Scientific Committee on Consumer Products

SCCP

OPINION ON

HC BLUE N°2

COLIPA N° B37

The SCCP adopted this opinion during its 10th plenary of 19 December 2006

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 Products (SCCP), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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

SCCP

Questions concerning the safety of consumer products (non-food products intended for the consumer).

In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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ACKNOWLEDGEMENTS

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Keywords: SCCP, scientific opinion, hair dye, HC Blue n°2, B37, Directive 768/76/EEC, CAS 33229-34-4

Opinion to be cited as:
Scientific Committee on Consumer Products (SCCP), 19 December 2006, Opinion on HC Blue n°2 COLIPA N° B37

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1. BACKGROUND

Submission I for HC Blue n° 2 with the chemical name N1,N4,N4-Tris-(2-hydroxyethyl)-1,4-diamino-2-nitrobenzene was submitted in January 1989 by COLIPA[1,2].

Submission II for HC Blue n° 2 was submitted in February 1991 by COLIPA[2].

The Scientific Committee on Cosmetology (SCC) adopted in its 46th plenary meeting on 19 February 1991 an opinion with the final conclusion that:

"The SCC does not see any possible health risk connected with the use of this dye."

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 47 on the List of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission III for this substance was submitted in July 2005 by COLIPA.
According to this submission HC Blue No. 2 is used in semi-permanent hair formulations at a maximum concentration of 2.8%.

Submission III 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 Products (SCCP) consider HC Blue n° 2 safe for use as a non- oxidative hair dye with a concentration of maximum 2.8 % taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use HC Blue n° 2 in non-oxidative hair dye formulations?*

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° 2

3.1.1.2. Chemical names

2,2´-{[4-(2-hydroxyethyl)amino-3-nitrophenyl]imino}bisethanol (IUPAC)

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

[2] According to records of COLIPA

Ethanol, 2,2´-{[4-(2-hydroxyethyl)amino-3-nitrophenyl]imino}bis-(CAS)
N1,N4,N4 -Tris-(2-Hydroxyethyl)-1,4-diamino-2-nitro-benzene
1-β-hydroxyethylamino-2-nitro-4-bis-(β-hydroxyethyl)aminobenzene

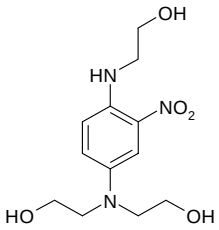
3.1.1.3. Trade names and abbreviations

Jarocol Blue 2
Immexine FAF
COLIPA N° B037

3.1.1.4. CAS / EINECS number

CAS: 33229-34-4
EINECS: 251-410-3

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{12}\ H_{19}N_3O_5$

3.1.2. Physical form

Brown powder
Dark-blue microcrystalline (75% pure) or blackish-blue amorphous powder (98% pure), with a copper cast (IARC)

3.1.3. Molecular weight

Molecular weight: 285

3.1.4. Purity, composition and substance codes

Purity and impurities in HC Blue n° 2

Description	**Batch number**		
	31394	**114B5**	**9233***
Appearance	Brown powder		Blackish blue powder
Identification /Characterisation	NMR, IR, MS, UV_Vis	Elemental analysis, IR NMR, MS, UV_Vis,	NMR, IR, UV_Vis
Titre by potentio-metry[1] (g/100 g)	99.5	98.7	102.9
Melting point	103.4°C	104°C	93-98°C
HPLC purity (% peak area)	98	98	98**
Impurities (g/100g, HPLC/MS) A and C B D	 ND <0.01 ND <0.02 0.08	 ND <0.01 ND <0.02 0.08	Detection of some impurities by HPTLC

Description	Batch number		
	31394	114B5	9233*
E F X Y (% peak area) Z (% peak area)	ND <0.03 0.12 0.12 1.24 0.17	ND <0.03 0.11 0.15 1.12 0.16	
Water content (w/w)	0.5	0.27	1.24
Loss on drying (w/w)	<0.5	<0.5	
Ash content[2]			
Metals		See 3.1.5	
Solvents	See 3.1.5		

* No supportive data, only an unsigned certificate of analysis is provided

** No value reported in the certificate, it is given in the table where different batches were compared

[1] Neutralisation of amine function by perchloric acid in an acetic acid medium

[2] Ash content is reported as <0.5% under Raw Material Presentation

Impurities:

A: 2-{[(4-Amino)2-nitrophenyl]-amino}ethanol (HC Red n° 3)
B: 4-[(2-Hydroxyethyl)amino]-2-nitroaniline (HC Red n° 7)
C: 4-[Di-(2-Hydroxyethyl)amino]-2-nitroaniline (HC Red n° 13)
D: N,N-dihydroxyethyl-4-fluoro-3-nitroaniline
E: 4-Fluoro-3-nitroaniline
F: 2-[4-(2-hydroxyethylamino)-2-nitrophenylamino]ethanol
X: 2-[{4-(2-[2-hydroxyethoxy]-ethylamino)-3-nitrophenyl}-[2-hydroxyethyl]-amino]ethanol
Y: 2[[2-{4-[Bis(2-hydroxyethyl)amino]-2-nitrophenylamino}ethoxy)-3-nitrophenyl-(2-hydroxyethyl)amino]ethanol
Z: 2-[{4-[(4-Amino-2-nitrophenyl)-(2-hydroxyethyl)amino]-3-nitrophenyl}-(2-hydroxyethyl)-amino]ethanol

X, Y and Z are proposed structures based on LC-MS analyses

3.1.5. Impurities / accompanying contaminants

See 3.1.4

Metal content in the Batch 114B5:

Fe: 35 ppm
Al: 2 ppm
Cr: 1ppm
Ag, As, Ba, Bi, Cd, Co, Cu, Mn, Mo, Ni, Pb, Pd, Pt, Sb, Se, Sn, Ti, V, Zn: each <1ppm
Hg: < 0.1 ppm

Residual solvents in Batches 31394 and 114B5:
No residual solvents such as methanol, ethanol, isopropanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone or monochlorobenzene was detected, detection limit 100 ppm for each

3.1.6. Solubility

Water: 4.57 ± 0.14 g/l at 20 ± 0.5 °C
Ethanol: < 1 g/100 ml
DMSO: ≥ 20 g/100ml

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow}: 0.72 at 23 ± 2°C, pH 7.45

3.1.8.	Additional physical and chemical specifications

Melting point:	103°C -104°C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis maxima:	263.6 nm and 535.0 nm

3.1.9.	Stability and Homogeneity

1 mg/ml and 200 mg/ml solutions of HC Blue n° 2 in 0.5% carboxymethylcellulose (CMC), at room temperature up to 6 hours and up to 9 days at 4°C, were stable (maximum deviation from original concentration = 6%) when stored protected from light and under inert gas atmosphere.

