Scientific Committee on Consumer Products

SCCP

OPINION ON

HC Blue n° 12

COLIPA n° B73

The SCCP adopted this opinion at its 14th plenary of 18 December 2007

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.

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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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http://ec.europa.eu/health/ph_risk/risk_en.htm

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TABLE OF CONTENTS

ACKNOWLEDGMENTS 3

1. BACKGROUND 5

2. TERMS OF REFERENCE 6

3. OPINION 7

4. CONCLUSION 30

5. MINORITY OPINION 30

6. REFERENCES 31

1. BACKGROUND

Submission I for HC Blue n° 12 with the chemical name 1-(ß-hydroxyethyl)amino-2-nitro-4-N-ethyl-N-(ß-hydroxyethyl) aminobenzene, earlier called Nitroblau, was submitted in March 1992 by COLIPA[1, 2].

The Scientific Committee on Cosmetology (SCC) adopted at its 53rd plenary meeting on 25 June 1993 an opinion (SPC/1077/93) with the final conclusion that:

"Nitroblau was found moderately toxic in the acute oral toxicity test and slightly toxic after dermal administration to rabbits. Nitroblau showed no signs of irritation. The sensitization test was carried out inadequately. In the 90-day study with rats, 30 mg/kg bw was considered to be the NOAEL. In the teratogenicity study, no irreversible structural changes were observed in the foetuses of the rat, after administration of 140 mg/kg bw.
Nitroblau is not genotoxic. The cutaneous absorption was 1% of the administered 14C for the hair dyeing formulation. For normal use of hair dye, the following calculation can be made: 750 mg nitroblau comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing 0.75% nitroblau). With a maximal penetration of 1%, this results in a dermal absorption of 7.5 mg per treatment, which is 0.125 mg/kg bw (assuming a body weight of 60 kg). 0.53 g nitroblau comes in contact with the human skin in semi-permanent hair dye condition (based on a usage volume 35 ml containing maximal 1.5% nitroblau). With a penetration of 1%; this results in a dermal absorption of 5.25 mg per treatment, which is 0.0875 mg/kg bw So a margin of safety of 240 can be calculated between the figure for human exposure to oxidative hair dye and the no adverse effect level found in rats in the 90-day study (limited study, only 1 dose tested). It should be noted that no effects were observed in the teratogenicity study at 140 mg/kg bw. For the semi-permanent hair dye a safety margin of 343 can be calculated. It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month and human exposure to semi-permanent hair dye is unlikely to be more than once a week."

Submission II for HC Blue n° 12 was submitted in December 1993 by COLIPA[2].

The Scientific Committee on Cosmetic products and Non-Food Products intended for Consumers (SCCNFP) adopted at its plenary meeting on 23 June 1999 an opinion (SCCNFP/0140/99) with the conclusion that:

"The SCCNFP is of the opinion that Nitroblau can be used safely in semi-permanent hair dyes and colour setting lotions at a maximum concentration of 1.5%. In permanent hair dyes the maximum concentration is 1.5%. Since the permanent hair dyes are mixed with hydrogen peroxide before application the use concentration is 0.75%. The sensitisation data in the dossier was generated with a method not conforming with OECD n° 406. However, no further sensitisation data should be requested provided that cosmetic products containing this substance carry a label warning of a risk of sensitisation."

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 16 on the List of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission III for HC Blue n° 12 was submitted by COLIPA in July 2005. According to this submission the substance is used in hair colouring formulations as:

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

a) a non-reactive hair colouring agent ("direct dye") in non-oxidative hair dye formulations at a maximum on-head concentration of 1.5%. It is common practice to apply 35 to 50 g of the product over a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at weekly intervals.

b) a non-reactive hair colouring agent ("direct dye") in oxidative hair dye formulations at a maximum on-head concentration of 0.75%. The colorant component and a developer (hydrogen peroxide) are mixed in ratios between 1:1 to 1:3. It is common practice to apply up to 100 g of the finished mixed product for a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at monthly intervals.

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° 12 safe for use as a non-oxidative hair dye with an on-head concentration of maximum 1.5% taken into account the scientific data provided?*

2. *Does the SCCP consider HC Blue n° 12 safe for use in oxidative hair dye with an on-head concentration of maximum 0.75% taken into account the scientific data provided?*

3. *Does the SCCP recommend any further restrictions with regard to the use of HC Blue n° 12 in any non-oxidative or 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° 12 (INCI)

3.1.1.2. Chemical names

Free Base
1-(beta-hydroxyethyl)amino-2-nitro-4-N-ethyl-N-(beta-hydroxyethyl)aminobenzene (INCI)
2-[[4-[ethyl(2-hydroxyethyl)amino]-2-nitrophenyl]amino-ethanol
2-[Ethyl-4-[(2-hydroxyethyl)amino]-3-nitroanilino]ethanol
1,4-di(beta-hydroxyethyl)amino-4-N-ethyl-2-nitrobenzene
4-N-ethyl-N-(beta-hydroxyethyl)amino-1-(.beta.-hydroxyethyl)amino-2-nitrobenzene

Hydrochloride
1-(beta-hydroxyethyl)amino-2-nitro-4-N-ethyl-N-(beta-hydroxyethyl)aminobenzene hydrochloride
2-[Ethyl-4-[(2-hydroxyethyl)amino]-3-nitroanilino]ethanol hydrochloride (IUPAC)
Ethanol, 2-[[4-[ethyl(2-hydroxyethyl)amino]-2-nitrophenyl]amino-, monohydrochloride (CA index name, 9CI)

3.1.1.3. Trade names and abbreviations

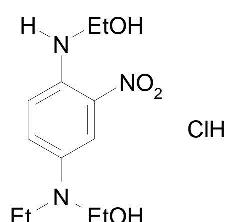
Nitroblau
COLIPA n° B73

3.1.1.4. CAS / EINECS number

Free base
CAS: 104576-93-0

Hydrochloride
CAS: 132885-85-9
ELINCS: 407-020-2

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{12}H_{19}N_3O_4.HCl$

3.1.2. Physical form

Yellow green powder

3.1.3. Molecular weight

Molecular weight: 269.25 (free base)
305.76 (hydrochloride)

3.1.4. Purity, composition and substance codes

Material placed on the market (deduced specification)

HPLC quantitative (hydrochloride):	> 98% w/w
1,4-Diamino-2-nitrobenzene:	< 100 ppm
Solvent content:	< 1%
Ash:	< 1%

The above specifications were deduced from the following data:

Purity

HPLC quantitative (hydrochloride):	> 92 % w/w
NMR quantitative (hydrochloride):	> 92 % w/w
Chloride:	< 5 % w/w
Solvent content:	< 1.5 %
Ash:	< 0.2 %
Element screening:	in all batches no significant inorganic impurities detected

Potential impurities

1,4-Di((2-hydroxyethylamino)-2-nitrobenzene (HC Violet No. 7):	< 1.5 %, w/w
4-((2-Hydroxyethyl)amino)-3-nitroaniline (HC Red No. 3):	≤ 900 ppm
4-Fluoro-3-nitroaniline:	not found (LOD = 140 ppm)
1,4-Diamino-2-nitrobenzene:	< 30 ppm

Solvent residues
Solvents, (i.e. solvents such as methanol, ethanol, isopropanol, n-propanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone and monochlorobenzene < 100 ppm) were not detected.

