EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

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

COLIPA n° B34

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 6th plenary meeting
of 23 March 2010

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 Evaluation Agency (EMEA), 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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ISSN 1831-4767 ISBN 978-92-79-12735-9
doi:10.2772/22740 ND-AQ-09-007-EN-N

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

ACKNOWLEDGMENTS

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This opinion has been subject to a commenting period of four weeks after its initial publication. All 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.

Keywords: SCCS, scientific opinion, hair dye, B34, N,N'-bis-(2-hydroxyethylamino)-2-nitro-p-phenylenediamine, CAS 84041-77-0, EC 281-856-4, directive 76/768/EEC

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on N,N'-bis-(2-hydroxyethylamino)-2-nitro-p-phenylenediamine, 23 March 2010

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1. BACKGROUND

Submission I of N,N'-Bis-(2-hydroxyethylamino)-2-nitro-p-phenylenediamine (CAS 84041-77-0) (B034) was submitted by COLIPA [1] in May 1994 according to COLIPA. The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) expressed its opinion (SCCNFP/0781/04) in the meeting on 23 April 2004.

Submission II was submitted by COLIPA in July 2005. Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Safety (SCCS) consider N,N'-Bis-(2-hydroxyethylamino)-2-nitro-p-phenylenediamine safe for use at concentrations on the head up to 1.5% in non-oxidative or up to 1.0% oxidative hair dye formulations taken into account the data provided?*

2. *Does the SCCS recommend any restrictions with regard to the use of N,N'-Bis-(2-hydroxyethylamino)-2-nitro-p-phenylenediamine in non-oxidative or oxidative hair dye formulations?*

[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

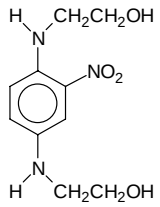
3.1.1.3. Trade names and abbreviations

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.:14

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)	/ λ_{max} 251 nm and 518 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 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 did not affect 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

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 however, did not fulfil the EU criteria for classification as eye irritant (R36).

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 that 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 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.

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 mg 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 <u>with</u> hydrogen peroxide (= 1.0 mg 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 <u>without</u> hydrogen peroxide (= 1.0 mg 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 mg 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 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*** ± *0.407*	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 ± 0.407 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. Therefore, 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

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), 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. 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

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

Reverse Mutation Testing Using Bacteria

Guideline:	OECD 471 (1983)
Species/Strain:	*Salmonella typhimurium* (TA98, TA98 NR)
Test item:	HC Violet B5
Batch:	/
Purity:	/
Replicate:	2 experiments
Doses:	100, 500, 1000, 2500, 5000, 7500, 10000 µg/plate
Metabolic Act.:	Phenobarbital / Naphthoflavone-treated rat liver homogenate
Positive controls:	2NF (-S9); 2AA (+S9)
GLP:	in compliance

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

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

Ref.: 11, subm I

Reverse Mutation Testing Using Bacteria

Guideline:	OECD 471 (1983)
Species/Strain:	*Salmonella typhimurium* (TA98 NR: his D 3052 rfa, uvrB-, R-factor)
Test item:	HC Violet B5
Batch:	/
Purity:	/
Replicate:	3 experiments
Doses:	50, 100, 500, 1000, 2500, 5000 µg/plate
Metabolic Act.:	Phenobarbital / Naphthoflavone-treated rat liver homogenate
Positive controls:	2NF (-S9); 2AA (+S9)
GLP:	in compliance

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

Conclusions
The test item is mutagenic on bacterial cells.

Ref.: 10, subm I

Reverse Mutation Testing Using Bacteria

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

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

Conclusions
The test item is slightly mutagenic on bacterial cells.

Ref.: 9, subm I

Reverse Mutation Testing Using Bacteria

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

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

Conclusions
The test item is mutagenic on bacterial cells.

Ref.: 8, subm I

Reverse Mutation Testing Using Bacteria

Guideline:	/
Species/Strain:	*Salmonella typhimurium* (TA98; TA100); *E. coli* (wP2uvrA-p)
Test item:	WS 1-75
Batch:	/
Purity:	/
Replicate:	1 experiment
Doses:	from 3.2 to 10000 µg/plate
Metabolic Act.:	Aroclor induced rat liver homogenate

Positive control: 2NF (TA98); Sodium Azide (TA100); MNNG (E. coli) and 2AA
GLP: /

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

Conclusion
The test item is mutagenic on bacterial cells.

Ref.: 7, subm I

Reverse Mutation Testing Using Bacteria

Guideline: /
Species/Strain: *E. coli* wP2; CH871
Test item: N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine
Batch: /
Purity: /
Replicate: 4 experiments
Metabolic Act.: /
GLP: /

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

Ref.: 12, subm I

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 and dose dependent 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

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

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

Results
Toxicity: (2 h treatment): 5000 µg/ml (-S9) induced 95% of lethality, whereas a lethality of 100% was induced in the presence of S9. The other doses did not show any lethality.
Mutagenicity
No dose related toxicity was observed in the absence of S9 up to 2500 µg/ml; the maximum dose (5000 µg/ml) induced 75% mortality; in the presence of S9 a dose reduction survival was observed starting from 1000 µg/ml (70%) to 4000 µg/ml (20%).
There was no counting of small and large colonies; there was no indication of induction of an increase of mutants compared to the control in both conditions. The positive controls induced significant increase in mutation frequency.