0.1 mg/ml and 500 mg/ml solutions of HC Blue n° 2 in DMSO were stable at room temperature up to 4 hours study period (maximum deviation from original concentration = 4%) when stored protected from light and under inert gas atmosphere.
10 mg/ml and 100 mg/ml solutions of HC Blue n° 2 in DMF were stable at room temperature up to 4 hours study period (maximum deviation from original concentration = 6%) when stored protected from light and under inert gas atmosphere.

The solutions of HC Blue n° 2 in CMC were found to be homogeneous during the 9 days storage period (CV maximum 5%), when stored at 4°C, protected from light and under inert gas atmosphere

General comments to physico-chemical characterisation

- No supportive data is submitted for the identification/characterisation and properties of HC Blue n° 2, batch 9233. Only an unsigned certificate of analysis is presented.
- Chemical structure of HC Blue n° 2 is comprised of both a secondary and a tertiary amino group. It is therefore prone to nitrosation. Nitrosamine content in HC Blue n° 2 is not reported.
- Stability of HC Blue n° 2 in marketed products is not reported.

3.2. Function and uses

HC Blue n° 2 is used in semi-permanent hair dye formulations at a maximum concentration of 2.8%

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 420
Species/strain:	rats, Sprague-Dawley Rj: SD (IOPS Han)
Test substance:	HC Blue N°2 (B037)
Batch:	3I394
Purity:	99.5%
Vehicle:	0.5% suspension of carboxymethylcellulose in purified water
GLP:	in compliance

The test item was administered by oral route (gavage) to one group of five fasted females, under a volume of 10 ml/kg. The test item was administered at the dose-level of 2000 mg/kg (sighting test) in one female and in the main test was administered at the same dose-level to one group of four additional females.

Results
Sighting test
No mortality occurred. Only hypoactivity, dyspnea and piloerection were observed within 4 hours of treatment. No other clinical signs were noted in this animal.

Main experiment
No mortality was recorded. Hypoactivity, piloerection and dyspnea were observed in all females within 4 hours of treatment. Blue spots were noted on the tail and/or on the fur of all females from day 1 up to day 8.
The overall body weight gain of the animals was not affected by treatment with the test item. At necropsy, no apparent abnormalities were observed.

Conclusion
Under the experimental conditions, the maximal non-lethal dose of the test item was 2000 mg/kg by oral route in rats.

Ref.: 1

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404
Species:	New Zealand White rabbits
Group:	3 males
Substance:	HC Blue n° 2
Batch:	114B5
Purity:	98.7%

Dose: 0.5 ml of 3% HC Blue n° 2 in a 0.5% suspension of carboxymethylcellulose in purified water
GLP: in compliance

0.5 ml 3% HC Blue n° 2 was applied for periods of 3 minutes, 1 hour and 4 hours to a single male New Zealand White rabbit. The substance 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 and then daily until the end of the observation period.
Since persistent purple coloration of the skin was noted in this animal, the study was then extended to include two additional animals by applying the test substance for 4 hours to the clipped flank. Observations for any cutaneous reaction were made at approximately 1 hour, 24, 48 and 72 hours after removal of the dressing.
Because of the discoloration, the animals were killed and skin samples were taken from the flanks of the animals (treated and untreated sites) for histological examination.

Results
The purplish discoloration may have masked any erythema. No associated cutaneous reactions (dryness of the skin, crusts or oedema) were observed.
The microscopic examination of the last two treated animals did not show any changes which could be attributable to the treatment with the test item.

Conclusion
Under the conditions of the study, 3% HC Blue n° 2 was not irritant to rabbit skin.

Ref.: 2

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405
Species: New Zealand White rabbits
Group: 3 males
Substance: HC Blue n° 2
Batch: 114B5
Purity: 98.7%
Dose: 0.1 ml of 3% HC Blue n° 2 in a 0.5% suspension of carboxymethylcellulose in purified water
GLP: in compliance

0.1 ml of 3% HC Blue n° 2 in a 0.5% suspension of carboxymethylcellulose in purified water was instilled into the conjunctival sac of the left eye 3 New Zealand White male rabbits. The right eyes served as controls. Observations were made at 1, 24, 48 and 72 hours and then daily until day 7.

Results
Chemosis was observed in 2 animals and slight conjunctival redness in 1 animal at 24 hours. Loss of hair beneath the eye of 1 animal was present from day 2 to day 7.

Conclusion
Under the conditions of the experiment, 3% HC Blue n° 2 caused transient irritation to the eye of rabbits.

Ref.: 3

3.3.3. Skin sensitisation

Local Lymph Node Assay

Guideline: OECD 429

Species: female mice, CBA/J
Group: 7 groups of 4 mice (28 animals) for each of 2 experiments
Substance: HC Blue n° 2
Batch: 114B5
Purity: 98.7%
Dose: 25 µl of 0.5, 1, 2.5, 5 and 10% in dimethylformamide
GLP: in compliance

In two independent experiments, 25 µl HC Blue n° 2 in dimethylformamide was applied to the dorsal surface of both ears at the following concentrations: 0.5%, 1%, 2.5%, 5% and 10%. A positive control group received 25% alpha-hexylcinnamaldehyde (HCA) in dimethylformamide and the negative control the vehicle alone.

In each experiment, during the induction phase, the test item, vehicle or positive control 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).

Treatment	Concentration (%)	Signs of local irritation	Stimulation Index (SI)
B37	0.5	no	1.37
B37	1	no	1.03
B37	2.5	no	1.30
B37	5	no	3.92
B37	10	no	1.55
HCA	25	-	9.97

In the first experiment, an increase in stimulation index was observed at 5% only.

Treatment	Concentration (%)	Signs of local irritation	Stimulation Index (SI)
B37	0.5	no	1.60
B37	1	no	0.99
B37	2.5	no	1.35
B37	5	no	1.50
B37	10	no	2.01
HCA	25	-	5.70

In the second experiment, a dose-related increase in the stimulation index was noted at concentrations between 1 and 10%, but the threshold positive value of 3 was not reached at any of the tested concentrations

Conclusion
While the data are equivocal, HC Blue n° 2 is considered to have a skin sensitising potential in mice.

Ref.: 4

3.3.4. Dermal / percutaneous absorption

Guideline: OECD draft 428
Species: Human
Group: 4 females, abdominal skin, 2 from each
Substance: HC Blue n° 2
Batch: 114B5
Purity: 98.7%
Radiolabelled: [ring-U-14C]- HC Blue n° 2
Batch: CFQ13908
Purity: 98.1%

Dose: 20 mg/cm2 of a formulation containing 2.8% HC Blue n°2
GLP: in compliance

The in vitro percutaneous absorption of HC Blue n° 2 from a typical semi-permanent hair dye formulation was determined in human dermatomed skin mounted in 9 mm flow-through diffusion cells using phosphate-buffered saline containing 0.01% sodium azide (w/v) as the receptor fluid.

