investigated the three endpoints of genotoxicity: gene mutations, structural chromosome aberrations and aneuploidy. N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was positive in the gene mutation assays in bacteria (predominantly strain TA98) but did not induce gene mutation in mammalian cells at the *TK* or *HPRT* locus of mouse lymphoma cells. Contradictory results were found *in vitro* UDS tests treatment of hepatocytes with N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine. In a well performed test, unscheduled DNA synthesis was not observed. In a further UDS test, no unscheduled DNA synthesis was observed. In a well-performed *in vitro* micronucleus test, N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine exposure resulted in an increase in the number of cells with micronuclei.

The positive findings of the *in vitro* micronucleus test could not be confirmed in *in vivo* micronucleus tests in mice and thus N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine has no clastogenic potential *in vivo*.

However, the positive findings found in the *in vitro* gene mutation assays in bacteria were not confirmed nor ruled out in an appropriate *in vivo* test on the same genotoxic endpoint.

As N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was positive in gene mutation tests in bacteria, additional tests were performed (Submission IV). N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine was further investigated in mammalian gene mutation test at the *HPRT* locus on mouse lymphoma cells L5178Y. Additionally, DNA

damage *in vitro* with 3D-Comet assay using a human reconstructed 3D-skin tissue model and under conditions relevant to the dermal exposure was performed (submission IV). Both the comet assay as well as the mouse lymphoma gene mutation test at *HPRT* locus in the absence and presence of metabolic activation were negative and over-ruled positive result on the Ames test. Thus, N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine can be considered to have no genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No new data submitted.

4. CONCLUSION

1) In light of the new data provided, does the SCCS consider N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylamine (B34) safe when used as an oxidative hair dye with a concentration on-head of maximum 1.0 % and as a non-oxidative hair dye with a concentration up to 1.5 %?

On the basis of new submitted data, the SCCS considers N,N'-bis(2-hydroxyethylamino)-2-nitro-pphenylenediamine safe for use as an oxidative hair dye with an on-head concentration of maximum 1.0% and as a non-oxidative hair dye with a concentration up to 1.5%.

2) Does the SCCS have any further scientific concerns with regard to the use of N,N'-Bis-(2-hydroxyethyl)-2-nitro-p-phenylamine (B34) in other cosmetic products?

N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is a secondary amine and prone to nitrosation. The nitrosamine content in the dye should be <50 ppb. It should not be used in the presence of nitrosating agents.

A selective analytical method, such as HPLC with PDA detection, should be used to demonstrate stability for up to 60 min. under oxidative conditions.

5. MINORITY OPINION

/

6. REFERENCES

Submission I, 1994

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