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

CONCERNING

HC BLUE N° 14

COLIPA n° C172

adopted by the SCCNFP during the 25th plenary meeting
of 20 October 2003

1. Terms of Reference

1.1. Context of the question

The adaptation to technical progress of the Annexes to Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products.

1.2. Request to the SCCNFP

The SCCNFP is requested to answer the following questions:

* Is HC Blue n° 14 safe for use in cosmetic products?

* Does the SCCNFP propose any restrictions or conditions for its use in cosmetic products?

1.3. Statement on the toxicological evaluation

The SCCNFP is the scientific advisory body to the European Commission in matters of consumer protection with respect to cosmetics and non-food products intended for consumers.

The Commission's general policy regarding research on animals supports the development of alternative methods to replace or to reduce animal testing when possible. In this context, the SCCNFP has a specific working group on alternatives to animal testing which, in co-operation with other Commission services such as ECVAM (European Centre for Validation of Alternative Methods), evaluates these methods.
The extent to which these validated methods are applicable to cosmetic products and its ingredients is a matter of the SCCNFP.

SCCNFP opinions include evaluations of experiments using laboratory animals; such tests are conducted in accordance with all legal provisions and preferably under chemical law regulations. Only in cases where no alternative method is available will such tests be evaluated and the resulting data accepted, in order to meet the fundamental requirements of the protection of consumer health.

2. Toxicological Evaluation and Characterisation

2.1. General

2.1.1. Primary name

HC Blue n° 14 (INCI name)

2.1.2. Chemical names

Chemical name : 1,4-bis[(2,3-dihydroxypropyl)amino]-9,10-anthracenedione
CAS name : 9,10-Anthracenedione, 1,4-bis[(2,3-dihydroxypropyl)amino]-
Synonyms : 1,4-bis[(2,3-dihydroxypropyl)amino]-anthraquinone

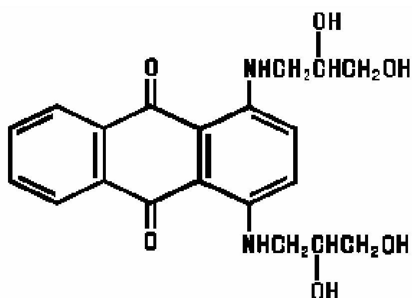
2.1.3. Trade names and abbreviations

Trade name : Imexine BJ (Chimex)
COLIPA n° : C 172

2.1.4. CAS No. / EINECS No.

CAS no : 99788-75-7
EINECS : /

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_{20}H_{22}N_2O_6$
Mol weight : 386.408

2.1.7. Purity, composition, and substance codes

All data refer to one batch (Pil 1).

Purity
Titre as determined by HPLC : 94.6 % (peak area)
Potentiometric titration : 97.3 % (amino function)

Water content	:	1.3 %
Ash	:	0.2 %

Impurities

1,4-Dihydroxyanthraquinone	:	220 ppm
Anthracene-1,4,9,10-tetraol	:	280 ppm
1,4-bis-(2,3-Dihydroxy-propylamino)-4-anthracene-9,10-diol	:	900 ppm
1-(2,3-Dihydroxy-propylamino)-4-hydroxy-anthraquinone	:	930 ppm
1-(2,3-Dihydroxy-propylamino)-4-hydroxy-anthraquinone dimer	:	210 ppm

Reagents and intermediate reaction products

2-Nitrobenzene-1,4-diamine	:	0.127 %
3-(4-Amino-3-nitro-phenyl)-oxazolidin-2-one	:	0.027 %
4-(Amino-3-nitro-phenyl)-carbamic acid 3-chloro-propyl ester	:	<0.025 %

Solvent residues (ethanol)	:	120 ppm
Chloride ions	:	0.26 %

2.1.8. Physical properties

Appearance	:	(navy)blue powder, agglomerated, almost odourless
Melting point	:	/
Boiling point	:	/
Density	:	/
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	–0.8 (calculated)

2.1.9. Solubility

Water	:	0.05 g in 100 ml partially insoluble (insufficient information)
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**	:	50µg/ml, which is higher than the amount penetrated

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

2.1.10 Stability

Stable in receptor fluid

General comments on analytical and physico-chemical characterisation

* Total impurities together with ash contents, water content, chloride content, residual solvent amount to 2.3 %. Considering the chromatographic purity to be 94.6%, some more impurities in the test material may be expected.
* 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 information provided on water solubility of HC Blue n° 14 is insufficient.
* The information on the stability of the test material is insufficient, protection from light is recommended.
* Melting point of HC Blue n° 14 is not reported.
* 1,4-diamino-2-nitrobenzene is classified as Carcinogenic category 3B according to MAK-Commission

2.2. 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.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	OECD n° 401
Species/strain	:	Sprague-Dawley Rat ICO/OFA-SD (IOPS Caw)
Group Size	:	5 rats each sex
Test substance	:	Imexine BJ
Batch no	:	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. 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. No abnormalities were observed at necropsy.

