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

CONCERNING

HC BLUE 15

COLIPA n° : /

adopted by the SCCNFP on 23 April 2004
by means of the written procedure

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 15 safe for use in cosmetic products as a hair dye ingredient?

* 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 Nº 15 (INCI)

Note: this compound is neither in the EU inventory, nor in the CTFA Dictionary.

2.1.1. Chemical names

Phosphoric acid compound with 4-[(2,6-dichlorophenyl) (4-imino-3,5-dimethyl-2,5-cyclohexadien-1-ylidene) methyl]-2,6-dimethylaniline (1:1)
Benzenamine,4-[(2,6-dichlorophenyl)(4-imino-3,5-dimethyl-2,5-cyclohexadien-1-ylidene) methyl] -2,6-dimethyl-, phosphate (1:1)
Phosphoric acid compound with 4-[(2,6-dichlorophenyl)(4-imino-3,5-dimethyl-2,5-cyclohexadien-1-ylidene)methyl]-2,6-dimethylaniline (1:1)

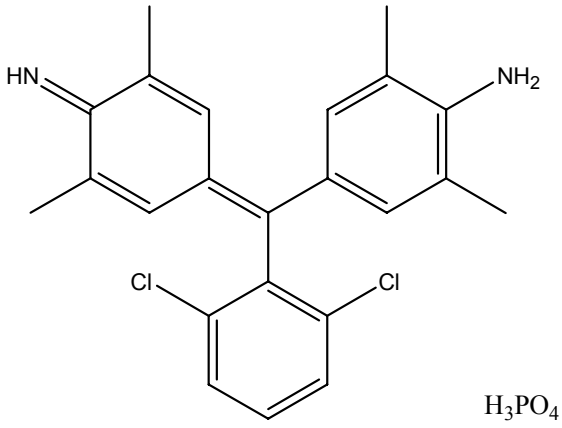
2.1.3. Trade names and abbreviations

COLIPA n°	:	/
Trade name	:	Jade Blue; Gardex Jade Blue; WR 802178; Jade Blue WR; 802178; A015892
Other names	:	Basic Blue 77 phosphate; Basic Blue 77

2.1.4. CAS / EINECS number

CAS n°	:	74578- 10- 2
EINECS n°	:	277- 929- 5

2.1.5. Structural formula



H_3PO_4

2.1.6. Empirical formula

Emp. Formula : $C_{23}H_{22}Cl_2N_2\ H_3O_4P$
Mol weight : 495.34

2.1.7. Purity, composition and substance codes

Substance code : /
Batches used : All analytical data are related to batch BB77-020420 (used in all toxicological studies).

Purity : 31.0 % by NMR (quantitative) (cationic part of the dye)

Phosphate content	:	12.7 %
Water content	:	8.4 % (w/w)
Ash content (sulphated)	:	15.6 % (5.1 % of sodium)
Heavy metals	:	< 124 ppm

Relative chromatographic purity (HPLC - UV/VIS peak area method): 95.7 % at 254 nm

Potential impurities

2-propanol	:	4.2 %
Starch	:	25-35 %
PEG 800	:	1.4 %
Boron	:	< 1.5 %
Ammonia	:	0.3 %

2.1.8. Physical properties

Appearance : Reddish brown powder
Melting point : /
Boiling point : 573 ± 40 ºC
Density : /
Rel. vap. dens. : /
Vapour Press. : 3.83E -13 Torr
Log P_{ow} : 5.916 ± 0.658 (calculated)
pK_a : 5.97± 0.20

2.1.9. Solubility

3.0 % soluble in water (pH 2.9), 6.4% soluble in acetone/water (1:1) (pH 3.8), 9.3% soluble in DMSO.

2.1.10. Stability

Stable in DMSO (5%) (recovery: 96.8-102.2%) and in acetone (0.2%) (recovery: 95.5-99.4%) for 8 days. In phosphate buffered solution (0.1%) at pH 7.6, a linear degradation was observed (a recovery of 43.5% after 8 days). In a formulation maintained 10 months at 25ºC in market

packaging, the dye content found was 0.013% (theoretical dye content: 0.027%). This means more than 50% loss. No information was given on the degradation products.