Batches used:
- L4/119, *free base*
- GHS111183 / L4/130, *free base*
- L4/154
- 29/30 (R00060907)
- GHS111183 / Drum 6113 / Lot 8 (R5077) / R5077
- GHS111183 / 13 / 1 / 2
- GHS111183 / 011-01 / 27-04
- Lot 8, WE87, Partie 1 92-11-18
- Lot 27/ Fass 7178 (R99053825)

Comparison of the different batches of HC Blue n° 12

		free base		L4/154	GHS 111183 / Drum 6113 / Lot 8 (R5077) / R5077	GHS 111183 / 13 / 1 / 2	GHS 111183 / 011-01 / 27-04	Lot 8, WE87; Partie 1; 92-11-18	Lot 27/ Fass 7178 (R99053825)	29/30 (R00060907)
		GHS 111183 / L4 / 130[#]	**L4 / 119[#]**							
Date of Entry		1984	1984	1984	1991	1991		1992	1999	2000
NMR content	%, w/w	96.0[#]	100[#]	93.2[##]	99.2[##]	96.9[##]	100[##]	92.8[##]	98.5[##]	97.9[##]
HPLC content *	%, w/w	96.5[#*]	104.1[#*]	94.6*	95.7°°	99.5°°	103.9°°	92.0*	97.2°°	98.4°°
HPLC purity / area%**	area%									
210 nm		94.5	94.8	92.1	93.0	95.7	97.2	92.5	93.6	93.3
254 nm		97.8	98.2	93.2	93.8	96.3	97.9	92.4	94.2	93.9
540 nm		99.7	99.6	94.0	95.1	97.8	98.2	95.9	96.9	96.5
Chloride	%, w/w	n.d.***	< 0.1	11.4	11.6	12.1	11.5	11.3	n.d.***	11.4
Water content	%, w/w	0.53	n.d.***	0.4	0.39	0.34	0.2	0.38	0.31	0.3
Loss on drying	%, w/w	n.d.***	n.d.***	0.13	0.09	1.5	1.2	0.18	0.04	0.02
Ash	%, w/w	n.d.***	n.d.***	0.19	0.06	< 0.1	< 0.1	0.04	0.04	0.06
Impurities:										
1,4-Di((2-hydroxyethyl amino)-2-nitrobenzene (HC Violet No. 7)	%, w/w	0.17	0.032	1.11	0.8	0.49	0.04	0.78	0.49	0.58
4-((2-Hydroxyethyl) amino)-3-nitro-aniline (HC Red n° 3)	ppm	10	7	801	465	262	59	240	130	84
4-Fluoro-3-nitroaniline	ppm	< 20°	< 100°	< 20°	< 140°	< 140°	< 140°	< 20°	< 140°	< 140°
1,4-Diamino-2-nitrobenzene	% w/w	< 3°	< 20°	15	22	27	Ca. 1	< 3°	Ca. 1	Ca. 1
Element screening	For all batches: no significant impurities were detected.									

[#] Calculated as free base
[##] Calculated as hydrochloride salt
*** not determined because lack of substance
° not detected; shown value indicates limit of detection
** HPLC conditions: Nucleosil RP C18, 5µm 250 x 4 mm with precolumn; Eluent: 35% acetonitrile / 75% 0,02M KH_2PO_4 buffer at pH3; Flow: 1 mL/min
* Refers to 29/30 (R00060907) with an NMR-content of 92.9 weight% (corrected for L4/119 in A2002/244)
°° Refers to Batch F518612 Fass 2 with a NMR content of 94.8 weight% (see A 9806/188)

Remark
The last two of the above footnotes indicate that the reported data were calculated from the data of another batch.

Based on the above comparative data, the following conclusion can be made:

The ^{1}H-NMR-spectra of all samples confirm the chemical identity of the test *object HC BLUE n° 12*. With the exception of the signals of *HC BLUE n° 12* the ^{1}H-NMR-spectra showed no other signals indicating organic impurities. The corresponding absolute *HC BLUE n° 12*

values ascertained by HPLC/DAD vary from 92%, w/w and over 100%, w/w depending on the batch.

Depending on the *HC BLUE n° 12* batch analysed the content of 1,4-Di((2-hydroxyethylamino)-2-nitrobenzene (HC Violet No. 7) is in the range of 300 mg/kg and 1.1%, w/w; ; the content of 1,4-Diamino-2-nitrobenzene between is in the range of 1 ppm to 30 ppm; and 4-((2-Hydroxyethyl)amino)-3-nitro-aniline (HC Red No. 3) is in the range of between 7 ppm to 800 ppm. Only 4-Fluoro-3-nitroaniline was never detected (LOD = 140 ppm).

Inorganic impurities do not play a role as side component in any sample, shown by a negligible the residue on ignition. Similarly, the loss on drying and water content varies just to a maximum amount of 1.5%, w/w.

The chloride content, except for batches GHS111183 / L4 / 130 and L4 / 119, which are not the hydrochloride form of *HC BLUE n° 12* but the free base, is in the range of 11.3 to 12.1%, w/w, which corresponds perfectly with the theoretical amount of 11.6%, w/w.

3.1.5. Impurities / accompanying contaminants

See above

3.1.6. Solubility

Soluble	in water	> 100 g/L (pH 2.4)
	in acetone/water 1:1	70 < S < 120 g/L (pH 2)
	in DMSO	> 100 g/L
	in ethanol	5 < S < 15 g/L

3.1.7. Partition coefficient (Log P_{ow})

P_{ow}: 20.9
log P_{ow} 1.32 (at pH 8.0 ; room temperature) (EU - A.8) *(Reference: 2)*

3.1.8. Additional physical and chemical specifications

pH-value:	1.80 (saturated aqueous solution, 20°C)		*(Reference: 3)*
pKa-value:	15.41 for R-CH_2OH (acidic) 15.51 for R-CH_2OH (acidic) 4.11 for R-(Ar)-N-(R1, R2) (basic) -0.46 for R-(Ar)-NH-R (basic) -2.80 for R-CH_2OH (basic) -4.03 for R-CH_2OH (basic) (calculated, Pallas Software)		*(Reference: 4)*
Melting point:	164° - 170 °C	(EU – A.1)	*(Reference: 5)*
Boiling point:	not detectable (decomposition at 205 °C)	(EU – A.2)	*(Reference: 6)*
Density:	1.363 g/cm^3 (20 °C)	(EU - A.3)	*(References: 7; 3)*
Vapour pressure:	1.92 exp -5 hPa (20 °C, extrapolated)	(EU - A.4)	*(References: 8; 3)*
Surface tension (in water): (c = 6 g/l); 20°C)	66.9 mN/m	(EU - A.5)	*(References: 9; 3)*
Water solubility:	> 200000 mg/l (20°C)	(EU - A.6)	*(References: 10; 3)*
Flammability (solids):	not highly flammable	(EU - A.10)	*(Reference: 11)*
UV-Vis spectrum:	/		

3.1.9. Homogeneity and Stability

No data submitted

General Comments to physico-chemical characterisation

- HC Blue n° 12 is both a secondary and a tertiary amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.
- -The physical form "yellow green powder" reported for both forms (free base and hydrochloride) is different from previously reported, "dark violet, fine grained powder" (for the free base) and "beige crystalline powder" (for the hydrochloride).
- No data submitted for the stability of the test substance in solutions or in the marketed products.

3.2. Function and uses

a) Semi-permanent Hair Colorants

HC Blue n° 12 is used as a non-reactive hair colouring agent ("Direct Dye") in semi-permanent hair dye formulations at a maximum on-head concentration of 1.5 %.

b) Oxidative Hair Colorants

HC Blue n° 12 is used as a non-reactive hair colouring agent ("Direct Dye") in oxidative hair dye formulations at a maximum on-head concentration of 0.75 %.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline: /
Species/strain: Wistar rats and CF1 mice
Group size: Rats: 6 females per dose
Mice: 10 per sex and dose
Test substance: Nitroblau in distilled water
Batch: L4/119
Purity: 98.2% (HPLC)
Dose: Mice (female): 1200, 1450, 1700, 1950 and 2200 mg/kg bw
Mice (male): 1200, 1500, 1800, 2100 and 2400 mg/kg bw
Rats (female): 1200, 1600, 2000 and 2400 mg/kg bw
Route: once, oral, gavage
GLP: in compliance

A 10% solution of the test substance in distilled water was given once by stomach tube to CF1 mice (10/sex) and Wistar rats (6 females). During an observation period of 14 days mortalities and clinical observations were recorded daily and the body weights were noted weekly. At the end of the observation period all surviving animals were sacrificed and the organs were examined.

