EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

HC Red n° 13

COLIPA n° B31

Scientific Committees
- on consumer products
- on emerging and newly identified health risks
- on health and environmental risk

The SCCP adopted this opinion at its 12th plenary meeting on 19 June 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.

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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Keywords: SCCP, scientific opinion, hair dye, B31, HC Red n°13, directive 76/768/ECC, CAS 29705-39-3, CAS 94158-13-1, EINECS 303-083-4

Opinion to be cited as: Opinion of the SCCP on HC Red n° 13, 19 June 2007

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1. BACKGROUND

Submission I for HC Red n° 13 with the chemical name 2,2'-[(4-Amino-3-nitrophenyl)imino]-bisethanol hydrochloride was submitted in July 1992 by COLIPA [1], [2].

Submission II for HC Red n° 13 was submitted in April 1995 by COLIPA [2].

The Scientific Committee on Cosmetology (SCC) adopted at its 62nd plenary meeting on 18 January 1996 an opinion (SCC/106/92) with the final conclusion that:

"*The irritation tests showed no harmful effects. The substance (C.P. [3]) can be classified as non-sensitizer. In the 90-day studies with rats, 5 mg/kg bw C.P. is considered to be the NOAEL. In the teratogenicity study, no signs of maternal or foetal toxicity were observed in the rat after administration of 30 mg/kg bw (NOAEL). 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. Percutaneous absorption of a formulation was about 0.05.*"

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 31 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 Red n° 13 was submitted by COLIPA in July 2005. According to this submission the substance is used as:

a) a non-reactive hair colouring agent ("direct dye") in non-oxidative hair dye formulations at a maximum on-head concentration of 2.5%. 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 1.25%. If used in oxidative hair dye formulations, the dye component and 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.

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA
[3] C.P. commercial product

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider HC Red n° 13 safe for use as a non-oxidative hair dye with an on-head concentration of maximum 2.5% taken into account the scientific data provided?*

2. *Does the SCCP consider HC Red n° 13 safe for use as an oxidative hair dye with an on-head concentration of maximum 1.25% taken into account the scientific data provided?*

3. *Does the SCCP recommend any further restrictions with regard to the use of HC Red n° 13 in any non-oxidative or oxidative hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

HC Red n° 13 is used in hair dyes as hydrochloride salt. In the past the free base of HC Red n° 13 was also used in market formulations as well as in some of the toxicological investigations presented in this opinion. Therefore, this opinion describes the available information for both the free base and the hydrochloride salt.

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Red n° 13 (INCI name)

Free base
2,2'-((4-Amino-3-nitrophenyl)imino)bisethanol

3.1.1.2. Chemical names

Free base
2,2'-((4-Amino-3-nitrophenyl)imino)bisethanol
Ethanol, 2,2`-[(4-amino-3-nitrophenyl)imino]bis- (CA index name, 9CI)
2-[(4-Amino-3-nitrophenyl)(2-hydroxyethyl)amino]ethanol
4-(Di(2-hydroxyethyl)amino)-2-nitroaniline
2-nitro-4-bis(β-hydroxyethyl)-p-phenylenediamine
1-amino-4-bis(β-hydroxyethyl)amino-2-nitrobenzene
1-amino-2-nitro-4-bis(β-hydroxyethyl)-aminobenzene
2-nitro-4-bis(β-hydroxyethyl)aminoaniline
4-bis(β-hydroxyethyl)amino-2-nitroaniline
4-amino-N,N-bis(β-hydroxyethyl)-3-nitroaniline
4-N,N-bis(2-hydroxyethyl)-2-nitro-p-phenylenediamine

Hydrochloride
2,2'-[(4-amino-3-nitrophenyl)imino]bisethanol monohydrochloride
Ethanol, 2,2`-[(4-amino-3-nitrophenyl)imino]bis-, monohydrochloride (CA index name, 9CI)
2-[(4-Amino-3-nitrophenyl)(2-hydroxyethyl)amino]ethanol monohydrochloride

4-(Di(2-hydroxyethyl)amino)-2-nitroaniline monohydrochloride
2-nitro-4-bis(β-hydroxyethyl)-p-phenylenediamine monohydrochloride
1-amino-4-bis(β-hydroxyethyl)amino-2-nitrobenzene monohydrochloride
1-amino-2-nitro-4-bis(β-hydroxyethyl)-aminobenzene monohydrochloride
2-nitro-4-bis(β-hydroxyethyl)aminoaniline monohydrochloride
4-bis(β-hydroxyethyl)amino-2-nitroaniline monohydrochloride
4-amino-N,N-bis(β-hydroxyethyl)-3-nitroaniline monohydrochloride
4-N,N-bis(2-hydroxyethyl)-2-nitro-p-phenylenediamine monohydrochloride