Under these experimental conditions, the LD_{50} of the test substance was higher than 2000 mg/kg in rats. No signs of toxicity were observed at this dose.

Ref. : 1

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

No data

2.3.5. Repeated dose dermal toxicity

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

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 no	:	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/day) -32%, -29%, -27%, -45%. In males, there was -32% reduction at 1000 mg/kg/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 and1000 mg/kg/day respectively) and in males, 9% at 1000 mg/kg/day dosing. There was a 2% increase in sodium levels at 1000 mg/kg/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/day (not reversible after 4 weeks recovery); blue coloration of the extremities and hair in animals given 300 and 1000 mg/kg/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/day was defined as the NOAEL.

Ref. : 5

2.3.8. Sub-chronic dermal toxicity

No data

2.3.9. Sub-chronic inhalation toxicity

No data

2.3.10. Chronic toxicity

No data

2.4. Irritation and corrosivity

2.4.1. Irritation (skin)

Guideline	:	OECD 404
Species/strain	:	white rabbits, New Zealand
Group size	:	3 male
Test substance	:	Imexine BJ
Batch number	:	Pil 1
Purity	:	94.6% (HPLC)
Dose	:	0.5g applied to $6cm^2$ of intact skin for 4 hours
GLP	:	In compliance

After clipping the back and flanks, 0.5g of the test material was applied to a 6 cm^2 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. : 3

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405
Species/strain	:	white rabbits, New Zealand
Group size	:	3 male
Test substance	:	Imexine BJ
Batch number	:	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 mild irritant.

Ref. : 2

2.5. Sensitisation

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 number	:	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. : 4

2.6. Teratogenicity

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 no	:	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 with10ml /kg 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 NOEL was defined as 1000 mg/kg/day.

Ref. : 10

2.7. Toxicokinetics (including Percutaneous Absorption)

Guideline	:	/
Test substance	:	IMEXINE BJ 1,4-bis-(2,3-dihydroxy-propylamino)-anthraquinone
Batch no	:	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
Specific 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 of contact	:	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 ingredient : 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 ± 19.87ng/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

2.8. Mutagenicity/Genotoxicity

2.8.1. Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline : OECD 471
Species/strain : *S. typhimurium,* TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA
Replicates : Triplicate plates, 3 independent tests
Test substance : Imexine BJ in DMSO
Batch no : Pil 1
Purity : 94.6% (HPLC)
Concentrations : *Salmonella typhimurium* and *E. coli*
Test #1 (direct plate incorporation method) :
With and without metabolic activation at 5 doses
(312.5, 625, 1250, 2500, 5000 µg/plate)

Test # 2 :
Without metabolic activation (direct plate incorporation method)
at 5 doses (312.5, 625, 1250, 2500, 5000 µg/plate)
With metabolic activation (preincubation assay)
at 5 doses (312.5, 625, 1250, 2500, 5000 µg/plate)

Test # 3 :
With metabolic activation (preincubation assay)

		at 5 doses (312.5, 625, 1250, 2500, 5000 µg/plate)
GLP	:	In compliance

Imexine BJ has been investigated for gene mutation in *S. typhimurium* and *E. coli* using the direct plate incorporation or the preincubation methods either with or without S9 mix.
Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guidelines.

Results
Dose range finding assay
No signs of toxicity was noted for the top dose of 5000 µg/plate in *E. coli*; a slight toxicity was observed for the 2 strains TA 98 and TA 100. No precipitate occurred at this top dose, therefore, the top dose has been selected to be 5000 µg/plate.

Test # 1, # 2 and # 3
In the presence of activation under the technical conditions of the preincubation method, a dose related and reproducible increase in revertant numbers has been observed in the *Salmonella* TA 1537 frameshift tester strain (mean revertant frequency factor compared with control : test # 2 – 1.2 to 5.3 x ; test # 3 – 1.8 to 10.2 x).

Conclusions
Based on the reversion rate, and under the conditions of the 2 preincubation assays performed in the presence of S9 mix , it is concluded that the test agent Imexine BJ dissolved in DMSO and/or one of its metabolites display mutagenic potential in the frameshift TA 1537 bacterial tester strain. It is therefore considered as positive under the conditions of the assays.

