Scientific Committee on Consumer Safety

SCCS

OPINION ON

N,N'-bis-(2-Hydroxyethyl)-2-nitro-p-phenylenediamine

COLIPA n° B34

The SCCS adopted this opinion at its 15th plenary meeting

of 26-27 June 2012

About the Scientific Committees

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

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

SCCS

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

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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. 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.
Revised opinions carry the date of revision.

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1. BACKGROUND

Submission I for N,N'-bis(2-hydroxyethylamino)-2-nitro-p-phenylenediamine (CAS 84041-77-0; EC 281-856-4) was submitted in May 1994 by COLIPA[1] according to COLIPA.

The first scientific opinion (SCCNFP/0781/04) was adopted by Scientific Committee on Cosmetic Products and Non-Food intended for Consumers on 23 April 2004 with 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 Pow 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."*

The second scientific opinion (SCCS/1228/09) was adopted by the Scientific Committee on Consumer Safety (SCCS) 23 March 2010 (revision after comments of 12 July 2010) with the opinion "*that a conclusion on the gene mutation potential of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine cannot be drawn without further testing.*
Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.
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 current submission III is an answer to the above mentioned opinion from 2010 and includes a new in vitro UDS study.

2. TERMS OF REFERENCE

1. *Does SCCS consider N,N'-bis(2-hydroxyethylamino)-2-nitro-pphenylenediamine safe for use 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% taken into account the scientific data provided?*

2. *And/or does the SCCS have any scientific concern with regard to the use of N,N'-bis(2-hydroxyethylamino)-2-nitro-p-phenylenediamine in oxidative or non-oxidative hair dye formulations?*

[1] COLIPA – The European Cosmetics 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

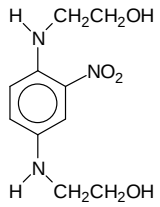
3.1.1.3. Trade names and abbreviations

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 (NDELA)	42ppb
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 content of the relevant nitrosamine, i.e. nitrosamine corresponding to N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in the raw material is not known. The nitrosamine content in the dye should be <50 ppb. It should not be used in the presence of nitrosating agents.

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.: 16

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

3.1.7. Partition coefficient (Log P_{ow})

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

Ref.: 15

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.

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 NDELA content in the raw material was 42 ppb. However, The content of the relevant nitrosamine, i.e. nitrosamine corresponding to N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in the raw material is not known. 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.
- No data on the stability of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in typical hair dye formulations has been provided

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

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 irritant to rabbit skin under the conditions of the experiment.

Ref.: 4

Comment
Purple staining of the skin was noted in all animals at all-time points. The study authors stated that this staining did not 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 condition of this experiment, B034 was irritant to rabbit eyes.

Ref.: 5

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

Concentration	**Stimulation Index**
Test item	
vehicle	/
2.5%	2.0
5%	2.1
10%	2.1
α-hexylcinnamaldehyde	
5%	1.5
10%	2.3
25%	8.4

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-sensitizer 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

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% B034) experiment B: B034 in oxidative dye cream with developer and hydrogen peroxide (= 1.0% B034) experiment C: B034 in oxidative dye cream with developer and without hydrogen peroxide (= 1.0% B034) experiment D: B034 in aqueous solution (= 0.7% 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.

Direct dye cream	Concentration in %
B034	1.50
Cetearyl alcohol	6.00
Fatty alcohol, C12-18	6.00
Ceteareth-12	3.00
Ceteareth-20	3.00
Methylparaben	0.30
Propylparaben	0.20
Phenoxyethanol	1.00
PEG-8	5.00
PEG-40 castor oil	1.00
Hydroxymethylcellulose	1.00
Sodium hydroxide	0.10 and for pH adjustment
Citric acid	for pH-adjustment
Water	ad 100

Oxidative dye formulation	Concentration in %
B034	2.00
Cetearyl alcohol	9.35
Sodium laureth sulfate	4.05
Cocoamidopropyl betaine	3.75
Fatty alcohol, C12-18	2.20
Ceteareth-20	0.75
Ascorbic acid	0.20
Sodium sulphite	0.20
Ammonium sulfate	0.40
Etidronic acid	0.12
Sodium silicate 38.2% active matter	0.50
Monoethanolamine	For pH adjustment
Water	ad 100

Ingredients of the two developer mixes:	**Developer with H_2O_2 in %**	**Developer without H_2O_2 in %**
Hydrogen peroxide	**6.00**	-
Dipicolinic acid	0.10	0.10
sodium propylphosphate	0.03	0.03
Etidronic acid	0.9	0.9
Sodium laureth sulfate	0.56	0.56
Silicone emulsion, 10% active substance	0.067	0.067
Acryl polymer	4.20	4.20
Ammonia, 25%	0.964	0.921
L(+)-Tartaric acid (pH adjustment)	0.22	-
Water	ad 100	ad 100

Results

Experiment A: B034 in direct dye cream (= 1.5% B034)