Results
The test substance caused a limitation of the animal activity, abdominal position and a blue discolouration of the extremities. No macroscopic organ damages were noted. The mortality rates are compiled in the Table.

female mice		male mice		female rats	
dose (mg/kg)	**mortality**	**dose (mg/kg)**	**mortality**	**dose (mg/kg)**	**mortality**
1200	1/10	1200	0/10	1200	1/6
1450	2/10	1500	2/10	1600	3/6
1700	3/10	1800	8/10	2000	4/6
1950	6/10	2100	6/10	2400	6/6
2200	10/10	2400	10/10		

Conclusion
Based on the mortality rates the following LD_{50} figures were calculated:

LD_{50} female mice: 1775 mg/kg bw
LD_{50} male mice: 1770 mg/kg bw
LD_{50} female rats: 1668 mg/kg bw

Ref.: 13

3.3.1.2. Acute dermal toxicity

Guideline: /
Species/strain: White New Zealand rabbits
Group size: 5 per sex and dose
Test substance: Nitroblau
Batch: R5077
Purity: 93.8 % (HPLC)
Dose: 2000 mg/kg bw
Route: single dermal application

GLP: /

The moistened test substance was once dermally applied to the shaven back of NZW rabbits (5 per sex). The applied dose was 200 mg/kg bw. The treated area was covered by a gauze patch and held in place for 24 h. During an observation period of 14 days clinical signs and the skin alterations were recorded daily. The body weights were measured at day 0 and 14. After sacrifice a post mortem examination was carried out on all animals.

Results
No mortalities were observed. Body weight gain revealed no treatment related effect. Skin alterations could not be evaluated because of the skin discolouration. No macroscopic organ changes were noted.

Conclusion
The LD_{50} of acute dermal toxicity is > 2000 mg/kg bw.

Ref.: 14

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404
Species/strain:	Rabbit, strain New Zealand White
Group size:	3 females
Test substance:	*Nitroblau*
Batch:	R5077 (not in original report but added later from laboratory notes)
Purity:	>98% in report as supported by sponsor
Dose level:	0.5 g (moistened with 1 ml water)
Route:	dermal
Application conditions:	Single administration, 4 h, occlusive
GLP:	in compliance

Nitroblau (0.5 g*)*, moistened with aqua distilled (1 ml), was applied onto the back skin (area about 6 cm²) of 3 female White New Zealand rabbits and kept in contact for 4 hours under occlusive conditions. The treated area was covered with gauze and with a self-adhesive dressing. The effect on the skin was evaluated 1, 24, 48 and 72 h after patch removal.

Results
No skin reactions at all were noted after application of the undiluted material to the rabbit skin.

Conclusion
In this *in vivo* study in rabbits, undiluted *Nitroblau* did not cause any signs of skin irritation.
Ref.: 15

A skin irritation assay with in female Pirbright white guinea pigs was submitted as part of a former submission. As this test had several limitations with regard to study design and reporting, it was not considered by the SCCP.

Ref.: 5 (subm. I)

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405
Species/strain: Rabbit, strain New Zealand White
Group size: 3 females
Test substance: *Nitroblau*
Batch: R5077 (not in original report but added later from laboratory notes)
Purity: >98% in report as supported by sponsor
Dose level: 0.1 ml (about 30 to 50 mg undiluted material)
Route: Ocular
Application conditions: Single application, no rinsing
GLP: In compliance

An equivalent of 0.1 ml (about 30 to 50 mg) of *Nitroblau* was applied into the conjunctival sac of the right eye of 3 female New Zealand white rabbits without rinsing, while the left eye served as control. The effects on the eyes were evaluated and scored 1, 24, 48 and 72 h after application.

Results
No effects on the cornea or the iris were noted at any reading. Mild conjunctival erythema was noted in two animals up to 48 h after instillation. For the same animals conjunctival oedema was also noted up to 1 hour after substance instillation.

Conclusion
Under the conditions of the test, undiluted *Nitroblau* caused transient eye irritation in rabbits.

Ref.: 16

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline: OECD 429
Species/strain: Mouse, strain CBA/J
Group size: 5 females per concentration
Test substance: *HC BLUE NO. 12 WR 20854*
Batch: 29/30
Purity: 97.2 % at 254 nm HPLC
Concentrations: A) 0.5, 1.5, 5.0 and 10.0 % (w/v) in DMSO
B) 0.5, 1.5, 5.0 and 10.0 % in water/acetone (1:1) mixed with olive oil (4:1)
GLP: in compliance

The skin sensitising potential of *HC BLUE NO. 12 WR 20854* was investigated in CBA/J mice by measuring the cell proliferation in the draining lymph nodes after topical application on the ear.

25 µl of 0 (vehicle only), 0.5, 1.5, 5 and 10 % of *HC BLUE NO. 12 WR 20854* in DMSO or in a mixture of water/acetone (1:1) with olive oil (4:1) (equal to the maximum solubility) were applied to the surface of the ear of five female CBA/J mice per group for three consecutive days. After application, the ears were dried by means of a hair dryer for about 5 minutes. A positive control, p-phenylenediamine (PPD) at a concentration of 1 % in DMSO, was investigated in parallel under identical test conditions.
On day 5, mice received an intravenous injection of 250 µl phosphate buffered saline containing 23.8 µCi of [H^3] methyl thymidine. Approximately five hours later, the mice were

killed by CO_2-inhalation and the draining auricular lymph nodes were removed and weighed. After preparing a single cell suspension from the lymph nodes of each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [H^3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
A) Stimulation indices of 1.4, 1.5, 3.0 and 2.7 were obtained for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively for *HC BLUE NO. 12 WR 20854* in DMSO. The EC3 value was estimated to be 5.0 %.
B) Stimulation indices were 1.4, 1.0, 0.8 and 0.9 for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively for *HC BLUE NO. 12 WR 20854 in* water/acetone/olive oil. An EC3 value was not calculated for *HC BLUE NO. 12 WR 20854* since all stimulation indices in this vehicle were below 3.

The positive control (PPD, 1 % in DMSO) caused a stimulation index of 7.4.

Conclusion
HC BLUE NO. 12 WR 20854 induced a biologically relevant immune response in the draining lymph nodes after dermal application to the mouse ear if applied in DMSO. The EC3 value was estimated to be 5.0 %. In the vehicle aqua/acetone/olive oil, however, no relevant immune response was observed. The concurrent positive control demonstrated the sensitivity of the assay.

Ref.: 17

The dermal sensitisation potential of *HC BLUE NO. 12* was evaluated by the Magnusson-Kligman Maximisation method in guinea pigs. Due to staining problems the evaluation was compromised and considered by the sponsors to be of limited value in evaluating the skin sensitising properties of *HC BLUE NO. 12*. but the result indicated weak to moderately sensitising effects (Bornatowicz, N., Skin sensitisation study with "Nitroblau" (Guinea Pig Maximisation Test); *SEIBERSDORF*; 1991).

Another skin sensitisation study was presented in a former dossier. This test, performed in 1985, was considered of limited validity (e.g. not in compliance with GLP, inadequate test conditions, etc.) and therefore not considered as relevant for the hazard assessment of *HC BLUE NO. 12* but the result suggested a non-sensitising property.

Ref.: 7 (subm. I)

Conclusion Skin Sensitisation
In local lymph node assay, *HC BLUE NO. 12* did induce a weak but biologically relevant immune response in local lymph nodes after dermal application to the mouse ear in DMSO, whereas the test substance revealed no skin-sensitising properties if water/acetone/olive oil was used as vehicle. According to the observed EC3 value of 5.0 % with the vehicle DMSO and according to the ECETOC skin sensitisation potency classification criteria *(Reference: 18)*, the test substance is classified as a moderate skin sensitising agent.

3.3.4. Dermal / percutaneous absorption

Guideline:	According to OECD–Draft Guideline Skin absorption: *in vitro* method" (2000)
Tissue:	Porcine back or flank skin (frozen/thawed; thickness: ≤ 1000 µm); 1 female donor
Method:	Diffusion Teflon-chambers
Integrity	tritiated water

Test substance:	*Nitroblau (WR20854)*
Batch:	Lot 27/ Fass 7178
Purity:	93.6 % (HPLC, 254 nm)
Concentration:	1.5 mg/cm² tested as part of a non-oxidative hair dye formulation (Batch 05.04.2004; composition not given) containing 1.5% *Nitroblau (WR20854).*
No. of chambers:	6 (five for the formulation containing the test item and one for the blank formulation)
Receptor fluid:	Physiological phosphate buffer containing NaCl and antibiotics.
Solubility in receptor fluid:	137.3 mg/ml
Stability in receptor fluid:	> 7 days
Analytical method:	HPLC
GLP:	In compliance

The skin absorption of *Nitroblau (WR20854)* was investigated at the maximum concentration intended for use in direct hair colorants using pig skin from one female donor (prepared 1000 µm thick). The dye (1.5 mg/cm²) was applied to the skin in a commercial hair dye formulation (400 mg aqueous cream formulation containing 1.5 % dye, applied to 4 cm² skin).
The integrity of each skin sample was demonstrated with tritiated water, resulting in penetration rates of 0.985 to 1.185 % of the applied dose. These figures were well within the limit of acceptance (≤ 1.5 %).
A diffusion Teflon-chamber was used. The receptor solution (physiological phosphate buffer containing NaCl and antibiotics) was pumped through the receptor chamber at a rate of 5 ml/h. Six chambers were investigated, one of which was the control.
Thirty minutes after substance application, the test item was removed by washing the skin twice with water, then once with washing solution (shampoo-formulation diluted to approximately 16.7 %) and again twice with water. The washing solutions were combined and the amount of dye was determined by HPLC.
Fractions of the receptor fluid were collected at 16, 24, 40, 48, 64 and 72 hours, concentrated directly after collection and stored at −20° C until analysis. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the dye content was quantified by means of HPLC.