The integrity of 20 skin samples was assessed by the tritiated water permeability coefficient. From these, 8 skin samples (2 from each of the 4 donors) were chosen which had a Kp of < 2.5 x 10-3 cm.h-1.

Twenty mg/cm2 of a formulation containing 2.8% HC Blue n° 2 was applied on the skin surface. After 30 minutes of exposure, the hair dye remaining on the skin surface was removed by washing. Twenty-four hours after application, skin wash, stratum corneum (isolated by tape stripping), skin samples and receptor fluid were analysed by liquid scintillation counting to assess the cutaneous distribution of the test substance.

Most of the hair dye remaining on the skin after the application period was removed in the washing procedure.

	µgeq/cm²									
Replicate no. **Donor no.**	**A-1** **3**	**A-2** **1**	**A-3** **2**	**A-4** **4**	**B-1** **1**	**B-2** **3**	**B-3** **4**	**B-4** **2**	**Mean**	**SD**
Skin wash	590.9	559.8	614.7	551.3	535.9	628.0	586.9	612.3	585.0	33.2
Cotton swabs	0.072	0.061	0.044	0.021	0.018	0.022	0.032	0.047	0.040	0.020
Donor Compartment	0.013	0.011	0.035	0.007	0.004	0.003	0.003	0.009	0.011	0.011
Dislodgeable dose [1]	590.9	559.8	614.7	551.3	535.9	628.1	586.9	612.4	585.0	33.2
Tape strips	0.032	0.105	0.030	0.031	0.031	0.021	0.028[4]	0.034[5]	0.039	0.027
Unabsorbed dose [2]	590.9	559.9	614.7	551.3	536.0	628.1	586.9	612.3	585.0	33.1
Skin	0.012	0.034	0.012	0.013	0.013	0.009	0.012	0.010	0.014	0.008
Receptor fluid + Receptor wash	0.002	0.003	0.003	0.002	0.011	0.002	0.010	0.003	0.005	0.004
Total recovery	591.0	560.0	614.8	551.4	536.0	628.1	587.0	612.4	585.1	33.1
Total absorption [3]	0.014	0.037	0.015	0.015	0.024	0.011	0.022	0.013	0.019	0.012

1 amount in skin wash, cotton swabs and donor compartment wash
2 amount in dislodgeable dose and tape strips
3 amount in receptor fluid, the receptor compartment wash and the skin (excluding tape strips)
4 the tape stripping procedure was stopped after 8 tape strips because the epidermis was disputed.
The tape strips containing (pieces of) the epidermis was pooled with the skin membrane. Stopping of the tape stripping procedure can cause a slight overestimation of the total absorption because not all of the stratum corneum may have been removed from the skin membrane
5 the tape stripping procedure was stopped after 9 tape strips because the epidermis was disputed.
The tape strips containing (pieces of) the epidermis was pooled with the skin membrane. Stopping of the tape stripping procedure can cause a slight overestimation of the total absorption because not all of the stratum corneum may have been removed from the skin membrane.

Virtually no penetration of the compound into the receptor fluid was observed (0.005 µgeq/cm² ± 0.004 or 0.001% of the applied dose). After 24 hours, 0.039 µgeq/cm² ± 0.027 (0.007%) of HC Blue n° 2 was retained in the stratum corneum and 0.014 µgeq/cm² ± 0.008 (0.003%) was found in the skin (living epidermis + dermis).

The mean total absorption (defined as the radioactivity present in the receptor fluid and the skin [excluding the stratum corneum]) or dermal delivery was 0.019 ± 0.012 µgeq/cm².

The maximum total absorption observed was 0.037 µgeq/cm² which should be used for calculating the MOS.

Ref.: 13

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

Guideline:	OECD 408 (1998)
Species/strain:	Rat, Sprague Dawley Crl CD (SD) IGS BR
Group size:	16 animals per sex at control and high dose (each 6 for recovery) 10 animals per sex at low and mid dose. Plus for all dose groups satellite groups of 6 animals/sex/dose
Test substance:	HC Blue n°2 (B037)
Batch number:	114B5
Purity:	98.7%
Vehicle:	0.5% suspension of carboxymethylcellulose in purified water
Dose levels:	0, 100, 300 and 1000 mg/kg/day
Route:	Oral (gavage)
Exposure period:	13 weeks
Recovery period:	4 weeks
GLP:	in compliance

A total of 140 Sprague-Dawley rats (70 males and 70 females) were allocated to three treated groups and one control group. Each group was composed of 10 males and 10 females. Recovery animals (six males and six females) were added to the control and high-dose groups for a 4-week treatment-free period. Additional satellite animals were allocated to the treated groups for toxicokinetic investigations.
The test item was administered daily by gavage as a suspension in the vehicle (0.5% carboxymethylcellulose), at the dose-level of 100, 300 or 1000 mg/kg/day and a constant volume of 5 ml/kg, for 13 weeks. Control animals received the vehicle alone under the same experimental conditions.
The animals were checked daily for mortality and clinical signs. Detailed clinical observations were carried out, weekly, on principal animals and during the treatment-free period. A Functional Observation Battery (including motor activity) was conducted at the end of the treatment period. Body weight and food consumption were recorded once a week during the study.
Ophthalmological examinations were performed before (all groups) and at the end (groups 1 and 4) of the treatment period.
Haematological and blood biochemical investigations as well as urinalysis were performed at the end of the treatment period.
Blood samples, for the determination of plasma levels of the test item, were taken in week 1 and in week 13 at designated time-points: 0.5, 1, 2, 4, 8 and 24 hours post-dosing.
On completion of the treatment or treatment-free periods, the animals were sacrificed and submitted to a full macroscopic *post-mortem* examination. Designated organs were weighed and specified tissues preserved. A microscopic examination was performed on selected tissues from animals of the control and high-dose groups and on macroscopic lesions from all the animals killed on completion of the treatment period.

Results
No unscheduled deaths occurred during the study.