	Amount recovered (µg/cm²)									
Chamber	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Mean**	**SD**
Donor	18				19					
Adsorption after 48h	1.915	4.328	4.820	1.743	2.036	1.310	3.345	5.381	*3.11*	*1.57*
Absorption after 48h	0.394	0.736	0.769	1.408	1.162	0.677	0.655	0.725	*0.816*	*0.319*
Penetration 0-48h	0.256	0.236	0.406	0.809	1.795	0.445	0.766	0.395	*0.639*	*0.513*
Skin rinsings	1345	1459	1453	1270	1559	1374	1480	1418	*1420*	*89*
Bioavailable (µg/cm²)	0.650	0.972	1.175	2.217	2.956	1.122	1.421	1.120	*1.454*	*0.758*
Bioavailable (%)	0.050	0.068	0.080	0.159	0.230	0.082	0.106	0.078	*0.107*	*0.060*
Recovery (%)	104	103	99	91	122	100	110	99	*104*	*9*

Experiment B: B034 in oxidative dye cream with developer and with hydrogen peroxide (= 1.0% B034)

	Amount recovered (µg/cm²)									
Chamber	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**Mean**	**SD**
Donor	18				19					
Adsorption after 48h	0.671	0.615	1.126	0.600	0.766	0.725	0.833	0.881	*0.78*	*0.17*
Absorption after 48h *	1.050	0.872	1.531	0.921	0.588	0.461	0.317	0.355	*0.762*	*0.413*
Penetration 0-48h	0.138	0.064	0.213	0.132	0.262	0.283	0.153	0.173	*0.177*	*0.072*
Skin rinsings	1005	876	827	922	877	853	886	765	*876*	*70*
Bioavailable (µg/cm²)*	1.188	0.936	1.743	1.053	0.850	0.743	0.470	0.528	*0.939*	*0.407*
Bioavailable (%) *	0.107	0.095	0.181	0.098	0.084	0.075	0.045	0.059	*0.093*	*0.041*
Recovery (%)	91	89	86	86	87	86	85	85	*87*	*2*

* Significant difference between donor n° 18 and 19

Experiment C: B034 in oxidative dye cream with developer and without hydrogen peroxide (= 1.0% B034)

	Amount recovered (µg/cm²)									
Chamber	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**Mean**	**SD**
Donor	18				19					
Adsorption after 48h	7.085	0.912	0.571	2.791	1.283	1.369	0.731	1.498	*2.03*	*2.15*
Absorption after 48h *	1.565	1.370	0.960	1.953	0.547	1.146	0.905	0.870	*1.164*	*0.448*
Penetration 0-48h	0.559	0.025	0.035	0.444	0.279	0.773	0.140	0.162	*0.302*	*0.268*
Skin rinsings	901	873	938	1000	998	964	974	923	*947*	*46*
Bioavailable (µg/cm²)	2.123	1.395	0.995	2.397	0.825	1.919	1.044	1.031	*1.466*	*0.599*
Bioavailable (%)	0.225	0.154	0.102	0.226	0.079	0.192	0.105	0.107	*0.149*	*0.059*
Recovery (%)	96	97	96	95	96	97	98	96	*96*	*1*

* Significant difference between donor n° 18 and 19

Experiment D: B034 in aqueous solution (= 0.7% B034)

	Amount recovered (µg/cm²)									
Chamber	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**Mean**	**SD**
Donor	18				19					
Adsorption after 48h	0.623	1.324	0.610	0.641	1.673	1.379	1.175	1.010	*1.05*	*0.40*
Absorption after 48h	0.493	0.613	0.653	0.583	0.357	0.316	0.120	0.909	*0.505*	*0.242*
Penetration 0-48h	0.003	0.041	0.017	0.030	0.218	0.002	0.014	0.022	*0.043*	*0.072*
Skin rinsings	511	619	628	718	635	649	661	644	*633*	*58*
Bioavailable (µg/cm²)	0.496	0.654	0.670	0.613	0.576	0.318	0.134	0.931	*0.549*	*0.240*
Bioavailable (%)	0.072	0.095	0.098	0.090	0.085	0.046	0.019	0.133	*0.080*	*0.035*
Recovery (%)	76	90	92	105	94	93	94	92	*92*	*8*

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 <u>with</u> hydrogen peroxide	***0.939*** ± *0.407*	0.470 - 1.743	***0.093*** ± *0.041*	1.0%
B034 in oxidative dye cream with developer and <u>without</u> 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 <u>with</u> 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 <u>without</u> hydrogen peroxide: 1.466 ± 0.599 µg/cm².

Ref.: 13

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 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

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 misdosing, 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 misdosing 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 and 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

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

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

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 Aroclor-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 without and with 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 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 tests in bacteria.

Ref.: 11, subm 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 or HC Violet BS
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 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. 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, subm 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 without and with S9-mix.

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

Ref.: 9, subm 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 without and with S9-mix
GLP:	in compliance
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 without and with 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 tests in bacteria.