Results
All samples/tissue extracts were analysed by HPLC. The limit of quantification of the applied method is not mentioned in this report. However, a LOQ of 17.2 ng/HPLC injection and a LOD of 1 ng/HPLC injection for the used method is provided in the study performed with hydrogen peroxide (see study below), which was performed in parallel to this study.
The total recovery was 96.1 ± 4.9 % of the applied dose. The major part of *Nitroblau (WR20854)* remained on the skin surface, representing 95.9 ± 4.9 % of the applied dose.
Under the conditions applied, the amount considered available (receptor fluid, epidermis, upper dermis) was 3.7 ± 1.0 µg/cm² (range 3.1 to 4.2) *Nitroblau (WR20854)*.

Ref.: 19

Comment
The A_{max} was 4.2 µg/cm² but only 5 chambers from 1 donor were used in the experiment which is not acceptable for calculating the MOS. Therefore, the percutaneous absorption from the toxicokinetics experiment (Reference 36) is used for calculating the MOS.

Percutaneous absorption *in vitro* (with hydrogen peroxide)

Guideline:	According to OECD–Draft Guideline Skin absorption: *in vitro* method" (2000)
Tissue:	Porcine back or flank skin (frozen/thawed; thickness: ≤ 1000 µm); 1 female donor
Method:	Diffusion Teflon-chambers
Integrity	tritiated water
Test substance:	*Nitroblau (WR20854)*
Batch:	Lot 27/ Fass 7178
Purity:	93.6 % (HPLC, 254 nm)
Concentration:	0.75 mg/cm² tested as part of an oxidative hair dye formulation containing 0.75% *Nitroblau (WR20854).*
No. of chambers:	6 (five for the formulation containing the test item and one for the blank formulation)
Receptor fluid:	Physiological phosphate buffer containing NaCl and antibiotics.
Solubility in receptor fluid:	137.3 mg/ml
Stability in receptor fluid:	> 7 days
Analytical method:	HPLC
GLP:	In compliance

The skin absorption of *Nitroblau (WR20854)* was investigated in the presence of hydrogen peroxide at the maximum concentration intended for use in oxidative hair colorants using pig skin from one female donor (prepared 1000 µm thick). The dye (0.75 mg/cm²) was applied to the skin in a commercial hair dye formulation (400 mg aqueous cream formulation diluted 1:1 with a formulation containing 6 % hydrogen peroxide and 3 mg *Nitroblau (WR20854)* (= 0.75 % dye), applied to 4 cm² skin).
The integrity of each skin sample was demonstrated with tritiated water, resulting in penetration rates of 0.85 to 1.3 % of the applied dose. These figures were within the limit of acceptance (≤ 1.5 %).
A diffusion Teflon-chamber was used. The receptor solution (physiological phosphate buffer containing NaCl and antibiotics) was pumped through the receptor chamber at a rate of 5 ml/h. Six chambers per experimental group were investigated.
Thirty minutes after substance application, the test item was removed by washing the skin twice with water, then once with washing solution (shampoo-formulation diluted to approximately 16.7 %) and again twice with water. The washing solutions were combined and the amount of dye was determined by HPLC.
Fractions of the receptor fluid were collected at 16, 24, 40, 48, 64 and 72 hours, concentrated directly after collection and stored at –20° C until analysis. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the dye content was quantified by means of HPLC.

Results
All samples/tissue extracts were analysed by HPLC. The limits of quantification and of detection of the applied method were 17.2 ng/HPLC-injection and 1 ng/HPLC-injection, respectively.
The total recovery was 95.5 ± 6.8 % of the applied dose. The major part of *Nitroblau (WR20854)* remained on the skin surface, representing 94.8 ± 6.9 % of the applied dose.
Under the conditions used, the amount considered available (receptor fluid, epidermis, upper dermis) was 5.4 ± 1.1 µg/cm² (range 4.4 to 6.1) *Nitroblau (WR20854)*.

Ref.: 20

Comment

The A_{max} was 6.1 µg/cm² but only 5 chambers from 1 donor were used in the experiment which is not acceptable for calculating the MOS. Therefore, the percutaneous absorption from the toxicokinetics experiment (Reference 36) is used for calculating the MOS.

Summary *in vitro* skin penetration
Too few chambers and only 1 donor were used in these experiments. Accordingly, an absorption of 21.7 µg/cm² obtained from the toxicokinetic study (reference 36) in the rat is used for calculating the MOS.

3.3.5. Repeated dose toxicity

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

Guideline: OECD 407 (1981)
Species/strain: Wistar rats Crl:Wi/Br (SPF)
Group size: 5 per sex per dose
Test substance: Nitroblau in distilled water
Batch: Drum Number 6113 Lot 8
Purity: 93.8% (HPLC)
Dose: 0, 100, 316 and 1000 mg/kg bw/d, additional 14 days recovery groups
Route: oral, gavage
Exposure: 28 day
GLP: in compliance

The test substance was administered at 100, 316 and 1000 mg/kg bw/d to 5 male and 5 female Wistar rats by oral gavage for 28 days. The control group received the vehicle distilled water. Two recovery groups were added (0 and 1000 mg/kg bw/d). The animals were checked daily for mortality and clinical signs. Body weight and feed consumption were determined weekly. Ophthalmoscopy was performed before the study and on day 27. Haematology and clinical biochemistry as well as urinalysis were carried out for all animals on day 0 and 28 and at day 42 for the recovery groups. At the end of the study necroscopy was done and organ weights were recorded. A number of tissues of the control and high dose group was investigated histopathologically.

Results
No test substance related mortality occurred. With the exception of diarrhoea of 1 animal no clinical signs were observed. Staining of the skin and fur in all mid and high dose animals was observed. In females a significant higher feed consumption and a higher body weight gain compared to controls was observed which was most pronounced in the high dose group. No abnormalities were found during ophthalmoscopy. During haematology reduced red blood cell counts and hemoglobin concentrations as well as increases in the rates of polychromatic erythrocytes were found were found in high dose animals of both sexes. In high dose females also the coagulation time was raised. Clinical chemistry showed changes in uric acid (lowered at high dose, both sexes) and bilirubin (raised at high dose, both sexes). Absolute and relative spleen weights were increased at the high dose but significance was reached only in males. Also in the high dose group liver weight was increased (females only). At the high dose with both sexes in the spleen lymphoid depletion and congestion as well as induction of haematopoesis were observed.

Conclusion
The NOAEL of the 28 day toxicity study is 316 mg/kg bw/d.

Ref.: 21

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

Rat Study 1

Guideline: OECD 408 (1981)
Species/strain: Wistar rats Bor.Wis.W. (SPF)
Group size: 15 per sex per dose
Test substance: Nitroblau hydrochloride in distilled water
Batch: L4/154
Purity: 93.2 % (HPLC)
Dose: 0, 15, 30 and 60 mg/kg bw/d, additional 14 days recovery groups
Route: oral, gavage
Exposure: 13 weeks
GLP: in compliance

The test substance was administered at 15, 30 and 60 mg/kg bw/d to 15 male and 15 female Wistar rats by oral gavage for 13 weeks. The control group received the vehicle distilled water. Two recovery groups were added (0 and 60 mg/kg bw/d). The animals were checked daily for mortality, clinical signs and motor activity. Body weight, feed and water consumption were determined weekly. Ophthalmoscopy was performed before the study and at term. Haematology and clinical biochemistry as well as urinalysis were carried out on day 0 and weeks 6, 12 or 13 and week 17 for the recovery groups with 10 animals per sex. At the end of the study necroscopy was done and organ weights were recorded. A number of tissues of 5 animals of each group was investigated histopathologically.