All treated animals produced purple colour of urine, coat, tail and urogenital area with a dose-related incidence. Ptyalism was seen in all treated animals. There were no effects on Functional Observation Battery and motor activity observations
No treatment-related effects were noted on body weight, food consumption, ophthalmology, haematology and blood biochemistry in both sexes.
Due to the urine coloration, the only urinary parameter that could be evaluated was the urine volume which was similar in all treated groups compared to controls.
HC Blue No. 2 (B037) was well absorbed following oral administration.
In groups 2, 3 and 4 and in weeks 1 and 13, plasma levels of the test item were quantifiable at the first time-point, and reached maximum levels between 0.5 and 4 hours post-dosing. The test item was no longer detectable after 24 hours post-dosing at any dose-level. The systemic exposure (as measured by AUC0-t) increased with dose-levels in a dose-proportional manner.
No significant changes in organ weight were observed at 100 and 300 mg/kg/day. Slightly higher absolute and/or relative liver and kidney weights were noted in males and females given 1000 mg/kg/day at the end of the dosing period and were still recorded in females at the end of the recovery period. The increase in liver and kidney weight was not accompanied by histopathological or blood biochemical changes/effects.
At the end of both treatment and treatment-free periods, purple colorations of the hair and extremities and some intestinal parts were observed at macroscopy. At microscopy no treatment-related findings were observed at any dose-level.
Under the experimental conditions of the study, the No Observed Adverse Effect Level (NOAEL) of HC Blue No. 2 (B037) was 300 mg/kg/day.

Ref.: 5

Comment
Ptyalism is considered an adverse effect for the following reasons:

a) it was observed in all animals in all treated groups;
b) it is seen early (from week 1) in the highest dose group and later (week 2 or 4) in the lower dose groups
c) it is not clear when the observations were performed (and therefore it cannot be assessed whether the effect is directly associated with dosing or is a systemic effect). Therefore the NOAEL in this study cannot be determined. The LOAEL is 100 mg/kg bw/day

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6. Mutagenicity/Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium*, TA 1535, TA 1537, TA 98, TA 100 and TA 102
Replicates:	Three plates per concentration in two independent experiments both in the presence and absence of Aroclor 1254 induced rat liver S9.
Assay conditions:	Direct plate incorporation method, apart from the second test with S9 mix, which was performed according to the pre-incubation method (1 hour at 37°C).
Test substance:	HC Blue n° 2
Batch:	114B5
Purity:	98.7%

Concentrations:	First experiment: Five concentrations from 1.6 to 5000 µg/plate in the presence or absence of S9 mix. Second experiment: Five concentrations from 156.25 to 5000 µg/plate in the presence or absence of S9 mix.
Solvent:	DMSO
GLP:	in compliance

HC Blue n° 2 was tested in a preliminary toxicity test (TA 100) and in two independent experiments in the absence and presence of metabolic activation (S9 mix prepared from the livers of rats given Aroclor 1254). The experiments were conducted according to the direct plating incorporation method, apart from the second test with S9 mix, which was performed according to the pre-incubation method with a pre-incubation period of 60 minutes at 37°C. Since HC Blue was freely soluble and non-toxic, it was tested up to the maximum recommended concentration, 5000 µg/plate, in experiments 1 and 2, both in the absence and presence of S9 mix. Negative (solvent) and positive controls were included in all experiments in accordance with OECD guidelines.

Results
No evidence of toxicity was observed in any of the tester strains in any of the experiments either in the absence or presence of S9 mix. HC Blue N°2 induced statistically significant, concentration-related (approximately 2-fold) increases in revertant numbers in strain TA 98 at the highest dose level tested in the absence of S9 mix in both experiments. No other compound-related increases in revertant numbers were noted.
All solvent controls were within the historical data range, and all positive controls used gave a distinct increase of induced revertant colonies.

Conclusion
Under the experimental conditions used in this study, HC Blue n° 2 was considered to be genotoxic (mutagenic) in the *Salmonella typhimurium* strain TA98 in the absence of metabolic activation.

Ref.: 6

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA 1535, TA100, TA 98, TA 97
Replicates:	Three plates per concentration in two independent experiments (only data from one experiment was shown) both in the presence and absence of Aroclor 1254 induced rat and Syrian hamster liver S9.
Assay conditions:	Pre incubation assay (20 minutes at 37°C)
Test substance:	HC Blue n° 2
Batch:	lot. No. 9233
Purity:	98%
Concentrations:	333, 1000, 3.333, 6.666 and 10.000 µg/plate
Solvent:	DMSO
GLP:	/

No preliminary toxicity assay was apparently performed, but no reduction in the revertants per plate was observed at the highest concentration tested, indicating that HC Blue n°2 was not toxic up to 10.000 µg/plate. The two highest concentration tested were above the maximum recommended concentration of 5000 µg/plate, both in the absence and presence of S9 mix. The test substance was dissolved in DMSO. Negative (solvent) control was included with all strains. No positive controls were included as required in the OECD guidelines.

Results

HC Blue N° 2 was not mutagenic in the base pair substitution strains TA100 and TA1535. A week concentration related effect was seen in TA98 without S9. With S9 a positive effect was seen at the highest tested concentration. A very week concentration related effect was seen in TA97 both without (1.6 fold compared to control at 10.000 µg/plate) and with S9 (1.4 fold compared to control at 10.000 µg/plate). No statistical analysis was performed.

Conclusion
It was concluded by the National Toxicology Programme (NTP) that HC Blue n° 2 was mutagenic in TA98 and TA97 but not in TA100 and TA1535

Remarks
This study was only shortly referred in ref 11 and not all relevant data were included. It was not mentioned whether a toxicity study was performed. Summary data was shown for one experiment only, although it was claimed that two tests were performed with similar results. Therefore it is not clear whether the positive results were reproducible. Only the positive effect in TA98 without S9 seems to have biological relevance. The positive effect in TA98 with S9 was only observed at a very high concentration level and might be due to toxicity. It is not known whether the very week effect observed in TA97 was reproducible.
Since the reporting of the test is limited and have deficiencies compared to the minimal current requirements for a genotoxicity test, this study has only supportive value.

Ref.: 11

Mammalian Cell Gene Mutation Test in Mouse Lymphoma Cells (*hprt* locus)

Guideline:	OECD 476
Species/strain:	Mouse lymphoma cell line L5178Y (*hprt* locus for 6-thioguanine (6-TG) resistance)
Replicates:	Duplicate cultures in two independent experiments
Metabolic act.:	Aroclor 1254 induced rat liver S9
Test substance:	HC Blue n° 2
Batch:	114B5
Purity:	98.7%
Concentrations:	1. experiment: 285, 275, 570, 1140, 1710, 2280 and 2850 µg/ml 2. experiment: 285, 275, 570, 1140, 1710, 2280 and 2850 µg/ml
Treatment:	Pulse (3h) treatment both in the absence and presence of S9 and 7 days expression period. Viability and mutant frequency was scored 7 and 11-12 days after the expression period, respectively.
Solvent:	DMSO
GLP:	in compliance

HC Blue n° 2 was evaluated for its ability to induce point mutations in the *hprt* locus in the mouse lymphoma cell line L5178Y. Two independent experiments using duplicate cultures each (single cultures for positive controls) was performed. Both experiments used a pulse (3-hour) treatment and were conducted in the absence and presence of metabolic activation (S9 mix prepared from the liver of rats given Aroclor 1254). According to the results in the pre-test HC Blue n° 2 was freely soluble and non-toxic, therefore the top concentration used in each experiment and test condition was 2850 µg/ml (equivalent to 10 mM). Six different concentrations were evaluated. Cells were treated for 3 hours followed by an expression period of 7 days to fix the DNA damage to stable *hprt*-mutations. At the end of the expression period, acceptable cultures were then plated for viability (2 plates per culture, 7 days) or 6-TG resistance (4 plates per culture, 11-12 days). Benzo[a]pyrene (BP), in the presence of S9 mix and 4-nitroquinoline 1-oxide, NQO, in the absence of S9 mix were used as positive controls. Negative controls consisted of cultures treated with the solvent alone (DMSO).

