EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Blue n° 14

COLIPA n° C172

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

The SCCS adopted this opinion at its 11th plenary meeting

of 21 June 2011

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

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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 HC Blue n° 14 with the chemical name 1,4-Bis[2,3-dihydroxyepropyl)amino]-9,10-anthracenedione was submitted in August 2001 by COLIPA [1, 2].

In the opinion SCCNFP/0734/03 the SCCNFP stated that: *the information submitted is inadequate to assess the safe use of the substance. Before any further consideration, the following information is required:*

* *Complete chemical characterisation of the impurities in HC Blue n° 14; stability of the test material in the test solutions and in the hair dye formulation; nitrosamine content of the test material.*
* *percutaneous absorption study in accordance with the Notes of Guidance.*
* *data on the genotoxicity/mutagenicity following the relevant SCCNFP-opinions and in accordance with the Notes of Guidance.*

Submission II of HC Blue n° 14 was submitted by COLIPA in July 2005. According to this submission the HC Blue n° 14 is used as an ingredient of semi-permanent hair colouring products with at maximum on-head concentration of 0.3%.

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 HC Blue n° 14 safe for use in non-oxidative hair dye formulations with an on-head concentration of maximum 0.3% taken into account the scientific data provided?*

2. *Does the SCCS recommend any further restrictions with regard to the use of HC Blue n° 14 in non-oxidative hair dyes?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Blue n° 14 (INCI name)

3.1.1.2. Chemical names

1,4-bis[(2,3-dihydroxypropyl)amino]-9,10-anthracenedione
9,10-Anthracenedione, 1,4-bis[(2,3-dihydroxypropyl)amino]-
1,4-bis[(2,3-dihydroxypropyl)amino]-anthraquinone

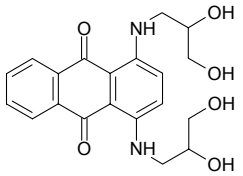
3.1.1.3 Trade names and abbreviations

Imexine BJ
COLIPA C172

3.1.1.4 CAS /EC number

CAS: 99788-75-7
EC: 421-470-7 (Imexine bj)

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_{20}H_{22}N_2O_6$

3.1.2 Physical form

(Navy)-blue powder, agglomerated, almost odourless

3.1.3 Molecular weight

Molecular weight: 386.4 g/mol

3.1.4 Purity, composition and substance codes

- Batch 0509393 was used in safety studies performed during 2004/2005 [Ref. 4, 7, 10-12, 16, 17]
- Batch Pil.1 was used in safety studies conducted in 1996/1997 [Ref. 1-3, 5, 6, 8, 9, 13-15]

- Batch 0509393 batch 0509524 of HC Blue n° 14 pigmentary paste (containing 18% HC Blue n° 14, batch 0509393) and batch CFQ14026 Batch 1 (radiochemical purity = 99.2%; [14C]-HC Blue n° 14) were used for the in vitro percutaneous absorption study using human skin [Ref. 16].

Characterisation and purity and impurity contents of various batches of HC Blue n° 14

	Batch			
	Pil.1	**0509393**	**0510310**	**0510599**
Characterisation	IR, NMR and MS	IR, NMR and MS		
Visible spectrum	The visible spectra are comparable			
HPTLC			HPTLC profile in conformance with specification	
Titre by potentiometry[1] (g/100g)	97.3	98.6	99.5	97.3
HPLC content (peak area%)	HPLC profile in conformance with specification	98.5%		
Loss on drying (g/100g)	1.3	0.9	0.3	0.3
Water content (g/100g)	1.3	1.2		
Ash content (w/w)	0.2	<0.1		
Impurity (µg/g) By HPLC				
A	220	<1000 (D)		
B	280			
C	210	<1000 (D)		
D	900	4900		
E	930	1000		
X		1900		
Y		900		
Residual solvents (µg/g) By GC				
Ethanol	120	<100 (D)		
Tetrahydrofuran		<100 (D)		
Isopropanol		<100 (D)		
Butanol		<100 (ND)		

D: detected, ND: not detected

[1]Neutralisation of amine (secondary) function with perchloric acid in an acetic acid medium

Impurities

A: 1,4-dihydroxyanthraquinone
B: 2,3-dihydro-9,10-dihydroxy-1,4-anthracenedione
C: 4,4'-bis[(2,3-dihydroxy-propyl)amino]-1,1'-dihydroxy-2,2'bianthracene-9,9',10,10'-tetrone
D: 1,4-bis-(2,3-dihydroxy-propylamino)-2,3-dihydroanthraquinone
E: 1-(2,3-dihydroxy-propylamino)-4-hydroxy-anthraquinone
X: 1-amino-4-(2,3-dihydroxy-propylamino)-anthraquinone
Y: 1-(2,3-dihydroxy-propylamino)-4-[(2,2-dimethyl-[1,3]dioxolan-4-ylmethyl9-amino]-anthraquinone

Comment

- Absolute concentration of HC Blue n° 14 in various batches is not reported. The purities described are based on measurements performed by potentiometric titration of amine function. No reference materials were used for the quantification of the dye. Thus, the reported purities also include amounts of impurities C, D, E, X and Y, which have amine functional groups. In addition, any salt content in HC Blue n° 14 will not be measured by the potentiometric titration performed.

- Conformance with HPLC profile and HPTLC profile with specifications is described for some batches of HC Blue n° 14, but specifications are not reported

3.1.5 Impurities / accompanying contaminants

See 3.1.4. Purity, composition and substance codes

3.1.6 Solubility

Water:	20.0 ± 1.9 mg/L at 20 ± 0.5°C according to EEC Method A6
Ethanol (96%):	0.05 g in 100 ml *
Dimethylsulfoxide:	0.05 g in 100 ml *
Dimethylformamide:	0.05 g in 100 ml *
Solubility in receptor fluid **:	50 µg/ml which is higher than the amount penetrated

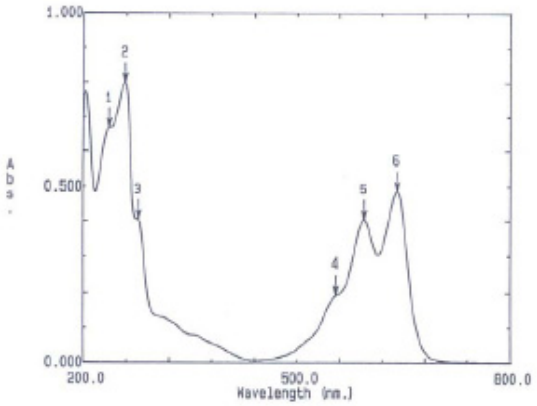
* soluble after ultrasonication (5 min) and magnetic stirring (30 min)
** Dulbecco phosphate buffer