General comments on analytical and physico-chemical characterisation

* It seems that the Log P_{ow} (calculated) of this substance is very high in respect to its solubility. Calculated values can not be accepted as estimates of the true physical constants without justification, indicating if the calculation has taken into consideration a pH value related to physiological conditions to the conditions of the percutaneous absorption studies; log P_{ow} is known to strongly depend from pH.
* In the analytical mass balance (93-94%), more than 30% corresponds to starch and PEG 800 content. This fact affects to the actual concentration of the dye (a.i.) used in toxicological studies.
* The degradation products of the dye in formulations are not reported.

2.2. Function and uses

HC Blue Nº 15 is intended for use in oxidative hair dyes as a non-reacting component at a maximum final concentration of 0.08% to 0.10 % after mixing with 1.0 to 1.5 volumes of hydrogen peroxide preparation respectively (0.20% in the dye formulation).

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

No data.

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

Guideline	:	OECD 407 (1995)
Species/strain	:	Wistar rat
Group size	:	10 animals (5 males & 5 females) / dose level
Observ. Period	:	28 days (no recovery group included)
Test substance	:	Basic Blue 77
Batch no	:	BB77-020420

Purity : 98.7 - 99.7%
Dose levels : 0, 5, 15, 45 mg/kg bw/day
GLP statement : not in compliance

The test item was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 5, 15 and 45 mg/kg bw/day for a period of 28 days. A control group was treated similarly with the vehicle, bidistilled water with 0.5 % 1,2-propylene glycol and 0.4 % Plantaren 2000 UP, only.
The group comprised 5 animals per sex, which were sacrificed after 28 days of treatment.
Clinical signs, food consumption and body weights were recorded periodically during pretest and treatment periods.
At the end of the dosing period, blood samples were withdrawn for haematology analyses. All animals were killed, prepared for necropsy and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose animals and on all gross lesions from all animals.

Results
Oral administration of the test item to Wistar rats at doses of 5, 15 and 45 mg/kg bw/day, for 28 days resulted in no mortalities, no clinical signs of toxicological relevance, no changes in food consumption or body weight and no changes in haematology parameters.

Test-related findings were

5 mg/kg bw/day : slightly pale faeces from d11 of treatment onwards.
15 mg/kg bw/day : blue discoloration of faeces from d6 onwards, discoloration of salivary glands, increased liver weights, coinciding with slight to minimal centrilobular hypertrophy in the liver
45 mg/kg bw/day : blue discoloration of faeces from d3 onwards and blue discoloration of body extremities from d6, discoloration of the liver, salivary glands, thymus (or discoloured foci) and exorbital lacrimal gland;
increased liver weights, coinciding with slight to minimal centrilobular hypertrophy in the liver (more severe in females than in males).

Conclusion
Based on the results of this study, the doses for the 90 days study were set on 0, 1, 4 and 15 mg/kg bw/day.

Ref.: 2

2.3.5 Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Subchronic oral toxicity

Guideline : OECD 408 (1998)
Species/strain : rat, HanBrl:WIST (SPF)

Group size	:	20 animals (10 males & 10 females) / dose level
Observ. Period	:	92 days (no recovery group included)
Test substance	:	Basic Blue 77
Batch no	:	BB77-020420
Purity	:	95.7 - 99.7%
Dose levels	:	0, 1, 4, 15 mg/kg bw/day
GLP statement	:	in compliance

The test item was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 1, 4 and 15 mg/kg body weight/day for a period of 91/92 days. A control group was treated similarly with the vehicle only.
Clinical signs, outside cage observations, food consumption, and body weights were recorded during the pre-experiment and the main experiment. Ophthalmologic examinations were performed both at the end of the pre-experiment and the main experiment. During week 13 the animals were evaluated according to a functional observational battery, including locomotor activity and grip strength.
At the end of the period, blood samples were withdrawn for haematology and plasma chemistry analyses. Urine samples were collected for urinalyses. All animals were scarified, prepared for necropsy and examined post mortem.

Histological examinations were performed on organs and tissues from all control and high dose animals and on all gross lesions from all animals.
From the animals of the low and middle dose groups, livers (females) and hearts (males) were examined to establish a no-effect-level.

Results
Oral administration of the test item to Wistar rats at doses of 1, 4 and 15 mg/kg bw/day, for 13 weeks resulted in no test item-related deaths, no clinical signs of toxicological relevance during daily or weekly (weeks 1 to 12) observations, no changes in the parameters of the functional observational battery (including grip strength or locomotor activity), no changes in mean food consumption or body weight development, no ophthalmoscopic changes, and no changes in haematology parameters.