Results

In the range finding toxicity test no marked toxicity was observed at any of the concentrations tested: at the highest concentration tested (2860 µg/ml, corresponding to 10 mM) the relative survival was 64% and 66% in the absence and presence of S9-mix, respectively.
When tested up to 10 mM, there were no increases in mutant frequency either in the presence or absence of S9 mix, in both experiments. A slight linear trend was observed in the presence of S9 in Experiment 2. However, as there were no statistically significant increases in mutant frequency for any concentration tested, and as all mutant frequencies were similar to the laboratory historical negative control mean value, this isolated linear trend was considered to have no biological relevance.
Mutation frequencies in solvent negative controls remained within normal ranges, and treatment with positive controls NQO and BP yielded distinct increases in mutant frequency.

Conclusion
Under the conditions of this study, HC Blue n° 2 was considered not to be genotoxic (mutagenic) in the mouse lymphoma assay (*hprt* locus) either in the absence or presence of metabolic activation.

Ref.: 7

Mammalian Cell Gene Mutation Test in Mouse Lymphoma Cells (*tk* locus)

Guideline:	/
Species/strain:	Mouse lymphoma cell line L5178Y (*tk* locus for trifluorothymidine resistance)
Replicates:	One experiment and one culture, 3 plates per concentration
Metabolic act.:	Aroclor induced male rat S9
Test substance:	HC Blue n° 2
Batch:	lot no. 9233
Purity:	98%
Concentrations:	75, 150, 200, 300, 400 and 600 µg/ml
Treatment:	Pulse (4h) treatment only in the presence of S9 and 2 days expression period.
Solvent:	DMSO
GLP:	/

No preliminary toxicity study was reported. Toxicity was measured in the main study as Relative Total Growth (RTG). HC Blue n° 2 was evaluated for its ability to induce point mutations/chromosomal aberration in the *tk* locus in the mouse lymphoma cell line L5178Y, but only in the presence of S9 mix. Six different concentrations were evaluated. Cells were treated for 4 hours followed by an expression period of 2 days to fix the DNA damage to stable *tk*-mutations. Three (3) plates were evaluated per concentration. No positive control in the presence of S9 mix was included; methyl methane sulfonate (MMS) was used as positive control in the absence of S9 mix. Negative controls consisted of cultures treated with the solvent (DMSO) alone.

Results
Severe toxic effect was observed at 300 µg/ml and above (RTG less than 10% survival). There was a concentration related increase in mutation frequency. However, the spontaneous mutation frequency was very low (19 mutants/10^6 clonable cells) and lower than the recommended spontaneous mutation frequency. In addition the toxicity of the 3 highest evaluated exceeds the recommended 20% survival.

Conclusion
HC Blue n° 2 was evaluated by NTP to be genotoxic (mutagenic and/or clastogenic) in the mouse lymphoma $TK^{+/-}$ assay in the presence of S9 mix.

Remarks
It was not possible to distinguish between the induction of point mutations and chromosomal aberrations because the number of small versus large colonies was not evaluated either for positive and negative controls or for any of the tested concentrations.
This study was only shortly referred in ref 11, not according to OECD guideline and not all relevant data were included. Therefore this study has only supportive value.

Ref.: 11

***In vitro* chromosome aberration test**

Guideline:	OECD 473
Cells:	Human lymphocytes from three healthy, non-smoking female donors.
Replicates:	Duplicate cultures in two independent experiments
Metabolic act.:	Aroclor 1254 rat liver S9
Test substance:	HC Blue n° 2
Solvent:	DMSO
Batch:	114B5
Purity:	98.7%
Concentrations:	1. experiment: 1467, 2293, 2866 µg/ml (with and without S9 mix) 2. experiment without S9: 64.18, 125.3 156.7 and 195.9 µg/ml (limit of cytotoxicity) 2. experiment with S9: 1824, 2280 and 2850 µg/ml (10 mM)
Treatment:	48 h mitogen stimulation (PHA) before treatment (both experiments) 1. experiment: 3 h exposure and 20 h harvest time (both with and without S9) 2. experiment without S9: 20 h exposure and harvest time 2. experiment with S9: 3 h exposure and 20 h harvest time
GLP:	in compliance

HC Blue N°2 was assessed for clastogenic potential in human lymphocytes. Two independent experiments were performed, with or without the metabolic activation (Aroclor rat liver S9).
For Experiment 1, duplicate cell cultures were exposed to the test substance for 3 hours in the absence and presence of S9 mix. The cultures were incubated for an additional 17 hours and harvested. For Experiment 2, cultures were exposed to the test substance for 20 hours in the absence of S9. Treatment in the presence of S9 was as described for Experiment 1. Two hours before harvest, colchicine was added to block cells in the metaphase stage of mitosis.
Concentrations selected for analysis were based on toxicity measured as reduction in mitotic index (mitotic inhibition) or highest recommended concentration (10 mM).
The chromosomal preparations were stained and evaluated microscopically for mitotic index and aberrations. Two hundred (200) well-spread metaphases per dose were read whenever possible. 4-Nitroquinoline 1-oxide (without S9) and cyclophosphamide (with S9) were used as positive controls.

Results
In the first experiment the highest tested concentration (2866 µg/ml) induced approximately 35% and 34% mitotic inhibition in absence and presence of metabolic activation respectively. In the second experiment the highest concentrations chosen for analysis, 195.9 µg/ml in absence of S9 and 2850 µg/ml in presence of S9 induced 56% and 27% mitotic inhibition respectively.
In both experiments both with and without S9 there was no concentration related increase in cells with structural or numerical chromosomal aberrations and the frequency of aberrant cells were within the historical laboratory negative control data. The positive controls yielded significant increases in chromosome aberrations in the treated cells.

Conclusion
Under the experimental conditions used HC Blue n° 2 was not genotoxic (clastogenic) in human lymphocytes *in vitro*.