3.1.7 Partition coefficient (Log P_{ow})

Log $P_{o/w}$: 2.09 (at 25 ± 1°C, pH 7.55) according to EEC Method A.8
Log $P_{o/w}$: -1.1 (calculated)

3.1.8 Additional physicochemical specifications

Melting point:	185 - 215°C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV/Visible spectrum:	see below



Point Pick

No.	Wavelength (nm.)	Abs.
1	236.00	0.667
2	258.50	0.798
3	277.00	0.401
4	555.00	0.191
5	594.50	0.402
6	641.00	0.483

[Spectrophotometer : Shimadzu UV-2101-PC]

3.1.9.	Stability

The homogeneity of the test item at 100 mg/mL in 0.5% methyl cellulose and at 0.1 and 5 mg/mL in culture medium on the day of preparation was satisfactory (coefficient of variation for top, middle and bottom samples <8%).

The stability of the test item in dosage forms at 100 mg/mL in 0.5% methyl cellulose, at 1 and 100 mg/mL in dimethylsulfoxide, at 0.1 and 5 mg/mL in culture medium and at 1 and 100 mg/mL in dimethylformamide was satisfactory (deviation ≤ 10% from the original concentration) over a 4-hour period at room temperature, protected from light and under inert gas atmosphere.
The radioactive HC Blue n° 14 used for dermal absorption study was shown to be stable (deviation <7%) in the test formulation during 24 h study period.

General Comments on Physico-chemical characterisation

- Absolute concentration of HC Blue n°14 in various batches is not reported. The purities described are based on measurements performed by potentiometric titration of amine function. Thus, the reported purities also include amounts of impurities C, D, E, X and Y, which have amine functional groups. In addition, any salt content in HC Blue n° 14 will not be measured by the potentiometric titration performed. The SCCS considers that potentiometric titration for the measurement of the purity and the impurity is not the state of the art.
- HC Blue n° 14 is a secondary alkanolamine and thus it is prone to nitrosation. No information is provided on the nitrosamine content of the test material.
- Stability of HC Blue n° 14 in typical hair dye formulations is not reported

3.2. Function and uses

HC Blue n° 14 is used as semi-permanent hair dye in hair dye formulation at 0.3%. 35 ml hair dye formulation is used per application.

3.3. Toxicological Evaluation

3.3.1.	Acute toxicity

3.3.1.1.	Acute oral toxicity

Taken from SCCNFP/0734/03

Guideline:	OECD 401
Species/strain:	Sprague-Dawley Rat ICO/OFA-SD (IOPS Caw)
Group Size:	5 rats each sex
Test substance:	Imexine BJ
Batch:	Pil 1
Purity:	94.6% (HPLC)
Dose:	2000 mg/kg bw in 10 ml/kg 0.5% aqueous methylcellulose
Observ. period:	14 days
GLP:	in compliance

Behaviour, clinical signs and deaths were monitored for 14 days after administration. The animals were weighed individually just before administration of the test substance on day 1 and then on days 8 and 15.
Macroscopical examination was performed after sacrifice.
The general behaviour and body weight gain of the animals were not affected by the treatment with the test substance. From day 8 onwards, spots of blue coloration were observed on the tail of the males. This was attributed to faecal elimination of the test substance, which is a dark blue dye. No deaths occurred at 2000 mg/kg bw. No abnormalities were observed at necropsy.

Under these experimental conditions, the LD50 of the test substance was higher than 2000 mg/kg bw in rats. No signs of toxicity were observed at this dose.

Ref.: 1

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402
Species/strain:	Sprague-Dawley Rat ICO/OFA-SD (IOPS Caw)
Group Size:	5 males and 5 females
Test substance:	Imexine BJ
Batch:	Pil 1
Purity:	94.6% (HPLC)
Dose:	2000 mg/kg bw
Observ. period:	14 d
GLP:	in compliance

The test substance was applied on a moistened compress under a semi-occlusive dressing at the dose of 2000 mg/kg bw. After 24 hours of exposure, any residual compound was removed using a dry compress. Animals were checked for mortality, clinical signs and body weight gain for 14 days following the single application of the test compound. A necropsy was performed on each animal at the end of the study.

Results
No deaths were noted during the study. There were no toxic effects. A slight blue colouration of the treatment site was observed up to day 10 in all animals. Body weight gain was not affected by compound administration. No macroscopic abnormalities were observed at necropsy.

Conclusion
Under the conditions of this study, the maximum non-lethal dose of HC Blue n° 14 following single dermal application to rats was higher than 2000 mg/kg bw.

Ref.: 2

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
Species/strain:	Male New Zealand White rabbits
Group size:	3 animals
Test substance:	HC Blue n° 14
Batch:	0509393

Purity:	98.6%
Dose:	0.5 ml of the test item at 10% in 0.5% CMC
Observation period:	1 hour, 24, 48 and 72 hours
GLP:	in compliance
Study date:	2005

A single dose of 0.5 mL of HC Blue n° 14 at the concentration of 10% (w/w) in 0.5% methylcellulose was applied to the closely-clipped skin of one flank of rabbits. The dosage form was held in contact with the skin by means of a semi-occlusive dressing.
Cutaneous reactions were observed approximately 1 hour, 24, 48 and 72 hours after removal of the dressing.
The mean values of the scores for erythema and oedema were calculated for each animal.

Results
Except a very slight erythema noted in 1/3 animals on day 1, after the 4-hour exposure, no cutaneous reactions were observed during the study. A slight blue coloration of the skin was noted in all animals (at all exposures) between day 1 and day 4.

Conclusion
Under the experimental conditions, the test item - HC Blue n° 14 at the concentration of 10% in 0.5% methylcellulose - is non-irritant when applied topically to rabbits.