Results
No treatment related mortality occurred. With the exception uf urine discolouration no clinical signs were observed. Motor activity measurement and ophthalmoscopic examination did not show a difference between control and test groups. Feed and water consumption and body weight gain were in the physiological range and no dose related changes were noted. Also neither haematology, biochemistry or urinalysis revealed test substance related changes. The histomorphological investigation revealed no substance-related alterations.

Conclusion
Under the conditions of the study the highest dose tested dose of 60 mg/kg bw/d was found as being the NOAEL dose. However, only a limited number of animals (5 per sex and dose) were analysed histopathologically.

Ref.: 22, 23

Rat Study 2

Guideline: /
Species/strain: Wistar rats Bor.Wis.W. (SPF)
Group size: 12 per sex
Test substance: Nitroblau hydrochloride in distilled water
Batch: L4/154
Purity: 93.2 % (HPLC)
Dose: 30 mg/kg bw/d
Route: oral, gavage
Exposure: 13 weeks
GLP: in compliance

The test substance was administered at 30 mg/kg bw/d to 12 male and 12 female Wistar rats by oral gavage for 13 weeks. No control group was included. The animals were checked

daily for mortality, clinical signs and motor activity. Body weight, feed and water consumption were determined weekly. Ophthalmoscopy was performed before the study and at term. Haematology and clinical biochemistry as well as urinalysis were carried out on day 0 and weeks 6 and 13 with 10 animals per sex. At the end of the study necroscopy was done and organ weights were recorded. A number of tissues of 10 animals per sex was investigated histopathologically.

Results
No treatment related mortality occurred. With the exception uf urine discolouration no clinical signs were observed. Motor activity measurement and ophthalmoscopic examination did not show a difference between control and test groups. Feed and water consumption and body weight gain were in the physiological range and no treatment related changes were noted. Also neither haematology, biochemistry or urinalysis revealed test substance related changes. The histomorphological investigation revealed no substance-related alterations.

Conclusion
Under the conditions of the study the tested dose of 30 mg/kg bw/d was found as not exerting systemic toxicity.

Ref.: 24, 25

Comment of the SCCP on sub-chronic toxicity
The dose of 60 mg/kg bw/d is considered the NOAEL dose of subchronic toxicity.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538
Replicates:	3 replicates in 2 individual experiments both in the presence and absence of S9-mix
Test substance:	GHS 111183
Solvent:	DMSO
Batch nr:	13/2/1
Purity:	96.9% NMR, 99.5% HPLC
Concentrations:	8 - 5000 μg/plate without and with S9-mix in both experiments
Treatment:	Direct plate incorporation (72 h treatment) method.
GLP:	In compliance

GHS 111183 (HC Blue n° 12) was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in a pre-experiment with TA100. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and/or a clearing of the bacterial background lawn. Since GHS 111183 was freely soluble and non toxic in this preliminary toxicity test, it was tested up to the prescribed maximum concentration of 5000 μg/plate. Negative and positive controls were in accordance with the OECD guideline.

Results

Precipitation of the test compound was not observed. A biological relevant and dose dependent increase in the number of revertant colonies following treatment with GHS 111183 (HC Blue no 12) was observed both in the absence and presence of metabolic activation in TA98 and TA1538 and in TA100 in the second experiment. An increase in revertant colonies was not seen in TA1535 and TA1537 and in TA100 in the first experiment.

Conclusion
Under the experimental conditions used GHS 111183 (HC Blue no 12) was mutagenic in the gene mutation tests in bacteria.

Ref. 27

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538
Replicates:	3 replicates in 2 individual experiments both in the presence and absence of S9-mix
Test substance:	Nitroblue HCl
Solvent:	sterile distilled water
Batch nr:	5077
Purity:	99.2 NMR, 95.7 HPLC
Concentrations:	Experiment 1: 8 - 5000 µg/plate without and with S9-mix Experiment 2: 312.5 - 5000 µg/plate without and with S9-mix
Treatment:	Direct plate incorporation (48 h treatment) method.
GLP:	In compliance

Nitroblue HCl was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in a preliminary toxicity study with TA100. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and/or a clearing of the bacterial background lawn. Since Nitroblue HCl was non toxic in this preliminary toxicity test, it was tested in the main test up to the prescribed maximum concentration of 5000 µg/plate. Negative and positive controls were in accordance with the OECD guideline.

Results
In both experiments with metabolic activation an increase in the number of revertants was found in the mid-doses between 200 and 1250 µg/plate in TA98 and TA1538. At the higher doses 2500 and 5000 µg/plate the number of revertants decreased again to the spontaneous background level.

Conclusion
Under the experimental conditions used Nitroblue HCl was mutagenic in the gene mutation tests in bacteria.

Ref. 26

***In Vitro* Mammalian Cell Gene Mutation Test (*tk* locus)**

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	duplicates in 2 independent experiments
Test substance:	HC Blue No. 12 (WR20854)
Solvent:	deionised water
Batch:	29/30

Purity:	97.2 %	
Concentrations:	Experiment I:	174.4 - 1041.7 µg/ml both without S9-mix 186.1- 666.7 µg/ml with S9-mix
	Experiment II:	72.6 - 260 µg/ml both without S9-mix
	Experiment IIA:	12.5 - 216 µg/ml both without S9-mix 25- 700 µg/ml with S9-mix
Concentrations:	Experiment I:	4 h both with S9-mix; expression period 72 h, selection growth 10-15 days.
	Experiment II:	24 h without S9-mix; expression period 48 h, selection growth 10-15 days.
	Experiment IIA	24 h without S9-mix; expression period 48 h, selection growth 10-15 days. 4 h both with S9-mix; expression period 72 h, selection growth 10-15 days.
GLP:	In compliance	

HC Blue n° 12 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth. In the main test, cells were treated for 4 h (experiment I, experiment IIA with S9-mix) or 24 h (experiment II, experiment IIA without S9) followed by an expression period of 48 h or 72 h to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was measured as percentage relative survival and total growth of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
Precipitation of HC Blue No. 12 was not observed. The level of toxicity was in both experiments in the absence and presence of S9-mix (almost) at the appropriate level of toxicity (10-20% survival after the highest dose).
After 4 h incubation with HC Blue No. 12 an increase in the mutant frequency was found in the cultures with metabolic activation. These increases were not dose dependent. In experiment I the increase only occurred at the two lowest doses and not at 6 higher doses. In Experiment IIA the increase in mutant frequency fell within the ranges of the positive controls. Therefore these increases were considered as not biologically relevant.
After 24 h treatment an increase in the mutant frequency was also observed in both experiments (II and IIA), being dose dependent in experiment IIA. However, the increases are small and only just outside the historical control ranges. The size distribution of induced colonies did not give a clear indication on the mechanism of the possible mutagenic potential.

Conclusion
Under the experimental conditions used, HC Blue No. 12 was (weakly) genotoxic (mutagenic and/or clastogenic) in the mouse lymphoma assay at the *tk* locus.

Ref. 28

In vitro Micronucleus Test

Guideline:	draft OECD 487 (2004)
Cells:	human lymphocytes from 2 healthy, non-smoking male volunteers
Replicates:	duplicates in 2 independent experiments
Test substance:	HC Blue n° 12 (WR 20854)
Solvent:	purified water
Batch:	29/30
Purity:	97.3%

Concentrations:	Experiment 1:	0, 100, 275, 325 and 350 µg/ml (without S9-mix) 0, 1200, 1500, 1700 and 1900 µg/ml (with S9-mix)
	Experiment 2:	0, 50, 300 and 400 µg/ml (without S9-mix) 0, 500, 1000, 1300 and 1400 µg/ml (with S9-mix)
Treatment	Experiment 1:	24 h PHA followed by 20 + 28 h treatment (without S9-mix) 24 h PHA followed by 3 + 45 h treatment (with S9-mix)
	Experiment 2:	48 h PHA followed by 20 + 28 h treatment (without S9-mix) 48 h PHA followed by 3 + 45 h treatment (with S9-mix)
GLP:	In compliance	

The test was performed in accordance with recommendations of IWTG workshop, draft OECD 487 (2004) and accepted scientific/regulatory principles described in current guidelines for clastogenicity testing *in vitro*.