Ref.: 8, subm I

Comment
The test was not performed according to the OECD guideline 471 and not in compliance with GLP. Purity and batch number are not reported. The value of this test is 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:	in compliance
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 without and with S9-mix. In the presence but not in the absence of S9-mix a concentration-dependent but not statistically significant 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 tests in bacteria.

Ref.: 7, subm I

Comment
The test was not performed according to the OECD guideline 471 and not in compliance with GLP. Purity and batch number are not reported. The value of this test is 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, without and with S9-mix
Treatment:	direct plate incorporation with 48 h incubation without and with 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 without and with 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 tests in bacteria.

Ref.: 7

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

Taken from 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:	in compliance
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 7 days, 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 at the next lower concentration no evidence of toxicity was observed, 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 without and with 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.: 14, subm I

Comment
The test was not performed according to the OECD guideline and not in compliance with GLP. Purity and batch number are not reported. The value of this test is 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 without and with 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. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
The appropriate level of toxicity (10-20% survival after the highest concentration) was reached in both experiments both without and with S9-mix.
In both experiment a reproducible, biologically relevant increase in the number of mutant colonies was not 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

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 without and with S9-mix; harvest time 24 h after start of treatment
GLP: in compliance
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 (without and with 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.: 17, subm I

Comment
The test was not performed according to the OECD guideline and not in compliance with GLP. Purity and batch number are not reported. The value of this test is 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 without and with 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.
Both in the absence and presence of S9-mix, a biologically relevant increase in cells with chromosome aberrations was not found.

Exclusively, at 200 µg/ml in the presence of metabolic activation endoreduplication was observed. As this was a single observation it is considered not biologically relevant.

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

Ref.: 18, subm I

Comment
Purity and batch number are 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, without and with 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 (without and with S9-mix). Harvest time was 24 h after the beginning of culture. For assessment of cytotoxicity also in the main test a XTT test was carried out 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, neither without nor with S9-mix, the XTT activity was reduced after 4 h treatment up to the highest concentration applied.
Both without and with 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

Comment

Since B 034 was genotoxic after 4 h treatment, a second experiment was according to international guidelines not performed

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 without and with S9-mix
Treatment	2.5 h treatment and cells were harvested immediately.
GLP:	in compliance
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. From the determined DNA concentration and the results of scintillation counting (dpm/ml) the UDS values (dpm/µg DNA) were calculated. 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.: 15, subm I

Comment
The test was not performed according to the OECD guideline and not in compliance with GLP. Purity and batch number are not reported. The value of this test is 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 both independent experiments a biologically relevant and reproducible concentration-dependent increase in the number of nuclear and net grain counts was not 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 and not 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.: 16, subm 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 weeks 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 18 h and 40 minutes (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 areas 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 precipitation of COLIPA B 034 was not found. At the highest concentration strong cytotoxicity was observed but only slight cytotoxicity at the next lower concentration. A biologically relevant increase in mean net nuclear grain count as compared to the untreated control was not 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 such increase was not found in experiment 2, this increase in cells in repair is considered not 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, subm III

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Taken from 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: in compliance
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 40 mg/kg bw and at 400 mg/kg bw (≤ 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.: 19, subm I

Comment
The study was 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 (1983)
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. Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found following treatment with B 034 at any time point.

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.: 20, subm I

Comment
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.
Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not 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

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

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 daily recorded throughout the study 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

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 the cage tray was 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.
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, litter weight and 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 significantly 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

3.3.9. Toxicokinetics

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:	in compliance
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 0.15% and other vehicle constituents 1.6%. 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

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Not applicable

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 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.
N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine is a secondary amine. NDELA content in the raw material was 42 ppb. However, the content of the relevant nitrosamine, i.e. nitrosamine corresponding to N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in the raw material is not known. The nitrosamine content in the dye should be <50 ppb. It should not be used in the presence of nitrosating agents.
No data on the stability of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in typical hair dye formulations has been provided.

Toxicity
No mortalities occurred up to 5 g/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 effects on kidney and urea at 720 mg/kg bw/day, 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/day.

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 program 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. In *in vitro* UDS tests treatment of hepatocytes with N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine contradictory results were found. In a well performed test unscheduled DNA synthesis was not observed whereas a test with very limited value was positive. In a newly submitted UDS test no unscheduled DNA synthesis was observed. Also in two *in vitro* chromosome aberration tests, both not performed according current standards and thus with limited value, contradictory results were found. In one test N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine treatment resulted in an increase in the number of cells with chromosome aberrations whereas the second test was negative. In a well performed *in vitro* micronucleus tests, measuring the same genotoxic endpoint, 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 and in *in vitro* tests measuring chromosome aberrations, a mutagenic potency cannot be excluded. Consequently, a conclusion on the genotoxic potential of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine cannot be drawn.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCS is of the opinion 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-pphenylenediamine not safe for use 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%.

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.

5. MINORITY OPINION

Not applicable

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