Ref.: 3

Taken from SCCNFP/0734/03

Guideline:	OECD 404
Species/strain:	white rabbits, New Zealand
Group size:	3 male
Test substance:	Imexine BJ
Batch:	Pil 1
Purity:	94.6% (HPLC)
Dose:	0.5g applied to 6cm² of intact skin for 4 hours
GLP:	In compliance
Study date:	1996

After clipping the back and flanks, 0.5g of the test material was applied to a 6 cm² moistened gauze pad and then applied to the right flank of the animals for four hours. The patches were removed after 4 hours, residual test article wiped off, and observations made at 1, 24, 48 and 72 hours after removal.

Results
No cutaneous reactions were observed during the study. Slight blue coloration of the test site was noted throughout the study in all animals. Imexine BJ was considered to be non-irritant to rabbit skin under the conditions of the study.

Ref.: 4

3.3.2.2. Mucous membrane irritation

Taken from SCCNFP/0734/03

Guideline:	OECD 405
Species/strain:	white rabbits, New Zealand
Group size:	3 male
Test substance:	Imexine BJ
Batch:	Pil 1
Purity:	94.6% (HPLC)

Dose: 100 mg
GLP: In compliance

A single dose of 100 mg of the test material was placed into the everted lower lid of the left eye of each animal. The right eye served as the untreated control. The eyes of the 3 animals remained unrinsed.
1, 24, 48, 72 and hours after instillation of the test material, the treated eyes of the rabbits were observed for signs of ocular irritation.

Results
In all animals, at the 1-hour reading, redness of the conjunctiva was masked by a blue coloration. A slight ocular discharge (grade 1) was the only effect noted in one animal. In the other 2 rabbits, slight chemosis (grade 1) was observed for 24 hours after treatment. Redness of the conjunctiva (grade 2 or 3) was noted on day 2 only in these 2 animals. These reactions were accompanied by a slight discharge (grade 1) at the 1-hour reading. No effects were observed on the iris or cornea. Reversibility of ocular lesions was observed on day 3.
Imexine BJ was considered a slight irritant.

Ref.: 5

3.3.3. Skin sensitisation

Taken from SCCNFP/0734/03

Magnusson and Kligman Guinea pig maximisation test

Guideline:	OECD 406	
Species/strain:	albino guinea pigs, Dunkin-Hartley	
Group size:	30 animals (10 males and 10 females test and 5 males and 5 females control)	
Test substance:	IMEXINE BJ	
Batch:	Pil 1	
Purity:	94.6% (HPLC)	
Dose:	Intradermal induction:	0.1 ml of 2.5% w/w in paraffin oil, Freund's Complete Adjuvant at 50% and equal parts of these two into either side of dorsal region.
	Topical induction:	0.5ml of a 10% dilution of test material in paraffin oil under occlusion for 48 hours. Controls received vehicle only. Skin pretreated with 0.5ml of 10% sodium lauryl sulphate in white soft paraffin.
	Challenge:	Performed on day 20 (12 days after epidermal applications) with 5% dilution in paraffin oil of the test substance (24 hours, occlusion).
GLP:	In compliance	

Animals were examined 24 and 48 hours after removal of the patches for signs of erythema and oedema.

Results
No cutaneous reactions were observed after the challenge application. Very slight blue coloration of the test sites were noted in all animals at the 24-hour reading and in most at the 48-hour reading. The coloration did not interfere with the evaluation of the reactions.

IMEXINE BJ was considered not to be a sensitiser under the test conditions.

Ref.: 6

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species/strain:	female CBA/J mice
Group size:	twenty-eight (4 per group: 5 treated, 1 negative, 1 positive)
Test substance:	HC Blue n° 14
Batch:	0509393
Purity:	98.6%
Dose levels:	0.5, 1, 2.5, 5 and 10%
Vehicle:	Dimethylformamide (DMF)
Route:	Topical
Radiochemical:	[3H] methyl-thymidine
Positive control:	α-hexylcinnamaldehyde 25% in DMF
GLP:	in compliance
Study date:	2005

The potential of the test item (HC Blue n° 14) to induce delayed contact hypersensitivity was studied using the murine Local Lymph Node Assay (LLNA). During the induction phase, the test item, vehicle or reference item was applied over the ears (25 µL per ear) for 3 consecutive days (days 1, 2 and 3). After 2 days of resting, the proliferation of lymphocytes in the lymph node draining the application site was measured by incorporation of tritiated methyl thymidine (day 6). The obtained values were used to calculate stimulation indices (SI).
The irritant potential of the test item was assessed in parallel by measurement of ear thickness on days 1, 2, 3 and 6.

Results
A blue coloration of the skin of the ears was observed in all test item treated animals on days 2, 3 and 6; this coloration could have masked a possible discrete erythema at 10% on day 6.
No increase in ear thickness was observed at any of the tested concentrations. No noteworthy lymphoproliferation was noted at any tested concentration

Treatment	Concentration (%)	Stimulation index
Test item	0.5	0.91
	1	0.69
	2.5	0.89
	5	1.21
	10	1.20
HCA	25	4.57

Conclusion
Under the experimental conditions, the test item HC Blue n° 14 (C172) does not induce delayed contact hypersensitivity in the murine Local Lymph Node Assay.

Ref.: 7

Comment
The highest concentration tested (10%) was too low for hazard identification. Therefore, a sensitising potential cannot be excluded

3.3.4. Dermal / percutaneous absorption

Taken from SCCNFP/0734/03

Guideline:	/
Test substance:	IMEXINE BJ 1,4-bis-(2,3-dihydroxy-propylamino)-anthraquinone

Batch:	Pil 1
Purity:	94.6% (HPLC)
Tissue:	human skin (absorption across isolated epidermis)
Skin integrity:	visual evaluation using a microscope before the test. At the end of the test, application of Chinese ink to verify the absence of leakage
Method:	*in vitro* static diffusion cell 2 cm²
Receptor fluid:	phosphate buffer (DUBELCCO)
Formulation:	standard commercial type formulation
Conditions:	application on the epidermis and on the epidermis covered by human hair (10 mg of finely cut bleached hair over 2 cm²)
Dose:	concentration tested 0.3%, application 40 mg of formulation over 2 cm² (i.e. 20 mg/cm²)
Replicate:	skin from 5 donors. Epidermis separated from dermis by heat. Two diffusion cells per skin donor for each condition of application (9 cells without hair, 11 cells with hair, - diffusion cells treated with a placebo)
Duration:	30 minutes followed by a washing of the epidermal surface. Diffusion monitored during 24 hours
Analyt. method:	HPLC – UV detection
Detection limit:	1 ng/ml
Stability:	no information
GLP:	in compliance