Test item-related findings were:

1 mg/kg bw/day	:	no substance related effects noted.
4 mg/kg bw/day	:	blue faeces and/or grey discoloration of fur, discoloration of salivary glands, and elevated mean sodium levels.
15 mg/kg bw/day	:	blue faeces and/or grey discoloration of fur, discoloration of salivary glands, discoloration of extraorbital lacrimal glands and preputial glands (no microscopic correlation found for the discoloration), elevated mean sodium levels, decreased mean glucose levels in females, elevated mean triglyceride levels, elevated mean cholesterol and mean phospholipid levels in females (considered to be indications of possible changes in non-specific metabolic pathways in the liver), increased urinary leukocytes, increased urinary erythrocytes in females, elevated liver weights, coinciding with minimal hypertrophy of centrilobular hepatocytes, elevated kidney weights; a marginally greater incidence and severity of focal myocarditis was

observed, but this was found to be more likely fortuitously than substance-related (statistically non-significant).

Conclusion
The results of this study indicate that 1 mg/kg bw/day of Basic Blue 77 was established as the no-observed-effect-level (NOEL), based upon passive effects at 4 mg/kg bw/day such as faecal discoloration, whereas 4 mg/kg bw/day was considered to be the no-observed-adverse-effect-level (NOAEL), based upon various changes in clinical biochemistry, urinalyses and higher kidney weights at 15 mg/kg bw/day.

Ref.: 3

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 & corrosivity

2.4.1. Irritation (skin)

Guideline	:	OECD 404 (1992)
Species/strain	:	New Zealand White Rabbit, SPF
Size	:	3 (both sexes)
Test item	:	HC Blue No. 15
Batch no.	:	BB77 -020420
Purity	:	See Comments on analytical characterisation
Dose	:	0.5 moistened with approx. 0.1 ml of purified water.
GLP	:	in compliance

The primary skin irritation potential of HC Blue No. 15 was investigated by topical semi-occlusive application of 0.5 g to the intact left flank of each of three young adult New Zealand White rabbits. The duration of the treatment was four hours. The scoring of skin reactions was performed 1, 24, 48 and 72 hours, as well as 7, 10 and 14 days after removal of the dressing.

Results
Erythema could not be fully evaluated during the first 24 hours after treatment due to marked staining at the application site. However, the mean score was calculated for each animal using the data available (24, 48 and 72 hours after patch removal) for erythema/eschar grades and for oedema grades. The mean erythema/eschar scores for the three animals were 1.67, 0.00 and 1.00, respectively and the mean oedema scores were 0.33, 0.00 and 0.00, respectively.

The application of HC Blue No. 15 to the skin resulted in mild signs of irritation such as erythema, oedema and scaling. These effects were reversible and were no longer evident 10 days after treatment. A light to marked blue staining was present at the application site of all animals throughout the entire 14 day observation period. No corrosive effects were noted on the treated skin of any animal at any of the measuring intervals.

Conclusion
Based upon the referred classification criteria (Commission Directive 2001/59/EC) HC Blue No. 15 is considered to be not irritating to rabbit skin.

Ref.: 4

2.4.2. Irritation (mucous membranes)

Study 1

Guideline	:	OECD 405 (1998)
Species/strain	:	New Zealand White Rabbit, SPF
Size	:	1 female
Test item	:	HC Blue No. 15
Batch no.	:	BB77-020420
Purity	:	See Comments on analytical characterisation
Dose	:	0.1 g (undiluted)
GLP	:	in compliance

The primary eye irritation potential of the test item was investigated by instillation of 0.1 g (undiluted due powder) into the left eye of a single young adult New Zealand White rabbit. Scoring of irritation effects was performed approximately 1, 24, 48 and 72 hours, as well as 7 and 10 days after treatment.

Results
The instillation of the test item into the eye of a single rabbit resulted in early-onset and severe signs of ocular irritation. The treated eye was washed with NaCl, 0.9 % 24 hours after treatment.