HC Blue n° 12 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. The suitable top concentrations were based on the results of a cytotoxicity range-finding experiment measuring replication index (RI). To determine the test concentrations for micronucleus analysis in each separate experiment the RI is measured in cultures treated with increasing concentrations of HC Blue n° 12. The top dose for micronucleus analysis was to be the one at which at least approximately 60% reduction in RI occurred or the highest dose tested. Two lower doses were selected so that a range of cytotoxicity from maximum (60%) to little or none is covered. Treatment periods were 20 h without and 3 h with S9-mix. Harvest times were 72 hours (experiment 1) or 96 hours (experiments 2) after the beginning of culture. The final 28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Negative and positive controls were in accordance with the draft guideline.

Results
Measurements on post-treatment media in the absence or presence of S9-mix indicated that HC Blue No. 12 had no effect on osmolarity or pH as compared to concurrent vehicle controls.
In both experiments, biologically relevant and statistically significant increases in the number of micronucleated binucleate cells compared to concurrent control values were found both in the absence and in the presence of S9-mix.

Conclusion
Under the experimental conditions used HC Blue No. 12 induced an increase in micronucleated binucleated cells and, consequently, is genotoxic (clastogenic and/or aneugenic) in cultured human peripheral lymphocytes *in vitro*.

Ref.: 29

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	NMRI mice
Group size:	5 male mice/group
Test substance:	HC Blue n° 12 (WR 20854)
Batch no:	29/30 R00060907
Purity:	97.2 %
Dose level:	0, 312.5, 625 and 1250 mg/kg bw
Route:	orally, once

Vehicle: 20% DMSO
Sacrifice times: 24h and 48h (highest dose only) after the treatment
GLP: In compliance

HC Blue n° 12 has been investigated for the induction of micronuclei in bone marrow cells of mice. The test concentrations were based on the result of a preliminary study on acute toxicity in which 2 mice were orally exposed to doses up to 2000 mg/kg bw HC Blue n° 12. Two animals per sex were examined for acute toxic symptoms at intervals of around 1, 2-4, 6, 24, 30, and 48h after administration of HC Blue n° 12. On the basis of these results 1250 mg/kg was selected as the maximum tolerated dose level. In the main experiment bone marrow cells were collected 24h and 48h (highest dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between normochromatic to polychromatic erythrocytes (PCE/NCE ratio). The animals were examined for acute toxic symptoms at intervals around 1, 2-4, 6 and 24 h after treatment. Bone marrow preparations were stained and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment for toxicity, animals treated with doses from 1250 mg kg/bw expressed toxic effects like reduction of spontaneous activity, abdominal position, eyelid closure, ruffled fur and purple urine. At 1250 mg kg/bw only reduction of spontaneous activity and ruffled fur remained but were lost after 6 h except for the purple urine. In the main experiment the similar toxic effects were observed; additionally some mice treated with 1250 mg kg/bw showed abdominal position sand eyelid closure. The urine of all treated animals was blue.
The ratio PCE/NCE was not substantially changed in the treated animals indicating that HC Blue n° 12 did not have cytotoxic properties in the bone marrow. However, the blue coloured urine of the treated animals together with the observed toxic effects indicate the systemic distribution and thus bioavailability in the bone marrow of HC Blue n° 12.
At the mid-dose a slightly higher percentage of micronucleated cells was found. However, this value as well as the individually numbers of the individual animals were within the historical control range. Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were therefore not found following treatment with HC Blue n° 12 at any time point.

Conclusion
Under the experimental conditions used HC Blue n° 12 did not induce micronuclei in bone marrow cells of treated mice and, consequently, HC Blue n° 12 was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 30

Comment
Up on the sponsors request the test was performed exclusively with male mice. The SCCP agrees since in the preliminary experiment on acute toxicity no differences in sensitivity towards HC Blue n° 12 were observed for both male and female mice.

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline: OECD draft guideline 486
Species/strain: male Wistar/WU rats
Group size: 4 rats per dose
Test substance: GHS 111183
Batch: 011-01/27-04
Purity: about 97.8%
Dose level: 800 and 1600 mg/kg bw

Route: oral gavage
Vehicle: deionised water
Sacrifice times: 2 h (highest dose only) and 16 h after dosing
GLP: in compliance

GHS 111183 has been investigated for the induction of unscheduled DNA synthesis in primary hepatocytes of F344 rats. Test concentrations were selected on the basis of a pre-experiment on acute toxicity. Hepatocytes for UDS analysis were collected 2 h (highest dose only) and 16 h after start of the treatment. Hepatocytes were isolated by *in situ* perfusion with the proteolytic enzyme collagenase. At least 90 minutes after plating the cells were incubated for 4 h with 5 µCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol) followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 12 - 14 days.
UDS was reported as net nuclear grain: the nuclear grain count subtracted with the cytoplasmic grain count. Negative and positive controls were in accordance with the draft OECD guideline.

Results
No toxic reactions of the animals occurred at any of the treatment periods or dose groups. The viability of the isolated hepatocytes was not substantially affected due to the *in vivo* pre-treatment with the test article. No dose level of the test compound revealed UDS induction in the treated primary hepatocytes as compared to the concurrent vehicle control. Neither the nuclear grains nor the resulting net grains were distinctly enhanced due to the *in vivo* treatment of the rats with the test compound.

Conclusion
Under the experimental conditions used the test compound GHS 111183 did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in primary hepatocytes of treated rats.

Ref.: 31

In vivo alkaline single cell gel electrophoresis (Comet) assay in mice

Guideline: / (according to an international accepted protocol for the in vivo Comet assay)
Species/strain: CRL (WI) BR Wistar rats
Group size: 5 male rats/group
Test substance: HC Blue n° 12 (WR20854)
Batch: 29/30
Purity: 97.9 % by NMR and 98.4 % by HPLC
Dose level: 0, 500, 1000 and 2000 mg/kg bw
Route: oral gavage, twice, 20 h apart.
Vehicle: deionized water
Sacrifice times: 23 h after start of the first treatment
Organs studied: liver, stomach and urinary bladder epithelium
GLP: in compliance

HC Blue n° 12 has been investigated for the induction of DNA damage in the alkaline single cell gel electrophoresis (Comet) assay in various tissues of rats. Test concentrations were based on the results of a dose range finding study with male mice treated with doses up to 2000 mg kg/bw for 20 hours. Samples of liver, stomach and urinary bladder epithelium were examined histopathologically. As a result the mice were exposed in the main experiment by oral gavage to 500, 1000 and 2000 mg/kg bw HC Blue n° 12. Mice were treated twice, 20 h apart, and sacrificed 3 h after the last treatment. Per organ 100 nuclei were examined. Tail length (the distance between the middle of the head and the end of the

tail) was used as assessment parameter. Additionally the tail moment and the % tail DNA were determined. Ethyl methane sulphate (300 mg/kg bw) was used as positive control.

Results
In the main test rats showed no clinical signs of toxicity. Just prior to sacrifice discoloured urine and /or piloerection were observed. Additionally the content of the bladder was discoloured. These findings demonstrate relevant systemic exposure to the test compound.
The viability of the isolated cells of the exposed liver, stomach and urinary bladder epithelium was comparable to the concurrent control values. After exposure to the test compound the tail lengths of liver, stomach and urine bladder cells of male exposed rats were comparable to values of the cells of the respective tissues collected from vehicle control rats.
Since the results of the comet assay were clearly negative, no histopathological investigation was needed.

Conclusion
Under the experimental conditions used HC Blue n° 12 did not induce DNA damage (measured as increase in tail length) in liver, stomach and urinary bladder of rats. Consequently, HC Blue n° 12 is not genotoxic in the Comet assay with rats under these conditions.