The skin penetration of IMEXINE BJ was evaluated in a static diffusion cell system across human isolated epidermis. The integrity of the epidermis was evaluated, the skin surface temperature was monitored (32 ± 1 °C). The formulation was applied in absence or in presence of human hair. The test substance was prepared at a concentration of 0.3% (97.3% of active material in the dye) in a "commercial type" formulation. Approximately 20 mg/cm² of the formulation (exactly measured) were applied to 2 cm² for 30 minutes. The excess from the skin surface removed by washing with water and with a SLS (2%) aqueous solution, then the skin was dried with a cotton swab. The substance was measured using HPLC in the receptor fluid after 4.5 hours and 24 hours the diffusion. IMEXINE BJ was not assayed in the washing fluids or in the epidermis at the end of the test. The mass balance of the experiment was not calculated.

Results
The quantity of IMEXINE BJ (cumulated amount) penetrating after a contact of 30 minutes through the epidermis to the receptor fluid during the 24 hours of the test, was higher in presence of hair (0.035 ± 0.026% of the applied dose, i.e. 25.03 ± 19.87 ng/cm²) than in absence of hair (0.015 ± 0.008% of the applied dose, i.e. 10.53 ± 5.82 ng/cm²).
Because (i) this study did not include determination of the recovery of the test substance, (ii) the amount of material present in the skin at the end of the test is unknown, it is considered inadequate.

Ref.: 11 (subm. I)

New study, 2005

Guideline:	OECD 428
Tissue:	human frozen abdominal skin (400 μm thickness)
Group size:	8 cells from 4 female donors
Skin integrity:	permeation coefficient for tritiated water < 2.5 x 10^{-3} cm/h
Diffusion cell:	flow-through diffusion cells
Test substance:	HC Blue n° 14
Batch:	0509393
Purity:	98.6%
Test item:	HC Blue n° 14, paste 18%
Dose:	20 mg/cm²

Dose of test substance: 0.6 mg/cm²
Receptor fluid: phosphate buffer saline with 0.01% sodium azide (w/v) and supplemented with 6% polyoxyethylene 20-oleyl ether (w/v)
Solubility receptor fluid: 169 μg/ml
Stability receptor fluid:
Method of Analysis: HPLC-UV analysis
GLP: in compliance
Study date: 2005

The in vitro percutaneous absorption of HC Blue n° 14 was studied through human skin membranes. The compound was tested as a direct hair dye in one formulation with a target concentration of 0.3%. An amount of 20mg/cm² of formulation were applied on the skin surface. The contact time was 30 min.

Results

Recovery of HC Blue n° 14 in human skin:

	$\mu g_{Eq}.cm^{-2}$									
Replicate no.	R 1	R 2	R 3	R 4	R 5	R 6	R 7	R 8		
Donor no.	1	2	3	4	2	3	1	4	**Mean**	**SD**
Skin wash	48.0	44.9	60.4	54.4	50.9	57.2	49.5	47.5	51.6	5.3
Cotton swabs	0.018	0.029	0.020	0.052	0.018	0.044	0.019	0.014	0.027	0.014
Donor compartment	0.002	0.003	0.003	0.040	0.005	0.011	0.003	0.007	0.009	0.013
Dislodgeable dose[1]	48.0	44.9	60.4	54.5	50.9	57.3	49.6	47.5	51.6	5.3
Tape strips	0.06	0.15	0.12	0.24	0.12	0.36	0.10	0.10	0.16	0.10
Unabsorbed dose[2]	48.1	45.1	60.5	54.7	51.0	57.6	49.6	47.6	51.8	5.3
Receptor fluid + receptor wash	0.001	0.001	0.002	0.003	0.001	0.001	0.004	0.002	0.002	0.001
Skin	0.010	0.012	0.037	0.048	0.074	0.052	0.017	0.013	0.033	0.024
Total absorption[3]	0.011	0.014	0.039	0.051	0.076	0.054	0.020	0.015	0.035	0.024
Total recovery	48.1	45.1	60.5	54.8	51.1	57.7	49.7	47.6	51.8	5.4

[1] Amount in skin wash, cotton swabs, and donor compartment wash
[2] Amount in dislodgeable dose and tape strips
[3] Amount in receptor fluid, receptor compartment wash and the skin (excluding tape strips)

The mean total absorption was 0.035 μg/cm² or 0.066% of the dose applied.

	% of dose applied	**μgeq/cm²**
Skin wash	97.6 ± 7.6	51.6 ± 5.3
Dislodgeable dose *	97.6 ± 7.7	51.6 ± 5.3
Stratum corneum	0.29 ± 0.17	0.16 ± 0.10
Skin (epidermis + dermis)	0.062 ± 0.046	0.033 ± 0.024
Receptor fluid	0.004 ± 0.002	0.002 ± 0.001
Unabsorbed dose **	97.9 ± 7.7	51.8 ± 5.3
Absorbed dose ***	0.066 ± 0.045	0.035 ± 0.024
Total recovery	98.0 ± 7.8	51.8 ± 5.4

* Dislodgeable dose is defined as the amount of test substance removable from the application site (skin wash, cotton swabs and donor compartment wash)
** Unabsorbed dose is defined as the dislodgeable dose including the amount recovered in the stratum corneum
*** Absorbed dose (dermal delivery) is defined as the amount in the receptor fluid, the receptor compartment wash and skin membrane, excluding tape strips

Ref.: 16

Comment
There is a high variability in the absorption of HC Blue n° 14, ranging from 0.011 to 0.076 μg/cm^2 with a CV of 68%, possibly due to the low absorption rate.
For the calculation of the Margin of Safety, the mean + 2SD (0.035 + 2 x 0.024 or 0.083 μg/cm²) will be used.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

No data submitted

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

Taken from SCCNFP/0734/03

Guideline:	OECD 408 (1981)
Species/strain:	Sprague Dawley rat, Crl:CD (SD) Br
Group Size:	16 each sex control and high dose; 10 each sex, low, mid and intermediate dose
Test material:	Imexine BJ suspended in 0.5% aqueous carboxymethylcellulose
Batch:	Pil 1
Purity:	94.6% (HPLC)
Dose:	0, 50, 125, 300 and 1000 mg/kg bw/day
Exposure period:	13 weeks
Recovery period:	4 week, control and high dose 6 each sex
GLP:	in compliance