3.1.1.3. Trade names and abbreviations

COLIPA n° B31
Jarocol Red 13 (Robinson)

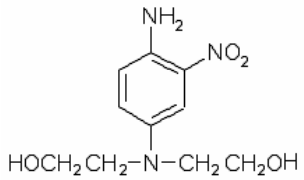
From previous submissions:
Kardinalrot
Kardinalrot (commercial Product), mixture of:
- 84% 1-amino-2-nitro-4-bis-(β-hydroxyethyl)-amino-benzene (B31)
- 13% 1-(ß-hydroxyethyl)-amino-2-nitro-4-bis-(β-hydroxyethyl)-amino-benzene (B37)
- 3% 1-amino-2-nitro-4-(β-hydroxyethyl)-amino-benzene hydrochloride

Substance code: A000774 (free base)
Substance code: A004820 (hydrochloride)

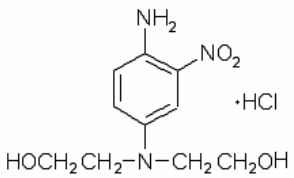
3.1.1.4. CAS / EINECS number

Free base:	CAS:	29705-39-3
	EINECS:	/
Hydrochloride:	CAS:	94158-13-1
	EINECS:	303-083-4

3.1.1.5. Structural formula



Free base



Hydrochloride

3.1.1.6. Empirical formula

Free base:	Formula:	$C_{10}H_{15}N_3O_4$
Hydrochloride:	Formula:	$C_{10}H_{15}N_3O_4.HCl$

3.1.2. Physical form

Yellow powder

3.1.3. Molecular weight

Free base:	Molecular weight:	241.3
Hydrochloride:	Molecular weight:	277.7

3.1.4. Purity, composition and substance codes

Material used in the market (deduced specifications)

Free base

HPLC qualitative (254 nm): > 99%
NMR quantitative: > 99% w/w
Solvent content: < 0.4% w/w
Ash: < 0.2% w/w

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

Hydrochloride

HPLC qualitative: > 98%
NMR quantitative: > 91.5% w/w
Solvent content (water): 4-8% w/w (< 7% w/w)
Ash: < 0.1% w/w

N-Nitrosodiethanolamine (NDELA) (EU CMR carcinogen category 2): < 20 ppb
4-(Di(2-hydroxyethyl)amino)-N-(2-hydroxyethyl)-2-nitro-aniline (HC Blue n° 2): < 1% w/w
4-((2-Hydroxyethyl)amino)-2-nitro-aniline (HC Red n° 7): < 1% w/w
1,4-Diamino-2-nitrobenzene (MAK carcinogen category 3b): < 1000 ppm
4-(Di(2-hydroxyethyl)amino)-2-nitro-phenol: < 300 ppm

Batch comparison

Description of sample	**4/5/6-1 Fass 3 (corresponds to 4/5/6/1 and R97006481)**	**R0011021**	**199/027**
	Hydrochloride	**Hydrochloride**	**Free base**
NMR content (%, w/w)	91.6#	91.8#	99.8##
HPLC purity (area %**)			
UV assay 210 nm	-	99.4	99.6
UV assay 254 nm	98.4	99.4	99.7
UV assay 520 nm	98.6	99.4	99.7
Potential Impurities			
N-Nitrosodiethanolamine (NDELA) (ppb)	< 5		< 4
4-(Di(2-hydroxyethyl)-amino)-N-(2-hydroxy-ethyl)-2-nitro-aniline (HC Blue n° 2) (ppm)	300	100	< 150°
4-((2-Hydroxyethyl)-amino)-2-nitro-aniline (Red HF) (%, w/w)	0.23	0.2	0.16
1,4-Diamino-2-nitrobenzene (ppm) °°	**1000**	20	40
4-(Di(2-hydroxyethyl)-amino)-2-nitrophenol (ppm):	100	30	ca. 70
4-((2-Hydroxyethyl)-amino)-5-methyl-2-nitroaniline (HC Violet No. 1) (ppm)	< 10°	< 1°	< 100°
Chloride (%, w/w	11.7	12.6	-
Water (%, w/w)	6.4	6.2	0.31
Loss on drying (%, w/w)	7.7	5.5	0.04
Ash (%, w/w)	< 0.1	0.02	0.14
Element screening	-		Na 709 ppm

The NMR content is based on the molecular formula of HC Red 13 with a molecular weight of 277.71 g/mol.