Ref.: 33

Comment
Using tail length as assessment parameter, the study authors concluded that HC Blue n° 12 did not induce DNA damage in liver, stomach and urine bladder. However, evaluation of the data for other parameters (tail moment and % tail DNA) revealed an increase in DNA damage. Therefore, the SCCP considers the results from this Comet assay as being equivocal.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline: /
Species/strain: Rat Wistar BOR:WISW-SPF TNO
Group size: 24 females per dose group
Test substance: Nitroblau hydrochloride suspended in deionised water
Batch: L4/154
Purity: 93.2 % (HPLC)
Dose: 0, 15, 60 and 140 mg/kg bw/d
Route: oral, gavage
Exposure: once daily, day 5 to 15 of gestation
GLP: in compliance

The test substance was given once daily by oral gavage to pregnant rats from day 5 to 15 of gestation at a dose of 15, 60 and 140 mg/kg bw/d. The controls received the vehicle only. Daily clinical observations were made on all animals with regard to sensoric and motoric behaviour considering hair coat, urine and faecal excretion and conditions of orifices.

Individual weights were recorded on day 0, 5, 10, 15 and 20 and food consumptions were measured for the given intervals. On day 20 the dams were sacrificed and a macroscopic evaluation of the organs was performed. The foetuses were developed by caesarean section and externally examined. 1/3 of the foetuses were evaluated for organic imperfections and the remainder was subjected to skeletal examination.

Results
With the exception of violet staining of urine (all treated animals) and fur, paw and tail (high dose group) no clinical signs were noted. Food consumption and body weight changes of the dams were not significantly different from controls. Foetal weight, sex ratio, placental weight, number of resorptions and corpora lutea were not influenced by treatment. Examination of skeleton and viscera revealed no relevant findings.

Conclusion
The NOAEL of maternal and developmental toxicity in this study is 140 mg/kg bw/d.

Ref.: 34

Comment
The highest dose of 140 mg/kg bw is too low for hazard assessment. For this, the highest dose used should be 1000 mg/kg bw and should induce toxicity.

3.3.9. Toxicokinetics

3.3.9.1. Toxicokinetics *in vitro*

Bioavailability across intestinal barrier in TC-7 (human intestinal epithelial) cells

Guideline:	/
Cells:	Human intestinal epithelial cell line TC-7
Test substance:	HC Blue n° 12
Batch:	29/30
Purity:	93.9 area% (HPLC)
Test concentration:	50 µM in HBSS buffer containing 1% DMSO
Incubation time:	60 min
Number of experiments:	two independent experiments
GLP:	not in compliance

The bioavailability of HC Blue n° 12 across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro*. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37 °C in 96-well transwell plates with shaking for a 60 min contact time. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of HLPC-MS/MS and the apparent permeability coefficient (P_{app}) was calculated for two independent experiments. ^{14}C-mannitol (about 4 µM) was used to demonstrate the integrity of the cell monolayer. Only monolayers revealing a permeability of < 2.5 x 10^{-6} cm/sec were used. Propranolol, atenolol, vinblastine and ranitidine were analysed concurrently to demonstrate the validity of the test system.
According to the laboratory’s classification system, a low permeability is considered for test items revealing a P_{app} < 2 x 10^{-6} cm/sec. A P_{app} of 2 - 20 x 10^{-6} cm/sec and a P_{app} ≥ 20 x 10^{-6} cm/sec classify a substance to have a moderate and a high permeability, respectively. As recommended by FDA, ranitidine (50 % absorption in humans) was used as the low permeability reference compound and propranolol (90 % absorption in humans) was used as the high permeability reference compound.
Results
The total recovery for the reference substances ranged from 56 to 191% and from 100 – 109% for HC Blue No. 12. The figures for the reference substances propranolol (P_{app} = 29.6 x 10^{-6} cm/sec) and ranitidine (P_{app} = 0.4 x 10^{-6} cm/sec) within the acceptance range of 20 –

45 x 10^{-6} cm/sec and 0.2 – 2 x 10^{-6} cm/sec, respectively. HC Blue No. 12 revealed a P_{app} of 65.9 x 10^{-6} cm/sec and was classified to be of high permeability in this test system.

Ref.: 35

Conclusion
Since absorption across the intestinal epithelium is considered to be a limiting factor of the uptake through the gastro-intestinal tract, the high permeability observed in this assay hints to a good absorption of HC Blue No. 12 after oral administration.

3.3.9.2. Toxicokinetics *in vivo*

Disposition and excretion following topical application in rats

Guideline:	/
Species/strain:	Rat, strain Long-Evans
Test substance:	*[^{14}C]* HC Blue No. 12 (ring labelled), radioactive purity 96 % specific activity: 2.01 µCi/mg mixed with non-radioactive HC Blue No. 12
Batch:	/
Dose:	1.667 mg/cm^2 test substance in a 1.5 % non-oxidative hair dye formulation, single cutaneous application, 0.5 h occlusive
Reference dose:	1.667 mg/cm^2 10 % (w/v) in DMSO single cutaneous application, 24 h occlusive
Duration:	72 h
GLP:	not in compliance

[^{14}C] HC Blue No. 12 was dermally applied to the clipped skin of 3 female and 3 males Long-Evans rats, either as a solution in DMSO (reference dose) or as a hair dye formulation. The contact time was 0.5 h for the hair dye formulation and 24 h for the reference. Radioactivity was determined in rinsing water, treated skin areas, urine, faeces, organ and carcass.

Results
95.85 – 96.52 % of the applied radioactivity was removed from the skin. In the urine 0.4 % (males) and 0.8 % (females) of the dose was excreted after 72 h, in the faeces 0.2 % (males) and 0.3 % (females) of the dose was excreted after 72 h; 2.36 % (males) and 2.92 % (females) remained in the skin. Of the radioactivity absorbed approximately 30 % was recovered in the faeces indicating biliary excretion. 60 – 79 % of the elimination via urine was within 24 h. The total residue in all tissues including the carcass and excluding the application site represented about 0.1% of the applied dose.

Ref.: 36

Conclusion of the SCCP
Summing up for females the percentages found in urine (0.8 %), faeces (0.3 %) and the residue in all organs and the carcass (0.1 %) the percutaneous absorption of HC Blue n° 12 was about 1.3% (21.7 µg/cm^2). But is has to mentioned that absorption was only determined under non-oxidative conditions.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(HC Blue n° 12)
(oxidative and non-oxidative)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**21.7 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**15.19 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.253 mg/kg**
No observed adverse effect level (13-week, oral, rat)	**NOAEL**	**=**	**60 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**237**

3.3.14. Discussion

Physico-chemical properties
HC Blue n° 12 is used as a non-reactive hair colouring agent ("Direct Dye") in semi-permanent hair dye formulations at a maximum on-head concentration of 1.5 %, as well as a non-reactive hair colouring agent ("Direct Dye") in oxidative hair dye formulations at a maximum on-head concentration of 0.75 %.
HC Blue n° 12 is both a secondary and a tertiary amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.
No data submitted for the stability of the test substance in solutions or in the marketed products.

General toxicity
In acute toxicity studies in mice and rats, based on the mortality rates LD_{50} figures from 1668 to 1775 mg/kg bw were calculated. The NOAEL of the 28 day toxicity study is 316 mg/kg bw/d. The dose of 60 mg/kg bw/d is considered the NOAEL dose of subchronic toxicity. The NOAEL of maternal and developmental toxicity is 140 mg/kg bw/d.

Irritation / sensitisation
Undiluted HC Blue n° 12 did not cause any signs of skin irritation in the rabbit. Under the conditions of the test, undiluted HC Blue n° 12 caused transient eye irritation in rabbits.

In local lymph node assay, the test substance was considered to be a moderate skin sensitising agent.

Dermal absorption
Considering the study results from the *in vitro* penetration assay without hydrogen peroxide, an A_{max} of 4.2 µg/cm² after application of 1.5 % (the intended maximum use concentration) HC Blue n° 12 was determined.
With an oxidative hair dye formulation containing the intended maximum concentration of 0.75 %, an A_{max} of 6.1 µg/cm² was obtained.
However, too few chambers and only 1 donor were used in these experiments. Accordingly, an absorption of 21.7 µg/cm² obtained from the toxicokinetic study (reference 36) in the rat is used for calculating the MOS. This *in vivo* study used only non-oxidative conditions. But the *in vitro* data indicate that the value of 21.7 µg/cm² is a worse case assumption.