On completion of the 13-week treatment period, the first six surviving animals of each sex in the control and high dose-level groups were kept for a 4-week recovery period.
The animals were examined for clinical signs daily and checked twice daily for mortality/viability. Food consumption and body weight were recorded once pre-test, and weekly thereafter including the recovery period and body weight at necropsy. Ophthalmoscopic examination was performed at pre-test and at week 13 (control and high-dose animals). A functional observational battery (modified Irwin screen test) was performed during pre-test and at week 12 on all rats and grip strength and locomotor activity were evaluated. At week 12, blood and urine were analysed. After 13 weeks, all animals were weighed and killed. Descriptions of all macroscopic abnormalities were recorded. The major tissues and organ were collected from all animals and absolute and relative weights were recorded at necropsy for adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thyroid, and thymus. A complete set of organs were examined by light microscopy.

Results
No treatment-related deaths were noted in any group. The mean bodyweight gain and food consumption of the dosed animals was similar to the controls.
No clinical signs of toxicological significance were observed during the study. During the treatment period, a blue coloration attributable to elimination of the dye or its metabolites was observed in the faeces in all dosed animals and in the urine of animals in the mid, intermediate and high dose groups with a dose-related incidence. The tail was stained in some females in the mid- and intermediate-dose group and in all animals at the high-dose. The fur was stained in some females at the high-dose. During the recovery period, only tail coloration was noted in the high-dose animals.
There were no treatment-related changes in the haematological parameters at the end of the treatment period.

There were some changes in blood biochemistry by the end of the treatment period, There was a statistically significant lower triglyceride level in females of all treated groups (50, 125, 300 and 1000 mg/kg bw/day) -32%, -29%, -27%, -45%. In males, there was -32% reduction at 1000 mg/kg bw/day that was not significant. After the recovery period, this lower triglyceride level was not reversed, remaining at the same low level.
Increases in inorganic phosphorus levels were noted in females (+16% and +18% at 300 and 1000 mg/kg bw/day respectively) and in males, 9% at 1000 mg/kg bw/day dosing. There was a 2% increase in sodium levels at 1000 mg/kg bw/day in both sexes. These differences were no longer seen after a 4-week recovery period and were considered to be treatment related. No treatment related findings were noted in the urinalysis.
No relevant differences in organ weights were noted between control and treated animals.
The following macroscopic findings were observed, and were considered to be related to the dyeing properties of the test substance: blue coloration of the tail in females of each treated group and in males given 1000 mg/kg bw/day (not reversible after 4 weeks recovery); blue coloration of the extremities and hair in animals given 300 and 1000 mg/kg bw/day; bluish or greenish discoloration of the gastrointestinal mucosa and contents in some animals of each treated group).
No treatment-related microscopic changes were noted.

Conclusion
Since only minor biochemical changes were noted, under these experimental conditions, the dose level of 1000 mg/kg bw/day was defined as the NOAEL.

Ref.: 8

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471 (1994)
Species/strain:	*S. typhimurium* TA98, TA100, TA1535, TA1537, *E. coli* WP2 *uvr*A
Replicates:	Triplicate plates, 3 independent tests
Test substance:	Imexine BJ
Solvent:	DMSO
Batch:	Pil 1
Purity:	94.6% (HPLC)
Concentrations:	0, 312.5, 625, 1250, 2500, 5000 µg/plate, without and with S9-mix
Treatment:	direct plate incorporation with 48 to 72 h incubation, experiment 1 and experiment 2 pre-incubation method with 60 minutes pre-incubation and 48 to 72 h incubation, experiment 2 (part with S9-mix only) and experiment 3
GLP:	In compliance
Study period:	10 October 1995 - 26 February 1996

Imexine BJ was investigated for the induction of gene mutations in strains of *S. typhimurium* and *E. coli*. 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 toxicity test with strains TA98, TA100 and WP2 *uvr*A. Toxicity was evaluated for 6 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. Experiment 1 and experiment 2 (part without S9-mix) were performed

according to the direct plate-incorporation test, experiment 2 (part with S9-mix) and experiment 3 with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
No signs of toxicity were noted for the top dose of 5000 µg/plate in *E. coli*; a slight toxicity was observed for the 2 strains TA98 and TA100. As no precipitation occurred, the top dose has been selected to be 5000 µg/plate.
In the presence of metabolic activation in both experiments using the preincubation method (experiments 2 and 3), a concentration related and reproducible increase in revertant numbers has been observed in *Salmonella* TA1537. A biological relevant increase in revertants was not observed in any of the 5 tester strains without metabolic activation or in the 4 remaining strains with metabolic activation.

Conclusions
Under the test conditions used, it is concluded that Imexine BJ is mutagenic in *Salmonella* strain TA1537 with metabolic activation.

Ref.: 9

***In vitro* Mammalian Cell Gene Mutation Test** (*hprt*-locus)

Guideline:	OECD 476 (1997)	
Species/strain:	L5178Y mouse lymphoma cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	HC Blue n° 14	
Batch:	0509393	
Purity:	> 98.6%	
Vehicle:	DMSO	
Concentrations:	experiment 1:	750, 1000, 1250, 1500, 2000, 2500, 3000 and 3864 µg/ml, without and with S9-mix
	experiment 2:	1000, 1250, 1500, 2000, 2500 and 3250 µg/ml without and with S9-mix
Treatment	3 h both without and with S9 mix; expression period 7 days and a selection period of 11 days.	
GLP:	in compliance	
Study period:	1 September 2004 – 25 October 2004	

HC Blue n° 14 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 cytotoxicity range-finding experiment measuring relative total survival using concentrations up to 1000 µg/ml. In the main test, cells were treated for 3 h followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Despite the results of the preliminary test, the highest concentration used in the main test was the prescribed maximum concentration (3864 µg/ml ≈ 10 mM) without S9-mix and 3000 µg/ml with S9-mix. Toxicity was measured as percentage relative survival of the treated cultures relative to the percentage relative survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the cytotoxicity range-finder experiment no evidence of toxicity or precipitation was observed up to the highest concentration tested, 1000 µg/ml. In experiment 2, precipitation occurred at 3250 µg/ml both without and with S9-mix. In both experiments in the absence and presence of S9-mix the appropriate level of toxicity (10-20% survival after the highest concentration) was not reached either because the prescribed maximum concentration was reached or precipitation occurred.