Ref. : 6

***In Vitro* Mammalian Chromosomal Aberration Test**

Guideline	:	OECD 473
Species/strain	:	Human lymphocytes (non pooled cultured blood samples)
Replicates	:	Duplicate cultures, 2 independent experiments
Test substance	:	Imexine BJ in water
Batch no	:	Pil 1
Purity	:	94.6% (HPLC)
Concentrations	:	Test #1 without S9 mix 3 h treatment – 20 h harvest : 10, 30, 100, 300, 900, 2700 µg/ml with S9 mix 3 h treatment – 20 h harvest 10, 30, 100, 300, 900, 2700 µg/ml Test # 2 without S9 mix 20 h treatment – 20 h harvest : 10, 30, 100, 300, 900 µg/ml 44 h treatment – 44 h harvest : 10, 30, 100, 300, 900 µg/ml with S9 mix 3 h treatment – 20 h harvest : 30, 100, 300, 450, 600 µg/ml 3 h treatment – 44 h harvest : 30, 100, 300, 450, 600 µg/ml

Test # 3
3 h treatment – 20 h harvest : 300, 450, 600 µg/ml
GLP : In compliance

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

Results
Structural chromosome aberrations

Test # 1
without S9 mix
A dose related decrease in Mitotic Index was noted (74, 76, 57, 47, 7 % MI from 30 to 2700 µg/ml compared with controls).
No statistically or biologically significant increase in the number of aberrant cells was observed compared with the corresponding solvent control.

with S9 mix
A sharp decrease in Mitotic Index was noted in a first assay, consequently another test has been performed (125, 108, 108, 7 % MI from 30 to 2700 µg/ml compared with controls).
No statistically or biologically significant increase in the number of aberrant cells was observed compared with the corresponding solvent control.

Test # 2
without S9 mix – 20 h exposure – 20 hours harvest.
A decrease in mitotic index was noted (114, 104, 27, 99, 59 % MI from 10 to 900 µg/ml compared with controls).
No statistically or biologically significant increase in the number of aberrant cells was observed compared with the corresponding solvent control.

without S9 mix – 3 h exposure – 20 hours harvest.
A dose related decrease in Mitotic Index was noted (74, 62, 65, 26, 18 % MI from 10 to 900 µg/ml compared with controls).
No statistically or biologically significant increase in the number of aberrant cells was observed compared with the corresponding solvent control.

with S9 mix – 3 h exposure – 20 hours harvest.
A dose related decrease in Mitotic Index was noted (114, 80, 68, 31, 33, % MI from 30 to 600 µg/ml compared with controls).
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).

with S9 mix – 3 h exposure – 44 hours harvest.
A significant decrease in Mitotic index was noted (85, 129, 54, 48, 13 % MI from 30 to 600 µg/ml compared with controls).

No statistically or biologically significant increase in the number of aberrant cells was observed compared with the corresponding solvent control.

Test # 3
with S9 mix – 3 h exposure – 20 hours harvest.
No decrease in Mitotic Index was noted (149, 102, 131 % MI from 300 to 600 µg/ml compared with controls).
While not statistically significant a trend for a dose response in the frequency of aberrant cells was found. Moreover, 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 doses. Similarly, this increase is due to the presence of chromatid and/or chromosome deletions 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 and/or aneugenic activity in human lymphocytes in the presence of activation under the conditions of the test.

Ref. : 7

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474 (1983)
Species	:	Swiss OF1 mice
Group sizes	:	5 males and 5 females
Test substance	:	Imexine BJ in 0.5% carboxymethylcellulose
Batch no	:	Pil 1
Purity	:	94.6 % (HPLC)
Dose levels	:	Maximum Tolerated Dose (MTD) A prelimiminary dose-range finding assays was conducted. According to the lack of clinical signs and toxic reactions of the mice, the top dose has been chosen to be 2000 mg/kg bw. Imexine BJ was administered by 1 single oral dose of 500, 1000 and 2000 mg/kg bw for a 24 h sacrifice time
GLP	:	In compliance

Imexine BJ has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined by a preliminary range finding study in which no toxic effects were seen at doses of 1000 and 2000 mg/bw. The substance was administered by a single intragastric gavage and the groups of animals sacrificed 24 hours after administration. Negative and positive controls were in accordance with the OECD guideline.

Number of cells scored : a total of at least 2000 erythrocytes were examined from each animal ; the incidence of micronucleated erythrocytes and the ratio of polychromatic erythrocytes to normochromatic erythrocytes were calculated.