Full assessment of the treated eye was prevented on a number of occasions due to the presence of a marked blue-green staining. However, examination of the eye 7 and 10 days after treatment revealed an opaque cornea and no light reflex in the iris. Effects observed in the conjunctivae consisted of reddening from 7 to 10 days after treatment and chemosis from 1 hour to 10 days after treatment. The maximum attainable score was achieved for both these parameters. Discharge was also present throughout the 10 day observation period and was noted to be of a thick mucus type from the 48-hour examination to termination. A light to marked blue-green staining was present in the treated eye throughout the observation period.

Based on these results the animal was prematurely sacrificed at the request of the Study Director 10 days after treatment. For ethical reasons no further animals were treated.

Based on the referred classification criteria (Commission Directive 2001/59/EC of August 6, 2001), the test item poses a risk of serious damage to eyes.

Ref.: 5

Study 2

Guideline	:	OECD 405 (1998)
Species/strain	:	New Zealand White Rabbit, SPF
Size	:	3 (both sexes)
Test item	:	HC Blue No. 15
Batch no.	:	BB77-020420
Purity	:	95.7 to 99.7 area % (HPLC) (See Comments on analytical characterisation)
Dose	:	0.1 ml of a 2% aqueous solution (pH adjusted to pH 6.30)
GLP	:	in compliance

The primary eye irritation potential of the diluted test item (2% in purified water) was investigated by instillation of 0.1 ml into the left eye of each of three young adult New Zealand White rabbit. Scoring of irritation effects was performed approximately 1, 24, 48 and 72 hours, as well as 7 and 10 days after test item application.

Results
The mean score was calculated across 3 scoring times (24, 48 and 72 hours after instillation) for each animal for corneal opacity, iris, redness and chemosis of the conjunctivae, separately. The individual mean scores for corneal opacity and iris were 0.00 for all three animals. The individual mean scores for the conjunctivae were 1.00, 1.00 and 0.67 for reddening and 0.00, 0.00 and 0.00 for chemosis, respectively.

The primary eye irritation score was calculated by totalling the mean cumulative scores at 24, 48 and 72 hours and then dividing the resulting total by the number of data points. The primary eye irritation score was 0.89 (max. 13).

The instillation of the test item into the eye resulted in mild, early-onset and transient ocular changes, such as reddening of the conjunctivae and sclerae, discharge and chemosis. These effects were reversible and were no longer evident 7 days after treatment, the end of the observation period for all animals. Corneal opacity due to abnormal findings was observed in the iris of any animal at any of the examinations. No corrosion was observed at any of the measuring intervals, blue staining of the treated eyes by the test item was observed in all animals at the 2-hour reading and was no longer evident 48 hours after treatment.

The test item did not induce significant or irreversible damage to the rabbit eye. Based on the referred classification criteria (Commission Directive 2001/59/EC), the diluted test item is considered to be not irritating to the rabbit eye.

Ref.: 6

Conclusion
The pure substance of HC Blue No. 15 is classified as irritant to mucous membranes (Ref. 5). However, at a concentration of 2% (in water) no irritation to mucous membranes was observed (Ref. 6). The concentration of HC Blue No. 15 in the final product is 0.2 %, therefore no irritating effect to mucous membranes of the final product is expected when applied as intended.

2.5. Sensitisation

Guideline	:	OECD 429 (2000)
Species/strain	:	Mouse: CBA/J
Size	:	5 females per concentration
Test item	:	HC Blue No. 15
Batch no.	:	BB77-020420
Purity	:	(See Comments on analytical characterisation)
Dose	:	0.5, 1.5, 3 and 5 % (w/v) in DMSO
GLP	:	in compliance

HC Blue No. 15 was tested in the local lymph node assay at different concentrations (0, 0.5, 1.5, 2.0, 5.0 % (w/v)) in DMSO (vehicle). On days 0,1 and 2 the animals received 2.5 µl of the test item formulation, positive control, or vehicle control on the dorsal surface of each pinnae.

Morbidity/mortality checks were performed twice daily. Clinical examinations were performed daily. Individual body weights were recorded on days -1 and 5. All animals were sacrificed on day 5 for the assessment of cell proliferation.

No mortality was observed during the study. There were no treatment-related clinical signs. There were no treatment-related effects on body weight or body weight gains.

Positive control (p-phenylenediamine) induced a positive response, as it elicited at least a 3-fold increase in isotope incorporation relative to the vehicle. The mean stimulation index was 3.9 at the concentration of 1%.