Biological relevant or statistically significant increases in mutant frequency were not found following treatment with HC Blue n° 14 at any dose level tested, either in the absence nor presence of S9-mix in both experiments with one exception. A statistically significant increase in mutant frequency was found in experiment 1 without S9-mix. However, since a concentration dependent increase was lacking and the positive result was not reproducible, it is considered not biologically relevant. In experiment 1 with S9-mix, a weak concentration dependent increase in the mutant frequency was found but as no statistically significant increases in mutant frequency were observed and the mutant frequencies were in the range of the historical control values, this result was considered not biologically relevant, as well.

Conclusion
Under the experimental conditions used, HC Blue n° 14 was considered not mutagenic in this *hprt* gene mutation assay in mouse lymphoma cells.

Ref. 10

In Vitro Mammalian Chromosomal Aberration Test

Guideline:	OECD 473	
Species/strain:	Human lymphocytes (non pooled cultured blood samples from one male and one female donor)	
Replicates:	Duplicate cultures, 2 independent experiments	
Test substance:	Imexine BJ	
Batch:	Pil 1	
Purity:	94.6% (HPLC)	
Vehicle:	water	
Concentrations:	experiment 1:	100, 300, 900 µg/ml, without S9-mix 30, 100, 300 µg/ml, with S9-mix
	Experiment 2:	100, 300, 900 µg/ml, without S9-mix and 20 h harvest 30, 100, 300 µg/ml, without S9-mix and 44 h harvest 100, 300, 600 µg/ml, with S9-mix and 20 h harvest 100, 300, 450 µg/ml, with S9-mix and 44 h harvest
	Experiment 3:	300, 450, 600 µg/ml, with S9-mix
Treatment:	Experiment 1:	3 h without or with S9-mix; harvest time 20 h after start of treatment.
	Experiment 2:	20 or 44 h without S9-mix; harvest time 20 or 44 h after start of treatment. 3 h with S9-mix; harvest time 20 or 44 h after start of treatment.
	Experiment 3:	3 h with S9-mix; harvest time 20 h after start of treatment.
GLP:	In compliance	
Study period:	24 April 1996 – 15 July 1996	

Imexine BJ in DMSO has been investigated for induction of chromosomal aberrations in human lymphocytes. The test concentrations were established on a basis on pH, osmolarity and solubility, no preliminary cytotoxicity test was performed (or data not presented). Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

Results
In experiment 1 and 2 but not in experiment 3 both without and with S9-mix, a concentration related decrease in mitotic index was noted.
In experiment 1 (without and with S9-mix) and in experiment 2 (without S9-mix and the 44 h harvest part with S9-mix) a biologically relevant increase in human lymphocytes with chromosomal aberrations was not found.

In experiments 2 and 3, an increase in human lymphocytes with chromosomal aberrations was found after treatment with Imexine BJ in the presence of S9-mix and a harvest time of 20 h. In experiment 2, while not statistically significant, a 4.5% change of aberrant cells was observed compared with the corresponding solvent control. This frequency, outside the historical control value, is due to the presence of chromatid deletions and occurred in the cultures originated from both donors (woman and man). In experiment 3, at the top dose, 5% increase of aberrant cells was observed compared with the corresponding solvent control. This frequency is statistically significant and should be considered as biologically relevant as it confirms the results from a previous study using the same concentrations. Similarly, this increase is due to the presence of chromatid and/or chromosome breaks and occurred in the cultures originated from both donors (woman and man).

Conclusions
The assay is acceptable for evaluation. Imexine BJ in DMSO is considered positive for clastogenic activity in human lymphocytes in the presence of activation under the conditions of the test.

Ref.: 7 (subm. I)

In Vitro Mammalian Chromosomal Aberration Test

Guideline:	OECD 473 (1997)	
Cells:	Human lymphocytes from 3 healthy, non-smoking male donors	
Replicates:	duplicates in two independent experiments	
Test substance:	HC Blue n° 14	
Batch:	0509393	
Purity:	99%	
Solvent:	DMSO	
Concentrations:	experiment 1:	518.6, 1978 and 3864 µg/ml without S9-mix 108.8, 810.3, 3091 and 3864 µg/ml with S9-mix
	experiment 2:	25, 100 and 250 µg/ml without S9-mix 400, 1500, 3000 and 3864 µg/ml with S9-mix
Treatment	experiment 1:	3 h treatment without and with S9-mix; harvest time 20 h after treatment
	experiment 2:	20 h treatment without S9-mix; harvest time 20 h after start of treatment 3 h treatment with S9-mix; harvest time 20 h after start of treatment
GLP:	in compliance	
Study date:	2 September 2004 – 1 December 2004	

HC Blue n° 14 has investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in human lymphocytes withdrawn from 3 healthy non-smoking, male donors. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Treatments covering a broad range of concentrations, separated by narrow intervals were performed both in the absence and presence of metabolic activation. The highest concentration used was 3864 µg/ml (equivalent to 10 mM, the prescribed maximum top concentration). Selection of concentrations for analysis was based on mitotic index. The highest concentration for chromosome analysis should be one at which at least 50% mitotic inhibition has occurred or should be the highest dose tested. At least 2 lower concentrations are selected such that a range of cytotoxicity from maximum to little or none is covered.
Cells were treated for 4 h and harvested 20 h after the start of treatment or for 20 h and harvested immediately after the end of treatment. Approximately 2 h before harvest, each culture was treated with colcemid (final concentration 1 µg/ml) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with 4% Giemsa in Gurr's

pH 6.8 buffer and examined microscopically for chromosomal aberrations and the mitotic index. Negative and positive controls were in accordance with the OECD guideline.

Results
In the absence of S9-mix, the frequencies of cells with chromosome aberrations were generally similar to those observed for the concurrent negative controls with one exception. The frequency of structural aberrations of this single culture treated with the intermediate concentration (1978 µg/ml in experiment 1) marginally exceeded the historical control range. Since the structural aberration frequency of the replicate culture and all other treated cultures fell within the normal range of the historical controls, this increase is not considered biologically relevant.
Treatment with HC Blue n° 14 in the presence of S9-mix resulted in more or less concentration-dependent increases in the number of cells with chromosomal aberrations. However, only the values found for the highest concentrations marginally exceeded the normal historical control range. These increases were associated with significant changes in osmolarity of treated cultures compared to concurrent negative control cultures. The authors, therefore, considered the increases seen of questionable biological significance.
In the presence of S9-mix increases in the number of cells with polyploidy were observed.