Ref.: 8

In Vitro Micronucleus Test in cultured Human Lymphocytes

Guideline:	OECD draft guideline 487 (2004)
Species/strain:	Human lymphocytes from two healthy, non-smoking female donors
Metabolic act.:	Aroclor 1254 induced rat liver S9.
Test substance:	HC Blue n° 2
Batch:	114B5
Purity:	98.7%
Concentrations:	Experiment 1: 382.5, 747.1, 1167 µg/ml (–S9); 1824, 2280, 2850 µg/ml (+S9) Experiment 2: 405.4, 561.1, 913.6 µg/ml (–S9); 2059, 2423, 2850 µg/ml (+S9)
Treatment:	Experiment 1: 24 hours mitogen (PHA) stimulation before treatment Experiment 2: 48 hours mitogen (PHA) stimulation before treatment Both experiments: With S9: 3 h treatment followed by 45 h recovery period Without S9: 20 h treatment follower by 28 h recovery period The last 28 hours of incubation in the presence of cytochalasin B
Solvent:	DMSO
GLP:	In compliance

HC Blue N°2 was evaluated for its ability to induce micronuclei (clastogenic and aneugenic potential) using duplicate cultures of human lymphocytes in two independent experiments in the absence and presence of metabolic activation. The highest concentration in each experiment and test condition was either 2850 µg/ml (equivalent to 10 mM) or was selected on the basis of cytotoxicity criteria (reduction in replication index, RI).
In experiment 1, cultures were incubated in the presence of the mitogen phytohaemagglutinin (PHA) for 24 hours and then received a 20 or 3-hour treatment in the absence or presence of S9 mix, respectively. Cells were harvested 72 hours after the beginning of incubation (the last 28 hours of incubation being in the presence of cytochalasin B). In experiment 2, a similar test procedure was used except that cultures were incubated in the presence of PHA for 48 hours prior to treatment (harvesting took place 96 hours after the beginning of incubation).
Lymphocytes were then harvested, fixed and placed on microscope slides for evaluation. Slides were examined for proportions of mononucleate, binucleate and multinucleate cells and the replication index (RI) calculated based on the analysis of 500 cells per replicate (1000 per dose) to determine the doses to be analysed for micronuclei.
One thousand binucleate cells from each culture selected (2000 per dose level) were analysed for micronuclei.
4-nitroquinoline-1-oxide, NQO, and vinblastine, VIN were used as positive control clastogene and aneugene respectively without S9 and cyclophosphamide, CPA, as clastogen positive control in the presence of S9. Solvent-treated cultures (DMSO, four replicates) were used as negative controls.

Results
In Experiment 1 (24-hour PHA stimulation prior to treatment), no compound-related increase in micronucleus formation was observed, either in the presence or absence of metabolic activation.

In Experiment 2 (48-hour PHA stimulation prior to treatment), a dose-related increase in micronucleus formation was observed in the absence of metabolic activation at all concentrations tested. A small, statistically significant increase observed in cultures treated at 2850 µg/ml in the presence of metabolic activation was within the historical control range and was not determined to be biologically relevantt. Treatment of cultures with positive controls NQO, CPA and VIN resulted in consistent significant increases in MNBN frequencies, compared to concurrent solvent controls. The negative control values were low, but within the historical negative control range.

Conclusion
Under the conditions of the study, HC Blue n° 2 was considered to be genotoxic (clastogenic and/or aneugenic) in cultured human lymphocytes in the absence of metabolic activation
Ref.: 9, 11

HC Blue No. 2 was mutagenic for strains TA97 and TA98 but not for strains TA100 or TA1535 of *Salmonella typhimurium* in the presence or absence of Aroclor 1254induced male Sprague-Dawley rat or Syrian hamster liver S9. HC Blue No. 2 was mutagenic in the mouse lymphoma L5178YPTK"-assay in the presence *of* Aroclor 1254-induced male F344 rat liver S9.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Rat bone marrow micronucleus test

Guideline:	OECD 474 (1997)
Species/strain:	Sprague-Dawley (SD) rats
Group size:	Five males and 5 females for the positive control and 500, 1000 mg/kg groups. Ten males and 10 females for the control and 2000 mg/kg groups
Test substance:	HC Blue n° 2
Batch:	114B5
Purity:	98.7%
Dose level:	0, 500, 1000 or 2000 mg/kg bw administered as single dose
Route:	Oral gavage
Vehicle:	0.5% aqueous carboxymethylcellulose
Sacrifice times:	24 hours after dosing. Additional negative control (0.5% aqueous carboxymethylcellulose) and high dose (2000 mg/kg) groups (5 rats/sex) were killed 48 hours after dosing.
GLP:	In compliance

The clastogenic/aneugenic potential of HC Blue n° 2 (batch no. 114B5; 98.7% pure) in 0.5% aqueous carboxymethylcellulose was tested in this study. A range-finding study was performed on groups of 3 male and 3 female rats to determine dose levels for the main study.

In the main study, a single dose of the vehicle, 500, 1000 or 2000 mg/kg HC Blue n° 2 was administered by oral gavage. The positive control compound (cyclophosphamide) was administered in a single oral dose of 60 mg/kg. Five (5) male and 5 female rats were used for the positive control, 500 and 1000 mg/kg groups; 10 males and 10 females were used for the vehicle control and the 2000 mg/kg groups.
The animals in the positive control, 500 and 1000 mg/kg groups were killed 24 hours after treatment. Five (5) males and 5 females were killed 24 hours after administration of the vehicle or 2000 mg/kg HC Blue n° 2; the remaining 5 males and 5 females were killed 48 hours after treatment. For each animal, bone marrow smears were prepared and the micronucleated polychromatic erythrocytes were counted in 2000 polychromatic erythrocytes. The polychromatic (PCE) / normochromatic (NCE) erythrocyte ratio was established by scoring a total of 500 erythrocytes per animal.

Results
No mortality occurred during the study. All animals in all dose groups showed purple urine and purple staining of one or more areas of the body on day 1 and day 2. This finding was not considered to be a sign of toxicity, but was attributed to the colour of the test compound. The maximum tolerated dose of 2000 mg/kg was achieved.

Statistically significant increases in micronucleus frequencies were observed in male rats at 500 and 2000 mg/kg bw. The increases were found to be within the laboratory historical control range and the MN frequency in the vehicle control males was quite low. Therefore, the increased MN frequency was not considered to be biologically relevant.
No statistically significant differences were observed in PCE / NCE ratio at any dose level. However, plasma analysis showed mean plasma levels of 18.9 µg/ml and 10.7 µg/ml, 1 hour and 4 hours after oral administration at 2000 mg/kg, respectively, and confirmed the systemic exposure of the test animals to the compound.
Positive controls yielded significant increases in micronucleus frequencies in PCEs.

Ref.: 10

Conclusion
HC Blue n° 2 did not induce biologically relevant micronucleus formation in the bone marrow cells of Sprague-Dawley rats treated orally up to the maximum recommended dose of 2000 mg/kg.