Results
The test substance induced a negative response, as it did not elicit a least a 3-fold increase in isotope incorporation relative to the vehicle. The mean stimulation indices were 1.0, 1.7, 2.3 and 1.9 at the concentrations of 0.5 %, 1.5 %, 3.0 % and 5.0 %, respectively.

Conclusion
Based of these results, the test substance is not a skin sensitizer under the defined experimental conditions.

Ref.: 7

2.6. Teratogenicity

Dose-range finding prenatal development toxicity study

Guideline	:	/
Species/strain	:	rat, HanBrl:WIST (SPF)
Group size	:	5 females / dose level
Observ. Period	:	21 days
Test substance	:	Basic Blue 77
Batch no	:	BB77-020420
Purity	:	95.4 - 98.7%
Dose levels	:	0, 5, 15, 150 mg/kg bw/day
GLP statement	:	/

Basic Blue 77 was administered by gavage in vehicle (1,2-propylene glycol, 50% aqueous decyl glycoside and water) at daily dosages of 0, 5, 15 and 150 mg/kg bw/day to 5 mated Wistar rats per group from day 6 to 20 of gestation inclusive, using a dosing volume of 10 ml/kg bw. Dams were killed on day 21 post coitum, just prior to expected delivery and foetuses were removed by caesarean section for examination.

Results
All animals in the 150 mg/kg bw/day dosage group showed bluish-green skin discoloration, dark faeces and blue discoloured urine. They showed reduced food consumption, body weight loss and all died before day 5 post coitum.
At 15 mg/kg bw/day, a slightly reduced body weight gain was noted, besides ruffled fur from day 8 till days 12 or 13 post coitum.
Neither the reproduction data, nor the foetal parameters were affected by substance administration.

Conclusion
Based on the results of this study, the doses for the prenatal developmental toxicity study were set on 0, 3, 10 and 30 mg/kg bw/day.

Ref.: 8

Prenatal development toxicity study

Guideline	:	OECD 414 (2001)
Species/strain	:	rat, HanBrl:WIST (SPF)
Group size	:	24 females / dose level
Observ. Period	:	21 days
Test substance	:	Basic Blue 77
Batch no	:	BB77-020420
Purity	:	95.4 - 98.7%
Dose levels	:	0, 3, 10 and 30 mg/kg bw/day
GLP statement	:	in compliance

Basic Blue 77 was tested for its embryotoxic, foetotoxic and teratogenic potential in rats. The test item was administered by gavage in vehicle (1,2-propylene glycol, 50% aqueous decyl glycoside and water) at daily dosages of 0, 3, 10 and 30 mg/kg bw to 24 mated Wistar rats per group from day 6 to 20 of gestation inclusive, using a dosing volume of 10 ml/kg bw. Dams were killed on day 21 post coitum, just prior to expected delivery and foetuses were removed by caesarean section for examination.

Results
There were no indications of an adverse effect of treatment with Basic Blue 77 on pregnant females on terms of mortality, clinical signs or necropsy observations.

Test item-related findings were :

3 mg/kg bw/day	:	no adverse effects noted.
10 mg/kg bw/day	:	blue discoloured content in urinary bladder, reduced food consumption (d6-d9 post coitum), slightly reduced body weight gain.

30 mg/kg bw/day: dark discoloured faeces, blue discoloured content in urinary bladder, blue discoloured content in stomach and intestines, reduced food consumption (d6-d18 post coitum), reduced body weight gain.

No treatment-related effects on reproduction data or foetal data were observed. Mean number of implantation sites, pre- and post-implantation losses and mean number of viable foetuses per litter and group were not affected by treatment. There were no dead or aborted foetuses.
Mean sex ratios and mean foetal body weights were not affected by treatment. There were no treatment-related foetal external, visceral or skeletal (bones and cartilage) findings.

Conclusion
Based on the results of this study, the no observable adverse effect level (NOAEL) for Basic Blue 77 was considered to be 3 mg/kg bw/day for females and 30 mg/kg bw/day for foetuses.