Conclusion
Under the experimental conditions used it can not be concluded whether HC Blue n° 14 was genotoxic (clastogenic) in this chromosome aberration test.

Ref.: 11

Comment
The authors considered the increases seen with S9-mix of questionable biological significance due to the fact that they were associated with significant changes in osmolarity of treated cultures compared to concurrent negative control cultures. The SCCS agrees and considers the result as inconclusive.

In Vitro Mammalian Chromosomal Aberration Test

Guideline:	OECD 473 (1997)	
Cells:	Human lymphocytes from 3 healthy, non-smoking male donors	
Replicates:	duplicates in two independent experiments	
Test substance:	HC Blue n° 14	
Batch:	0509393	
Purity:	99%	
Solvent:	culture medium	
Concentrations:	experiment 1:	50.21, 100.4 and 502.1 µg/ml without S9-mix 100.4, 502.1 and 1004 µg/ml with S9-mix
	experiment 2:	62.35, 498.6 and 2501 µg/ml without S9-mix 62.32, 498.6 and 2001 µg/ml with S9-mix
Treatment	experiment 1:	3 h treatment without and with S9-mix; harvest time 20 h after treatment
	experiment 2:	20 h treatment without S9-mix; harvest time 20h after start of treatment 3 h treatment with S9-mix; harvest time 20 h after start of treatment
GLP:	in compliance	
Study date:	6 January 2005 – 16 March 2005	

HC Blue n° 14 has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in human lymphocytes withdrawn from 3 healthy non-smoking, male donors. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. A cytotoxicity range finding study was performed to

select suitable top concentrations for experiment 1. In the main experiments, treatments covering a broad range of concentrations, separated by narrow intervals were performed both in the absence and presence of metabolic activation up to the highest suitable concentration found in the cytotoxicity range finding study. Selection of concentrations for analysis was based on mitotic index.
Cells were treated for 4 h and harvested 20 h after the start of treatment or for 20 h and harvested immediately after the end of treatment. Approximately 2 h before harvest, each culture was treated with colcemid (final concentration 1 µg/ml) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with 4% Giemsa stain in Gurr's pH 6.8 buffer and examined microscopically for chromosomal aberrations and the mitotic index.

Results
With S9-mix in experiment 1, precipitation was found at the higher concentrations (502.1 and 1004 µg/ml); in experiment 2, precipitation was observed both without and with S9-mix at 498.6 µg/ml and above.
Both in experiment 1 and 2, a biological relevant increase in the number of cells with chromosomal aberrations was not found. The number of aberrant cells with chromosomal aberrations in the majority fell within the historical control range. Two single cultures were marginally outside this range but as these increases were not reproducible they were considered as not biologically relevant.
In both experiments, a biologically relevant increase in the number of cells with polyploidy was not observed either.

Conclusion
Under the experimental conditions used, HC Blue n° 14 was not genotoxic (clastogenic) in this chromosomal aberration test with human lymphocytes either in the absence or in the presence of S9-mix.

Ref.: 12

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

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

Guideline:	OECD 474 (1983)
Species:	Swiss OF1/ICO:OF1 mice
Group sizes:	5 mice/sex/group
Test substance:	Imexine BJ
Batch:	Pil 1
Purity:	94.6% (HPLC)
Vehicle:	0.5% aqueous carboxymethylcellulose
Dose levels:	0, 500, 1000 and 2000 mg/kg bw/day
Route:	oral gavage, two applications 24 h apart
Sacrifice times:	24 h after treatment.
GLP:	In compliance
Study period:	March 1996 – May 1996

Imexine BJ has been investigated for induction of micronuclei in bone marrow cells of male or female mice. Dose levels were based on the results of a preliminary toxicity test in male and female mice on toxic signs and mortality recorded over a period of 48 h.
In the main experiment mice were exposed orally to 0, 500, 1000 and 2000 mg/kg bw/day twice, 24 h apart. Erythrocytes were collected 24 h after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Bone marrow preparations were stained with Giemsa and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the preliminary toxicity test, toxic effects were seen at doses of 1000 and 2000 mg/kg bw/day. Therefore, the top dose for the main study has been chosen to be 2000 mg/kg bw/day. In the main study clinical signs and mortality were not found. A more or less dose dependent decrease in the PCE/NCE ratio has been observed indicating cytotoxicity of Imexine BJ and thus exposure of the target cells.
A dose dependent but not statistically significant increase in the number of polychromatic erythrocytes with micronuclei over the concurrent vehicle control values was observed. However, the individual values for the different measure points are all well within the range of the historical control data. Therefore, the increase in the number of polychromatic erythrocytes with micronuclei is considered not biologically relevant.

Conclusions
Under the experimental conditions used, Imexine BJ did not induce an increase in the number of micronucleated polychromatic erythrocytes of treated mice and, consequently, Imexine BJ is not genotoxic (clastogenic and/or aneugenic) in polychromatic erythrocytes of mice

Ref.: 8 (subm. I), 13 (subm. II)

Unscheduled DNA Synthesis (UDS) Test

Guideline:	OECD draft guideline 486 (1991)
Species/strain:	Wistar HanIbm: WIST (SPF) rats
Group size:	4 males rat/dose
Test substance:	Imexine BJ
Vehicle:	0.5% carboxymethylcellulose
Batch:	Pil 1
Purity:	94.6% (HPLC)
Dose levels:	0, 200 and 2000 mg/kg bw.
Route:	oral gavage
Sacrifice times:	2 h (high dose only) and 16 hours after start of the treatment
GLP:	In compliance
Date:	18 June 1997 – 3 September 1997

Imexine BJ was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test concentrations were based on a pre-experiment for toxicity measuring acute toxic symptoms at intervals of 1 h and 24 h after administration of Imexine BJ. In the main experiment the highest dose was 2000 mg/kg bw which is the prescribed maximum concentration in the OECD guideline. The animals were starved before treatment.
Hepatocytes for UDS analysis were collected approximately 2 h (high dose only) and 16 h after administration of Imexine BJ. At least 90 minutes after plating the cells were incubated for 4 h with 5 µCi/ml ^{3}H-thymidine followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 14 days. UDS was reported as net grains per nucleus: the nuclear grain count subtracted with the number of grains in a nuclear sized area adjacent to each nucleus. Unscheduled DNA synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat. Only one positive control in accordance with OECD guideline has been used.