3.3.7. Carcinogenicity

Oral administration, mice

Guideline:	/
Species/strain:	B6C3F1 mice
Group size:	50 Animals per sex and dose
Test substance:	HC Blue n° 2
Batch:	Southland Corp. (Dallas, Texas), lot 9233
Purity:	98 %
Dose:	Males: 0, 5000, and 10000 ppm in the diet; Females: 0, 10000, and 20000 ppm in the diet
Route:	Oral
Exposure:	104 weeks
GLP:	in compliance

A 2-year oral dietary feeding carcinogenicity bioassay was conducted on HC Blue n° 2 using treatment and control groups of 50 male and 50 female B6C3F1 mice (7 weeks old). The low and high dietary concentrations of HC Blue n° 2 were for 5000 and 10000 ppm (equivalent to approximately 1320 and 2240 mg/kg/day) for males and 10000 and 20000 ppm (equivalent to approximately 2330 and 5600 mg/kg/day) for females. HC Blue n° 2 were administered for 104 weeks. The treatment period was followed by a treatment free observation period of about 2 weeks. Animals were observed for signs of toxicity and tissue masses or lesions throughout the study. Necropsy and histopathology were conducted on all animals.
The survival of high dose male mice was better than that for controls. The survival of high dose female mice was reduced ($P<0.05$) relative to that of the controls (control, 35/50; low dose, 27/50; high dose 19/50); this reduced survival was attributed to a reproductive tract infection. Final mean body weights for dosed male mice were within 5% of control values, but final mean body weights for dosed females were 15% (low dose) and 22% (high dose) lower than that of controls.
A marginal ($P = 0.05$) positive trend occurred in the incidence of lymphomas in male mice (1/50; 5/48; 8/49); the incidences in the dosed groups were not significantly greater than that in the controls when survival differences were taken into account. The incidence of

hyperostosis of the skull was detected in 1/49 high dose male and 4/50 high dose female mice.

US National Toxicology Program concluded under the conditions of these studies, there was no evidence of carcinogenicity in male and female B6C3F1 mice receiving HC Blue No. 2 in the diet at concentrations of 0.5% and 1.0% for males and 1.0% and 2.0% for females for 2 years.

Ref.: 11

Oral administration, rats

Guideline:	/
Species/strain:	F344 rats
Group size:	50 Animals per sex and dose
Test substance:	HC Blue n° 2
Batch:	Southland Corp. (Dallas, Texas), lot 9233
Purity:	98 %
Dose:	Males: 0, 5000, and 10000 ppm in the diet; Females: 0, 10000, and 20000 ppm in the diet
Route:	Oral
Exposure:	103 weeks
GLP:	in compliance

A 2-year oral dietary feeding carcinogenicity bioassay was conducted on HC Blue n° 2 using treatment and control groups of 50 male and 50 female Fischer 344 rats (6 – 7 weeks old). The low and high dietary concentrations of HC Blue n° 2 were for 5000 and 10000 ppm (equivalent to approximately 195 and 390 mg/kg/day) for males and 10000 and 20000 ppm (equivalent to approximately 465 and 1000 mg/kg/day) for females. HC Blue n° 2 were administered for 103 weeks. The treatment period was followed by a treatment free observation period of about 2 weeks. Animals were observed for signs of toxicity and tissue masses or lesions throughout the study. Necropsy and histopathology were conducted on all animals.
The survival of high dose male rats was better than that for controls, and the survival of dosed female rats was comparable to that of the controls. Final mean body weights relative to those of the controls were depressed less than 10% in dosed male rats, whereas depressions of 13% and 22% were observed in the low dose and high dose groups of female rats. Animal survival in all groups was sufficient to be able to detect late-developing tumours.
A dose-related increase in the incidence of hyperostosis of the skull was detected in rats (male, 5/50, 8/50, 25/49; female, 2/50, 19/50, 49/50). The hyperostosis consisted mainly of an increase in the number or thickness of the lamellae of the compact bone tissue of the calvaria, as compared with the calvaria of rats in the control groups. Mixed mesenchymal neoplasms of the kidney were detected in 2/50 high dose female rats; none was observed in any other group of female or male rats. This tumor is considered uncommon and has not been found in 1863 historical control female F344/N rats. A negative trend in fibroadenomas of the mammary gland was seen in female rats (20/50,10/50,4/50).

US National Toxicology program concluded that under the conditions of these studies, there was no evidence of carcinogenicity* in male and female F344/N rats receiving HC Blue No. 2 in the diet at concentrations of 0.5% and 1.0% for males and 1.0% and 2.0% for females for 2 years. HC Blue No. 2 administration caused a dose-related increase in the incidence of hyperostosis of the skull in male and female rats.

Ref.: 11

After the above study ended, the dye sample used for the 2-year studies was found to contain approximately 22 ppm of nitrosamines. Five discrete nitrosamines were found in the sample, and only one (N-nitrosodiethanolamine, 2.7 ppm) was identified. Based on total nitrosamine content of the dye and concentrations of the dye in the diet, high dose male rats and mice received an estimated 220 ppb of total nitrosamines and high dose female rats and mice received 440 ppb. Since there was no evidence of carcinogenicity attributable to the administration of HC Blue No. 2, the presence of the nitrosamines is not considered to be a significant factor in this study.

Ref.: 11

Comment
HC Blue n° 2 did not induce tumours in mice or rats after oral administration under the conditions of an US National Toxicology Program. However, as there were no signs of toxicity in the male rat and male mice studies, it is possible that the compound was not administered to male animals at the maximum tolerated concentration. The reduced survival of female mice precluded adequate evaluation of possible carcinogenic effect among female mice.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	OECD 414
Species/strain:	Sprague-Dawley Crl CD® (SD) IGS BR,
Group size:	24 females
Test substance:	HC Blue n° 2 (B037)
Batch number:	3I394
Purity:	99.5
Vehicle:	0.5% carboxymethylcellulose
Dose levels:	0, 100, 300 or 1000 mg/kg/day (oral, gavage)
Treatment period:	day 6 - 19 post-coitum, inclusive
GLP:	in compliance

Three groups of 24 mated females Sprague-Dawley derived rats were dosed with HC Blue n° 2 (B037), batch No. 3I394, once daily, by the oral route (gavage) from day 6 to day 19 *post-coitum* inclusive at 100, 300 or 1000 mg/kg/day. Another group of 24 mated females received only the vehicle, 0.5% carboxymethylcellulose, following the same regimen as the other groups. A constant dose volume of 5 ml/kg was used. Body weight, food consumption and clinical signs were recorded.
On day 20 *post-coitum*, the females were sacrificed and a gross macroscopic necropsy examination was performed. The number of corpora lutea, implantations, early and late resorptions, live and dead foetuses were recorded. The foetuses were removed from the uterus, weighed, sexed and examined for external, soft tissue or skeletal malformations and variations.