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

Ref.: 14, subm I

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 dose) 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

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

Guideline:	/
Species/Strain:	CHO cells
Test item:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (dark blue powder)
Batch:	/
Purity:	/
Replicate:	two experiments
Doses:	1250, 2500, 5000 µg/ml (2 hours of treatment)
Metabolic Act.:	Aroclor-1254 induced rat liver homogenate
Positive control:	MMS (-S9); CPA (+S9)

GLP: in compliance

Results
Toxicity
A preliminary study demonstrated that a dose of 5000 µg/ml in the presence of S9 induced a 66% reduction of Mitotic Index. No toxicity was observed in the absence of S9 at the same dose.
Clastogenicity
The dose of 5000 µg/ml (± S9) in both cultures induced a significant increase in the frequency of chromosome aberrations (± gaps).

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

Ref.: 17, subm I

In Vitro Mammalian Chromosome Aberration Test

Guideline:	OECD 473 (1983)
Species/Strain:	Human peripheral lymphocytes from healthy people (two individuals: 1+S9; 1-S9)
Test item:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (dark/blue/violet powder)
Batch:	/
Purity:	/
Replicate:	two cultures each donor
Doses:	15, 100, 200, 400, 600, µg/ml (+S9) 12, 25, 50, 100, 200 µg/ml (-S9) (2h; +S9; 24h: -S9)
Metabolic Act.:	Aroclor-1254 induced rat liver homogenate
Positive controls:	Bleomycin (+S9); B(a)P (-S9)
GLP:	in compliance

Results
Toxicity
In a preliminary study it was shown that the Mitotic Index was reduced by 5% starting from 50 µg/ml; at 400 µg/ml there was no survival in the absence of S9; whereas in the presence of S9 the almost complete lethality was observed at a dose of 800 µg/ml.
Clastogenicity
No induction of clastogenicity was observed except on endoreduplication at the dose of 200 µg/ml in the presence of metabolic activation.

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

Ref.: 18, subm I

Submission II, 2005

In vitro Micronucleus Test

Guideline:	draft OECD 487 and OECD 473 (*in vitro* chromosomal aberration test)
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 dose applied.
Both without and with S9-mix, a statistically significant and dose-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

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

Guideline:	/
Species/Strain:	HeLa S3 cells
Test item:	N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine (dark blue powder)
Batch:	/
Purity:	/
Replicate:	3 replicate/dose
Doses:	500, 100, 20, 4, 0.8; 0.16; 0.032; 0.0064 µg/ml (+S9) (1 hour of treatment)
Method:	Extraction of DNA and evaluation by count in a liquid scintillation counter (LSC)

Metabolic Act.: Aroclor-1254 induced rat liver homogenate (S9)
Positive controls: 4NQO (-S9); B(a)P (+S9)
GLP: in compliance

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

Ref.: 15, subm I

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

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

Results
Toxicity
A preliminary study with 10 doses (from 8.0 to 8000 µg/ml) indicated that a reduction of survival to 80% (53.33 µg/ml); for the remaining doses, a precipitation was observed.
UDS Induction
1st experiment
The positive control (2-AAF) induced 116.40 net grains per nucleus (untreated= -2.48 grains/nucleus – grains/Cytoplasm). All the five treated doses induced negative UDS, as the untreated control.
2nd experiment
The positive control (2-AAF) induced 43.86 net grains/nucleus (untreated control: -5.47). The concentration of 266.67 µg/ml of the test item induced 1.09 net grains per nucleus, in the presence of a precipitate of the test item.

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

Ref.: 16, subm I

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Taken from SCCNFP/0781/04

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

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

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

GROUP	***24h***	***48h***	***72h***
CONTROL	0.16%	0.25%	0.06%
TREATMENT	0.11%	0.15%	0.16%

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

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

Ref.: 19, subm I

In Vivo Mammalian Erythrocyte Micronucleus Test

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

Results
Toxicity: no experiment
MN: CPA induced MN and cytotoxicity. The test item did not induce either MN or cytotoxicity.

Conclusions
The test item did not induce micronuclei in mice.

Ref.: 20, subm I

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/NCE). 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/NCE 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/NCE 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

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

No data submitted

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. 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. No data on the stability of N,N'-bis-(2-hydroxyethyl)-2-nitro-p-phenylenediamine in typical hair dye formulations has been provided.

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/day, based on effects on kidney, 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.
No two generation reproduction study was submitted.

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.

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 assay in bacteria (in the well performed test only in strain TA98) but did not induce gene mutation in mammalian cells at the *tk* locus of mouse lymphoma cells. In an *in vitro* micronucleus tests, 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 an *in vivo* micronucleus test 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 assay in bacteria were not confirmed nor ruled out in an appropriate *in vivo* test on the same genotoxic endpoint.

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.

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.

5. MINORITY OPINION

Not applicable

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