Results
In the pre-experiment for toxicity at 2000 mg/kg bw, the highest dose tested, in both treated rats a reduction of spontaneous activity was observed 1 h but not at 24 h after treatment. On the basis of these data 2000 mg/kg bw was estimated to be a suitable dose.
The viability of the hepatocytes determined by means of the trypan blue dye exclusion assay, was not substantially affected by the treatments. Treatment with 200 and 2000 mg/kg bw Imexine BJ yielded average net nuclear grain counts of less than 0 for both

experiment times and caused no significant increases, as compared to the concurrent controls. The percentage of cells in repair did not significantly differ from the control group.

Conclusions
Under the experimental conditions used, Imexine BJ did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 9 (subm. I), 14 (subm. II)

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Taken from SCCNFP/0734/03

Prenatal Developmental Toxicity Study

Guideline:	OECD 414
Species/strain:	Sprague Dawley rat, Crl: (SD)BR
Group Size:	25 mated females
Test substance:	Imexine BJ suspended in 0.5% aqueous carboxymethylcellulose
Batch:	Pil 1
Purity:	94.6% (HPLC)
Dose:	0, 100, 300 and 1000 mg/kg bw/day
Treatment period:	Days 6 to 15 post coitum
GLP:	in compliance

The animals were dosed with 10ml/kg bw by gavage once daily. The control group received only the vehicle (double distilled water).
Food consumption was recorded for the following periods: days 0-6, 6-12, 12-18 and 18-21 *post coitum*; body weight was recorded daily from day 0 until day 21 post coitum. Clinical observations and mortality were recorded at least twice daily. At post mortem, on day 21 post-coitum, necropsy, all internal organs were examined with emphasis on the uterus, uterine contents, position of foetuses in the uterus and number of corpora lutea. The uteri of all females with live foetuses were weighed; the foetuses were removed from the uterus, weighed, sexed, and examined for gross external abnormalities.
Maternal deaths did not occur during the study and the only clinical signs were blue coloration of faeces at all doses. Mean post-implantation loss and mean number of foetuses per dam were similar between treated and control dams.
The mean foetal body weights were similar in all groups to the controls. The sex ratio for foetuses was similar in all groups. Any abnormal findings noted were not considered related to the test substance, as they were within the range for historical controls.
Under the experimental conditions, Imexine BJ was not toxic to embryo or foetus and was not teratogenic. There was no evidence of maternal toxicity. The NOAEL was defined as 1000 mg/kg bw/day.

Ref.: 15

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

HC Blue n° 14
(non-oxidative conditions)

Absorption through the skin	**A (mean + 2SD)**	**=**	**0.083 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.05 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.0008 mg/kg bw**
No Observed Adverse Effect Level (13-week, oral, rat)	**NOAEL**	**=**	**1000 mg/kg bw/d**
Adjusted NOAEL		**=**	**500 mg/kg bw/d**
MOS		**=**	**625 000**

Since no data on oral bioavailability were given, the NOAEL was adjusted assuming 50% bio-availability according to the SCCS Notes for Guidance.

3.3.14. Discussion

Physico-chemical specification
HC Blue n° 14 is used as semi-permanent hair dye in hair dye formulation at 0.3%. 35 ml hair dye formulation is used per application.
Absolute concentration of HC Blue n° 14 in various batches is not reported. The purities described are based on measurements performed by potentiometric titration of amine function. Thus, the reported purities also include amounts of impurities C, D, E, X and Y, which have amine functional groups. In addition, any salt content in HC Blue n° 14 will not be measured by the potentiometric titratrion performed.

HC Blue n° 14 is a secondary alkanolamine and thus it is prone to nitrosation. No information is provided on the nitrosamine content of the test material.
The stability of HC Blue n° 14 in typical hair dye formulations is not reported.

General toxicity
The LD50 of the test substance was higher than 2000 mg/kg bw in rats. No signs of toxicity were observed at this dose.
Only minor biochemical changes were noted in a 13-week oral toxicity study. The dose level of 1000 mg/kg bw/day was defined as the NOAEL.
HC Blue n° 14 was not toxic to embryo or foetus and was not teratogenic. There was no evidence of maternal toxicity. The NOEL was defined as 1000 mg/kg bw/day.

Irritation, sensitisation
HC Blue n° 14 is not irritant to the skin and a minor irritant to the eyes. It was considered not to be a sensitiser. In the LLNA, the highest concentration tested (10%) did not elicit a SI >3. However, this concentration was too low for hazard identification and therefore, a sensitising potential cannot be excluded.

Dermal absorption
There is a high variability in the absorption of HC Blue n° 14, ranging from 0.011 to 0.076 μg/cm² with a CV of 68%, possibly due to the low absorption rate.
For the calculation of the Margin of Safety, the mean + 2SD (0.035 + 2 x 0.024 or 0.083 μg/cm²) has been used.

Mutagenicity
Overall, the genotoxicity of HC Blue n° 14 is investigated in genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. HC Blue n° 14 did induce frame shift-mutations in *Salmonella* strain TA 1537 but did not induce an increase in the mutant frequency at the *hprt* locus of mammalian cells. In an older chromosome aberration test HC Blue n° 14 induced an increase in cells with chromosome aberration. A second chromosome aberration was inconclusive whereas in a third test a biological relevant increase in cells with chromosome aberration was not observed. The positive *in vitro* findings with HC Blue n° 14 could not be confirmed in *in vivo* assays: a micronucleus tests in mice and an *in vivo* UDS test were both negative.
As the *in vitro* results were not confirmed in *in vivo* tests, HC Blue n° 14 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data submitted

4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of HC Blue n° 14 at a maximum on-head concentration of 0.3% in non-oxidative hair dye formulations does not pose a risk to the health of the consumer.

HC Blue n° 14 is a secondary amine, and thus is prone to nitrosation and formation of nitrosamines. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submission I, 2001

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