Mutagenicity / genotoxicity
Overall, the genotoxicity program on HC Blue n° 12 investigated the three types of mutation: gene mutation, structural chromosome mutation and aneuploidy. Under *in vitro* conditions HC Blue n° 12 induced gene mutations in the gene mutation assay in bacteria and in mammalian cells at the *tk* locus of mouse lymphoma cells. It also induced chromosome aberrations and/or aneuploidy in a micronucleus test.
An *in vivo* UDS test was negative. In an *in vivo* micronucleus assay, HC Blue n° 12 did not induce an increase in micronucleated erythrocytes in mice. However, an *in vivo* Comet assay did not lead to a clear negative result. Although no increase in tail length as the measure for DNA migration was found, small increases were measured when using other image analysis parameters. The SCCP considered the result of this Comet assay as equivocal. Consequently, clarification of this test result is required. It is suggested to repeat the *in vivo* Comet assay including slight protocol modifications.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCP is of the opinion that the information submitted is insufficient to allow a final risk assessment to be carried out.

Before any further consideration, the equivocal result obtained with the *in vivo* Comet assay has to be clarified. It is suggested to repeat the Comet assay taking appropriate protocol modifications into consideration.

Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

6. REFERENCES

1. Simon, G. A.; Maibach, H. I.; The pig as an experimental animal model of percutaneous permeation in man: Qualitative and quantitative observations - An overview; SKIN PHARMACOL. PHYSIOL.; 13, 229-234; 2000
2. Lange, J.; Partition Coefficient (n-octanol / water): Shake flask method; NOACK; 2005
3. Jonas, W.; Physikalisch-chemische Untersuchungen mit der Prüfsubstanz Nitroblau; NATEC; 1991
4. Lange, J.; Prediction of the pKa-value; NOACK; 2004
5. Dommröse, A.-M.; Bestimmung des Schmelzpunktes bzw. des Schmelzbereiches der Prüfsubstanz Nitroblau gem. OECD-Richtlinie 102 bei Normaldruck; NATEC; 1990
6. Jonas, W.; Bestimmung des Siedepunktes oder der Zersetzungstemperatur Prüfsubstanz Nitroblau bei Normaldruck; NATEC; 1991
7. Dommröse, A.-M.; Bestimmung der Dichte der Prüfsubstanz Nitroblau bei 293 K (20°C) gemäß OECD-Prüfrichtlinie 109; NATEC; 1990
8. Dommröse, A.-M.; Bestimmung des Gesamtdampfdrucks der Prüfsubstanz Nitroblau bei 293 K (20°C) gemäß OECD-Prüfrichtlinie 104; NATEC; 1990
9. Dommröse, A.-M.; Bestimmung der Oberflächenspannung wässriger Lösungen der Prüfsubstanz Nitroblau gemäß OECD-Prüfrichtlinie 115; NATEC; 1989
10. Dommröse, A.-M.; Bestimmung der Wasserlöslichkeit der Prüfsubstanz Nitroblau bei 293 K (20°C) nach OECD-Richtlinie No. 105; NATEC; 1990
11. Lieske, M.; Untersuchung und Beurteilung des Stoffes "Nitroblau" für eine Anmeldung nach dem Chemikaliengesetz (ChemG); BAM; 1989
12. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers concerning HC Blue no. 12; SCCNFP; 1999
13. Weide, J.; Akute orale Toxizitätsprüfung von 1-(ß-Hydroxyethyl)-amino-2-nitro-4,N-ethyl-N-(ß-hydroxyethyl-amino)-benzol; WELLA AG; 1985
14. Sterner, W.; Akute dermale Toxizität an Kaninchen mit Nitroblau (in Anlehnung an die OECD-Richtlinien)-Limit-Test-; IBR; 1988
15. Bornatowicz, N.; Acute dermal irritation/corrosion study with Nitroblau; SEIBERSDORF; 1990
16. Borantowicz, N.; Acute eye irritation/corrosion study with Nitroblau; SEIBERSDORF; 1990
17. Ravel, G.; HC Blue No. 12 WR 20854 - Local lymph node assay; MDS PHARMA SERVICES; 2004
18. Contact sensitisation: Classification according to potency; ECETOC; 2003
19. Beck, H.; Cutaneous absorption of HC Blue No. 12 (=WR20854) in «"basic formulation for ringtest" with 1,5% Nitroblau» (derived from a typical hair dye formulation) through pig skin in vitro; COSMITAL SA; 2004
20. Beck, H.; Cutaneous absorption of 0.75% HC Blue No. 12 (= WR20854) in a typical formulation for oxidative hair dyes through pig skin in vitro; COSMITAL SA; 2004
21. Bornatowicz, N.; 28-Day toxicity study with "Nitroblau" in rats; SEIBERSDORF; 1991
22. Weide J.; Range finding study Three-month toxicity test in rats with the test substance Nitroblue (Original report: Reference 23); WELLA AG; 1986
23. Weide, J.; Range finding study 3-monatige Toxizitätsprüfung an Ratten mit der Testsubstanz Nitroblau (Translation: Reference 22); WELLA AG; 1986
24. Weide, J.; Three-month toxicity test in rats with the test substance Nitroblue (Original report: Reference 25); WELLA AG; 1988
25. Weide, J.; 3-monatige Toxizitätsprüfung an Ratten mit der Testsubstanz Nitroblau (Translation: Reference 24); WELLA AG; 1988
26. Thompson, P. W.; Nitroblue HCl: Reverse mutation assay "Ames Test" using Salmonella Typhimrium; SAFEPHARM; 1991
27. Grötsch, W.; Mutagenicity evaluation of GHS 111183 in the Ames Salmonella/Microsome plate-assay; L+S GmbH; 1991
28. Wollny, H.-E.; Cell mutation assay at the Thymidine Kinase Locus TK (+/-) in mouse lymphoma L5178Y cells with HC Blue No. 12 (WR 20854); RCC-CCR; 2005

29. Clare, G.; HC Blue No. 12 (WR 20854): Induction of micronuclei in cultured human peripheral blood lymphocytes; COVANCE; 2005
30. Honarvar, N.; Micronucleus assay in bone marrow cells of the mouse with HC Blue No. 12 (WR20854); RCC-CCR; 2005
31. Fautz, R.; In vivo / in vitro unscheduled DNA synthesis in rat hepatocytes with GHS 111183; CCR; 1993
32. Hartmann, A.; Agurell, E.; Beevers, C.; Brendler-Schwaab, S.; Burlinson, B.; Clay, P.; Collins, A.; Smith, A.; Speit, G.; Thybaud, V.; Tice, R. R.; Recommendations for conducting the in vivo alkaline Comet assay; MUTAGENESIS; 18, 45-51; 2003
33. Wirnitzer, U.; WR20854 - Comet Assay in vivo in male rat liver, stomach and urinary bladder epithelium; BAYER; 2005
34. Sterner, W.; Teratogenicity study on "Nitroblau" by oral application to Wistar rats; IBR; 1985
35. Kennedy, K. J.; ADME: A-B permeability - Study of HC Blue No. 12; CEREP; 2005
36. Hawkins, D. R.; The disposition and excretion of ^{14}C-Nitroblue following topical application; HUNTINGDON; 1986
37. König, P.; Data base search for references for B073 - HC BLUE NO. 12: 132885-85-9; WELLA AG; 2005

Submission I

3. Weide, J.; Augen- und Augenschleimhautverträglichkeit am Albinomeerschweinchen mit 1-(ß-Hydroxyethyl)-amino-2-nitro-4,N-ethyl-N-(ß-hydroxyethyl-amino)-benzol; WELLA AG; 1985
5. Weide, J.; Prüfung der primären Hautirritation am Albinomeerschweinchen gemäß den OECD-Richtlinien, Nr.404 - Testsubstanz: 1-(ß-Hydroxyethyl)-amino-2-nitro-4,N-ethyl-N-(ß-hydroxyethyl-amino)-benzol; WELLA AG; 1986)
7. Sterner, W.; Nitroblau - Delayed contact hypersensitivity in guinea-pigs, modified method of B. Magnusson and A. M. Kligman; *IBR*; 1985

Other references

Bornatowicz, N.; Skin sensitisation study with "Nitroblau" (Guinea Pig Maximisation Test); SEIBERSDORF; 1991