Ref.: 9

2.7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous absorption *in vitro*

Guideline	:	/
Tissue	:	Pig skin
Method	:	Franz diffusion cells
Test substance	:	HC Blue 15 (1.67% dye in final formulation)
Batch No	:	BB77-020420 formulated in batch 6746 11.06.2002
Dose levels	:	100 mg/cm² of the oxidative formulation; 1.67mg/ cm² of the dye active principle.
Receptor fluid	:	0.14 M NaCl, 2mM K2HPO4, 0.4mM KH2PO4,100 IU Penicillin/ml and 97 µg Streptomycin/ml.
Replicate cells	:	6 cells
Analytical method	:	HPLC (Detection at 606 nm)
GLP	:	In compliance

The skin penetration of HC Blue 15 was evaluated in a static Franz diffusion cell system using pig skin (thickness: 1000µm and exposure area: 4cm²). The integrity of the skin samples was demonstrated with tritiated water. The solubility of the dye in the receptor fluid was not studied.
The dye formulation (100mg/cm²) equivalent to 1670µg/cm² of the dye active principle was applied on the skin surface for 30 min. Then, the skin excess was washed off with shampoo and water and left unoccluded for a 72 hour exposure period. At 16,24, 40, 48, 64, and 72 hours, the dye content was analysed by HPLC as well as after 72 hours in the skin compartments (epidermis and upper dermis separated).

Results
Under the present experimental conditions, a total recovery of the dye of 109.8% has been obtained. Most of the hair dye applied on the skin surface was removed with the washing procedure (1829 µg/cm² or 109.7% of the applied dose). The amounts of HC Blue 15 detected in the separated skin layers were: 0.539µg/cm² (0.032% of the applied dose) in the epidermis, and 0.098µg/cm² (0.006% of the applied dose) in the upper dermis.
The content of HC Blue 15 in the receptor fluid was below 161ng/cm² adding up all 6 fractions. Considering the detection limit as upper limit, the amount of HC Blue 15 in the receptor fluid

can be considered as < 0.161 µg/cm² (<0.01% of the applied dose). The total percutaneous absorption value (receptor fluid, epidermis and upper dermis) is 0.798µg/cm² (0.048% of the applied dose).

Ref.: 10

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Reverse Mutation Testing Using Bacteria

Guideline	:	OECD 471 (1997)
Species/Strain	:	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 and TA102
Test item	:	HC Blue 15
Batch No.	:	WR802178
Purity	:	HC Blue 15: 38.5% (Starch: 30%; water 8.8%) isopropyl alcohol: 4.2%; PEG 800: 1.4% (Received: 14.06.2002, stable)
Doses	:	1-500 µg/plate for all strains (1st exp) 1-300 µg/plate for all strains (2nd exp) 2 exp.
Replicate	:	2 experiments
Positive controls	:	According to OECD Guideline
Metabolic Act.	:	S9 from Aroclor 1254-induced rat liver homogenate.
GLP	:	in compliance

Results
The test item results toxic to all strains of *Salmonella* up to 1000 µg/plate in the first experiment; in the second experiment doses were set up to 300 µg/plate. In this experiment the dose of 300 µg/plate was also toxic.

In the first experiment an increase in the number of revertants was observed in all doses up to 100 µg/plate on TA102 strain in the presence of metabolic activation. The increase did not reach the doubling of the control.

In the second experiment no dose-related increase in the number of revertants induced on TA102 strain was observed, in the presence of metabolic activation. For this strain a third experiment was performed with doses from 0.3 to 100, and again no induction of reversion was observed.
Conclusions
The test item might be considered non-mutagenic in this test assay: however, the toxicity observed might have influenced the results, by considering that the maximum tested dose (100 µg/plate) contains only 38.5% of the test item). The study is inadequate and cannot be used for the evaluation.

Ref.: 11

***In Vitro* Mammalian cell gene mutation test**

Guideline	:	OECD 476 (1997)
Species/Strain	:	Mouse lymphoma L5178Y cell line (Forward mutations at TK locus)
Doses	:	+ S9: 0.7 to 11-0 µg/ml (6 doses) - S9: 1.4 to 44.0 µg/ml (6 doses)

		Second experiment: +S9: 0.2 to 6.0 µg/ml (6 doses)
Replicate	:	2 experiments +S9; 1 exp. –S9 (2 cultures/each exp)
Metabolic Act.	:	S9 from Phenobarbital + β-Naphthoflavone-induced rat liver homogenate
Positive control	:	MMS (without metabolic activation) 3MC (with metabolic activation) (according to OECD Guidelines)
Substance	:	HC Blue 15; Jade Blue WR802178
Batch No	:	BB77-020420
Purity	:	HPLC: 99.76 % (area) byproducts: Starch: 30%; PEG: 1.4% isopropyl alcohol: 4.0%; Sodium Phosphate 17.8%; Ammonia 0.3% Water 8.4%.
Stability	:	not indicated by the Sponsor
GLP	:	in compliance