All females given HC Blue n° 2 (B037) had blue coloured urine from approximately day 7 *post-coitum* until necropsy and blue coloured extremities (except in one female at 100 mg/kg/day) from approximately day 14 *post-coitum* until necropsy. This finding was associated with blue coloured fur in the urogenital area of one female given 300 mg/kg/day and of 12/24 females given 1000 mg/kg/day towards the end of gestation. Excessive salivation (ptyalism) was observed in 10/24 females given 300 mg/kg/day and 17/24 females given 1000 mg/kg/day, for a few days after start of dosing until the end of the treatment period.
1000 mg/kg/day caused maternal toxicity, shown by significantly lower body weight gains and food consumption, while there were no indications of any effects on the pregnancy parameters or embryo-foetal development.
The No Observed Adverse Effect Level (NOAEL) for maternal toxicity was
300 mg/kg/day and the No Observed Effect level (NOEL) for developmental toxicity was 1000 mg/kg/day.

Ref: 12

Comment
At 1000 mg/kg/day, there was a statistically significant increase in the foetal incidence of incomplete ossification of the interparietal, when compared to controls (22 vs. 11 in controls, $p<0.05$). Also the (dose-related) excessive salivation in maternal animals at 1000 and 300 mg/kg bw is considered adverse.
As a consequence, SCCP derives from this study a foetal NOAEL of 300 mg/kg bw/day in the presence of maternal toxicity. The NOAEL for maternal toxicity is 100 mg/kg bw/day.

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

Maximum absorption through the skin	**A**	**=**	**0.037 µg/cm^2**
Skin area surface	**SAS**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.026 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.0004 mg/kg**
No observed adverse effect level (rat, oral, maternal toxicity)	**NOAEL**	**=**	**100 mg/kg**

Margin of Safety	**NOAEL/SED**	**=**	**250000**

3.3.14. Discussion

Physico-chemical properties
HC Blue No.2 is used in semi-permanent hair dye formulations at a maximum concentration of 2.8%. Stability of HC Blue n° 2 in marketed products is not reported. Chemical structure of HC Blue No.2 is comprised of both a secondary and a tertiary amino group. It is therefore prone to nitrosation. Nitrosamine content in HC Blue n° 2 is not reported. HC Blue n° 2 should not be used together with nitrosating agents

General toxicity
In an acute oral (gavage) toxicity study in rats the maximum non-lethal dose of HC Blue n° 2 was 2000 mg/kg.
A 13-week oral toxicity (gavage) study clinical signs related to the test compound consisted of ptyalism and purple discolouration of urine, urogenital region, extremities, coat and tail at all dose levels. Increased liver and kidney weights were observed in males and females at 1000 mg/kg/day; these changes were also observed in females at the end of the treatment-free period. The NOAEL could not be determined in this study, since ptyalism is considered a treatment related adverse effect. The LOAEL in this study was 100 mg/kg/day.
Gavage administration of 100, 300 or 1000 mg/kg/day HC Blue n° 2 to pregnant female Sprague-Dawley rats produced ptyalism as well as blue discolouration of urine, extremities and fur at 300 and 1000 mg/kg/day. Lower mean body weight gain and food consumption were observed at 1000 mg/kg/day At 1000 mg/kg/day, there was a statistically significant increase in the foetal incidence of incomplete ossification of the interparietal. Therefore, 100 mg/kg/day was considered the NOAEL for maternal toxicity, while the NOEL for developmental toxicity was set at 300 mg/kg/day.

Irritation / sensitisation
3% HC Blue n° 2 was not irritant to rabbit skin. It caused transient irritation to the eye of rabbits.
While the data are equivocal, HC Blue n° 2 is considered to have a skin sensitising potential in mice.

Dermal absorption
The maximum total absorption observed was 0.037 µgeq/cm² which should be used for calculating the MOS.

Mutagenicity / Genotoxicity
HC Blue n° 2 was investigated for its ability to induce gene mutations in bacteria and mammalian cells. Two non-guideline studies were poorly described and did not fulfil the requirements in OECD guidelines. Therefore, the value of these studies is limited and the results can only be used as supportive evidence.
HC Blue n° 2 was (weakly) positive exclusively in a single *Salmonella* strain (TA98) in the gene mutation tests in bacteria. This result was supported by results from a non-guideline study. A study of limited value showed positive effect at the *tk* locus in mammalian cells, but only at very toxic concentrations. These positive results could not be confirmed in a gene mutation tests in mammalian cells (*hprt* locus).
HC Blue n° 2 induced micronuclei but not chromosomal aberrations in human peripheral lymphocytes *in vitro*. An *in vivo* rat bone marrow micronucleus test was negative for clastogenic/aneugenic potential.
A positive effect in a non-guideline study indicating genotoxic activity of HC Blue n° 2 at the *tk* locus in mammalian cells was of
Although HC Blue n° 2 showed mutagenic activity in the *Salmonella*/microsome assay, this activity was observed only in the tester strain TA98 in the absence of S9 and was relatively weak (less than two fold and only at the highest concentration). Given that this activity was not confirmed in an *in vitro* gene mutation test in mammalian cells (*hprt* mouse lymphoma assay) HC Blue n° 2 was considered to have no mutagenic potential. Apparently, the clastogenic/aneugenic potential of HC Blue n° 2 observed *in vitro* does not lead to clastogenic/aneugenic effects in experimental animals under appropriate test conditions.
Plasma analysis showed mean plasma levels of 18.9 µg/ml and 10.7 µg/ml, 1 hour and 4 hours after oral administration at 2000 mg/kg, respectively, and confirmed the systemic exposure of the test animals to the compound.
Therefore additional tests with HC Blue n° 2 itself are not deemed necessary.

Carcinogenicity
HC Blue n° 2 did not induce tumours in mice or rats after oral administration under the conditions of an US National Toxicology Program. However, as there were no signs of toxicity in the male rat and male mice studies, it is possible that the compound was not administered to male animals at the maximum tolerated concentration. The reduced survival of female mice precluded adequate evaluation of possible carcinogenic effect among female mice.

4. CONCLUSION

Based on the information provided, the SCCP is of the opinion that the use of HC Blue n° 2 itself in semi-permanent hair dye formulations at a maximum concentration of 2.8% in the finished cosmetic product does not pose a risk to the health of the consumer, apart from its sensitising potential.

However, HC Blue n° 2 is comprised of both a secondary and a tertiary amino group, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

6. REFERENCES

References in *italics* were not submitted as full reports in the present dossier. They consist of reports on preliminary toxicity studies [15] and reports for studies considered to be inadequate [16-26]. These can be provided upon request.

1. G. Sire. HC Blue N°2: Acute Oral Toxicity in Rats. "Fixed Dose Method. CIT Study No. 29770 TAR, 2005
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