Results
PCE/NCE ratio.
A significant change of the ratio has been observed for the dose of 500 and 2000 mg/kg, 0.6 and 0.7 respectively. Control value are 0.9.
Micronucleated PCE : no statistically significant increase in the incidence of micronucleated polychromatic erythrocytes over the concurrent vehicle control values were observed for any dose levels. It should be noted that the authors have scored 2000 cells and expressed the mean value with the standard deviation. However, in the summary table 2, values were expressed as micronucleated PCE per thousand. The authors divided the mean value by 2 that is correct but also the SD by 2 which is mathematically incorrect.
The biological relevance of the variation in the PCE/NCE ratio is questionable due to the fact that generally speaking a very large inter-individual variation is found and because a decrease at the 24 h time point is not necessarily expected because of the relatively long replication time of erythroid cells.

Conclusions
Under the conditions of the test it can be concluded that Imexine BJ in DMSO at doses at which no sign of clinical toxicity were recorded, but some variation in the PCE/NCE ratio was observed does not induce statistically significant increase in the frequency of μ PCE. However, only a 24h sacrifice time was evaluated. The study is inadequate, because there is no demonstration that the compound reached the target cells.

Ref. : 8

Unscheduled DNA Synthesis (UDS) Test With Mammalian Liver Cells *in vivo*

Guideline	:	OECD draft guideline 486
Species/strain	:	Wistar rat, HanIbm: WIST (SPF) strain
Group size	:	4 males rats
Test substance	:	Imexine BJ in carboxymethylcellulose
Batch No	:	Pil 1
Purity	:	94.6% (HPLC)
Dose levels	:	A single oral dose was given to a group of male rats at dose levels of 200 and 2000 mg/kg. Two sampling times were selected : 2 h & 16 h post-treatment.
Exposure time	:	2 h and 16 hours: all dose groups
GLP	:	In compliance

Imexine BJ has been investigated for induction of unscheduled DNA synthesis in rats hepatocytes at 2 doses 200 and 2000 mg/kg bw.
Only one positive control in accordance with OECD guideline has been used and UDS analyzed by autoradiography. 3 males were used per dose/time sampling

Results
The viability of the hepatocytes was not substantially affected by the treatments.
Treatment with Imexine BJ at doses of 200 & 2000 mg/kg yielded group mean NNG values less than 0 for both experiment time and caused no significant increases, as compared to control, in the mean nuclear grain counts.
The percentage of cells in repair did not significantly differ from the control group.

Conclusions
Data indicate that single oral gavage treatment of male rats dosed once with 200 & 2000 mg/kg of Imexine BJ did not induce increased unscheduled DNA synthesis in hepatocytes isolated approximately 2 or 16 hours after dosing.
Under the experimental conditions, it is concluded that Imexine BJ did not display DNA repair activities detectable by this assay.

Ref. : 9

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

Not applicable

2.12. Conclusions

The reported purity of the HC Blue n° 14 (9,10-Anthracenedione, 1,4-bis[(2,3-dihydroxypropyl)amino) is approximately 95 %. It is a secondary alkanolamine and thus it is prone to nitrosation. No information is provided on the nitrosamine content of the test material. Some impurities have been characterised and quantified but there are also unidentified impurities. The information on solubility and stability of the test material is insufficient.

The LD_{50} of the test substance was higher than 2000 mg/kg 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/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/day.

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.

The percutaneous absorption study was considered inadequate.

HC Blue n° 14 has been tested in prokaryotic and mammalian cells for gene mutation, and in mammalian cells for chromosomal aberration in vitro. Two in vivo tests have been performed (bone marrow micronucleus and UDS tests).
The *in vitro* test for gene mutation in prokaryotes has been found positive in the presence of a metabolic activation system.
The *in vitro* test for clastogenicity in human lymphocytes is positive in the presence of activation.
The *in vivo* micronucleus test in mice gave negative results. The study is however inadequate.
The *in vivo/in vitro* UDS on rats hepatocytes is negative for the treatment of 2 and 16 hours.

The data on mutagenicity/genotoxicity are insufficient for an adequate evaluation.

2.13. References

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9. In vivo/in vitro Unscheduled DNA Synthesis in Rat Hepatocytes with Imexine BJ. Cytotest Cell Research GmbH and Co. KG (CCR), D-64380 Rossdorf, Germany. Report N° 587902, 23 September 1997
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11. Penetration in vitro du Colorant Imexine BJ Colipa CI72 a Travers I'Epiderme Humain Isolé Monté sur Cellules de Diffusion Type Franz. Recherche Evaluation Sécurité Produits, Département de Chimie Analytique, L'OREAL, Aulnay-soul-boll, France. Tests N° 30/01/97, 12/02/97. 19/03/97 et 14/05/97, 23 rd June 1997.

3. Opinion of the SCCNFP

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

Before any further consideration, the following information is required :

* 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.

4. Other considerations

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5. Minority opinions

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