Results
There was no change in osmolality and pH of the maximum dose used in the study compared to the solvent alone (DM50).
Pre-test for toxicity was performed ±S9 with doses from 5.5 to 700 µg/ml: relevant toxic effects were observed at the lowest concentration (5.5 µg/ml) in the absence of metabolic activation following 4 and 24h treatment; in the presence of S9 toxic effects were observed at a concentration of 21.9 µg/ml.
The concentrations for the mutagenicity assay were set on the base of observed toxicity: the cloning efficiency at the maximum tested concentration ± S9 ranged between 6 and 23%. The number of small size colonies (possibly chromosome aberrations) and of large size colonies (gene mutant colonies) was recorded during all the experiments.
There was an increase in the frequency of small size colonies at the maximum dose of 44.0 µg/ml (268 against 54 of the control) only in the second culture of the first experiment in the presence of S9 (higher than the positive control). Any increase was observed in the absence of S9 in both cultures of the first and the second experiment.
The experiment in the presence of S9 was not repeated. The observed mutagenic effect was not dose related.

Conclusion
HC Blue 15 is considered non mutagenic in this assay, in spite of some inadequacy and toxic effect of the substance.

Ref.: 12

2.8.2 Mutagenicity/Genotoxicity *in vivo*

in vivo Mammalian Erythrocyte Micronucleus

Guideline	:	OECD 474 (1997)
Species/Strain	:	NMRI Mice
Group size	:	5 males /5 females / group dosed
Doses	:	12.5-25-50 mg/Kg
Test substance	:	Jade Blue 802178
Batch No.	:	BB77-020420
Purity	:	HPLC: 99.7% (area). Byproducts: Starch: 30%; PEG: 1.4%; isopropyl alcohol: 4%; Sodium phosphate: 17.8% ammonia 0.3%; water: 8.4%
Stability	:	/

Positive controls : CPA 40 mg/Kg, 24 Hours
Negative control : Deionized water
Administration : Intraperitoneal injection
Sacrifice time : 24 and 48h
GLP : in compliance

Results
A toxicity study was made by treating 2 males and 2 females i.p with the item substance and following them for 24h.
A dose of 2000 mg/kg resulted toxic after 1h; a second experiment at a dose of 200 mg/kg showed also a 100% mortality of the animals after 1 hour; a third experiment with 20 mg/kg, showed also extreme toxicity. In a fourth experiment 4 animals were treated with 100 mg/kg, which expressed toxic reactions after 2 hours. Two more experiments were made with 50 and 75 mg/kg. It was decided to use doses of 50 mg/kg, 25 mg/kg and 12.5 mg/kg; they were sacrificed 24h and 48h after treatment; for 48h only the maximum concentration was performed.
There was no increase in the number of MN depending on treatment; CPA produced a significant positive effect.
The number of mature erythrocytes has not been evaluated and therefore a correct evaluation of the positive toxic effect could not be made. By considering the percentage of PCE/2000 scored erythrocytes, there are no differences among the control and the treated animals. Therefore, there is no demonstration that the test item has reached the bone marrow cells.

Conclusions
The study is not adequate because there is no demonstration of the presence of the test item in the target cell.

Ref.: 13

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

Not applicable

2.12. Conclusions

The information submitted on the physico-chemical properties of HC Blue 15 is incomplete.

Toxicity
1 mg/kg bw/day of Basic Blue 77 was established as the no-observed-effect-level (NOEL), based upon passive effects at 4 mg/kg bw/day such as faecal discoloration, whereas 4 mg/kg bw/day was considered to be the no-observed-adverse-effect-level (NOAEL), based upon various changes in clinical biochemistry, urinalyses and higher kidney weights at 15 mg/kg bw/day.

The NOAEL for Basic Blue 77 was set at 3 mg/kg bw/day for females and at 30 mg/kg bw/day for foetuses (teratogenicity study).

Irritation and sensitisation
HC Blue No. 15 is considered to be not irritating to rabbit skin.
Pure HC Blue No. 15 is classified as irritant to mucous membranes. However, at a concentration of 2% (in water) no irritation to mucous membranes was observed. The concentration of HC Blue No. 15 in the final product is 0.2 %, therefore no irritating effect to mucous membranes of the final product is expected when applied as intended.
Based of these results, the test substance is not a skin sensitizer under the defined experimental conditions.

Percutaneous absorption
The total percutaneous absorption (receptor fluid, epidermis and upper dermis) was set at 0.798µg/cm² (0.048% of the applied dose).

Mutagenicity/genotoxicity
HC Blue 15, as a sample containing approximately 30-40% of the active ingredient, has been tested in a bacterial reverse mutation test, in a gene mutational mammalian cell *in vitro* test and in the micronucleus test on mice.
The test item has been found very toxic in all the three systems, thus reducing the evaluation potential of the test assays.
In the bacterial reverse mutation assay, the maximum tested concentration (100 µg/plate) did show a mutagenic effect: However, the real tested dose should be considered around 50 µg/plate.
In the *in vitro* mammalian gene mutation system (TK forward mutations) a significant positive effect in the induction of putative chromosomal structural/numerical aberrations was observed in the presence of a metabolic activation system; this effect was observed only in one culture at the maximum tested dose (ca. 20 µg/ml of a.i.).
The experiment in this case was not repeated.
The *in vivo* micronucleus test on mice was performed at the maximum dose of 50 mg/kg in the presence of toxic effects. There was no demonstration that the compound had reached the target cells.

The data presented are not sufficient to make an evaluation of the potential of the test item to induce mutagenic/clastogenic effects.

2.13. References

1. Identity, purity and stability test of Gardex Jade Blue (Basic Blue 77); Wella AG, D-64274 Darmstadt, Germany; Study No.: G 2002/006; September 13, 2002
2. Basic Blue 77: 28-Day oral toxicity (gavage) study in the Wistar rat; RCC Ltd, CH-4452 Itingen, Switzerland; Study number 844693; December 4, 2002
3. Basic Blue 77: 13-Week oral toxicity (gavage) study in Wistar rats; RCC Ltd, CH-4452 Itingen RCC Ltd, Switzerland; Study number 846106; June 30, 2003
4. Basic Blue 77, 802178: Primary skin irritation study in rabbits (4-hour semi-occlusive application); RCC Ltd, CH-4414 Füllinsdorf, Switzerland; Study number 844723; September 17, 2002
5. Basic Blue 77, 802178: Primary eye irritation study in rabbits; RCC Ltd, CH-4414 Füllinsdorf, Switzerland; Study number 844724; January 29, 2003

6. Jade Blue WR 802178 dilution: Primary eye irritation study in rabbits; RCC Ltd, CH-4414 Füllinsdorf, Switzerland RCC Ltd, Switzerland; Study number 846544; April 14, 2003
7. 802178 (Jade Blue) – Local lymph node assay; MDS Pharma Services, F-69210 Saint Germain sur l'Arbresle, France; Study number 762/019
8. Basic Blue 77: Dose range-finding prenatal developmental toxicity study in the Han Wistar rat; RCC Ltd, CH-4452 Itingen, Switzerland; Study number 844582; (June 26, 2003
9. Basic Blue 77: Prenatal developmental toxicity study in the rat; RCC Ltd, CH-4452 Itingen, Switzerland; Study number 846263; June 30, 2003
10. Cutaneous absorption of WR802178 in formulation through pig skin in vitro; Cosmital SA, CH-1723 Marly 1, Switzerland; Study number KP 076; August 20, 2002
11. Assessment of the potential mutagenicity of WR802178 in the Ames reversion assay with Salmonella typhimurium; Cosmital SA, CH-1723 Marly 1, Switzerland; Study number AT 780; September 18, 2002
12. Cell mutation assay at the thymidine kinase locus ($TK^{+/-}$) in mouse lymphoma L5178Y cells with Jade Blue WR 802178; RCC – CCR GmbH, D-64380 Rossdorf, Germany; Study number 749402; November 8, 2002
13. Micronucleus assay in bone marrow cells of the mouse with Jade Blue WR 802178; RCC – CCR GmbH, D-64380 Rossdorf, Germany; Study number 749401; November 8, 2002
14. Stability in formulation; Sept.22, 2003. Wella AG; D-64274 Darmstadt

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 physico-chemical characterisation of the test substances used, including data on stability.

* 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