The NMR content is based on the free base of *HC Red No. 13* with a molecular weight of 241.25 g/mol.
** HPLC conditions: Purosphere RP-C18 250*4 mm, 5µm; eluent: 15% acetonitrile/85% 0,02 mM KH_2PO_4 pH 5.1
° Not detected, shown value indicates limit of detection
°° MAK classification carcinogenic category 3

No documentation was provided to support the data presented in the above table.

3.1.5. Impurities / accompanying contaminants

See above (point 3.1.4.)

3.1.6. Solubility

Water solubility**:** Using the official EEC method (EU - A6) the solubility of HC Red n° 13 (hydrochloride, batch R97006481) was determined to be 197 mg/l at pH 1.70 at 20 °C.

Soluble in:

acetone / water 1:1	> 100 g/l (pH 1.4)
DMSO	> 100 g/l

3.1.7. Partition coefficient (Log P_{ow})

P_{ow}	4.17		
log P_{ow}	0.62 (at pH 7.22, room temp.)	(method EU - A.8)	*Ref. 1*

3.1.8. Additional physical and chemical specifications

Melting point:	95.2 – 97.2°C (decomposition)	(method EU – A.1)	*Ref. 5*
Boiling point:	decomposition at 93°C	(method EU – A.2)	*Ref. 6*
Flash point: (rel. self-ignition temp.)	> 400 °C	(method EU - A.16)	*Ref. 12*
Vapour pressure:	/		
Density:	1.457 g/ml (20°C)	(method EU - A.3)	*Ref. 7*
Vapour pressure:	< 10^{-7} hPa (20°C, extrapolated)	(method EU - A.4)	*Ref. 8*
Surface tension (in water):	71.01 mN/m (20°C)	(method EU - A.5)	*Ref. 9*
Viscosity:	/		
Flammability (solids):	not highly flammable	(method EU - A.10)	*Ref. 10*
Explosive properties:	not explosive	(method EU - A.14)	*Ref. 11*
	> 400 °C(method EU - A.16)	*Ref. 12*	
Oxidising properties:	not oxidising	(method EU - A.17)	*Ref.13*
pH-value:	1.70 (saturated aqueous solution, 20°C)		*Ref. 3*
pKa-values (calc. Pallas Software)	15.09 for R-CH_2OH (acidic) 15.69 for R-CH_2OH (acidic) 3.24 for R-(Ar)-N-(R_1R_2) (basic) -0.38 for R-C_6H4-NH_3^+ (basic) -2.59 for R-CH_2OH (basic) -3.20 for R-CH_2OH (basic)		*Ref. 4*
UV_Vis spectrum:	λ_{max} = 210, 254, 520 nm		

3.1.9. Homogeneity and Stability

Homogeneity and stability was verified in several (not all) the toxicological studies, with the following indicative results:

comet Assay *in vivo*: The stability of the applied formulations of the test substance in water was demonstrated by analysis. The homogeneity and correctness of the dosing solutions was also analytically verified. The mean concentrations of the test samples were 93.4% and 93.2% of the nominal values of the two dose groups, confirming the proper dosing.

1-Generation reproduction toxicity: Stability, homogeneity and content of the solutions of HC Red n° 13 in the vehicle were analytically confirmed. Dosing solutions were prepared once per week as stability in the vehicle for up to 7 days could be demonstrated. The mean concentrations of the test samples were 98 and 103%, 99.9 and 101.6% as well as 100.4 and 99.2% of the nominal values for the three dose groups analysed twice at the beginning and at the end of the administration period. These values confirmed proper dosing for the entire study period. The slight variation (-2 to 1%) noted for the three samples taken from each dosing solution demonstrates the homogeneity of the test solutions used.

Teratogenicity study: Stability, homogeneity and content of the solutions of HC Red n° 13 was analytically confirmed. Dosing solutions were prepared once per week as stability in the vehicle for up to 7 days could be demonstrated. The mean recovery rates of the test samples were 95.4 and 98.4%, 98.6 and 97.6% as well as 98.7 and 96.7% of the nominal values for the low, mid and high dose groups, respectively, analysed twice at the beginning and at the end of the administration period. These values confirmed proper dosing for the entire study period.

General Comments to physico-chemical characterisation

- Data on characterization of the substance by NMR and HLPC are not provided.
- The stability of the test substance in marketed products is not reported.
- The test substance is 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 documentation was provided to support the data presented in the table "Batch comparison"

3.2. Function and uses

a) Semi-permanent Hair Colorants

HC Red n° 13 is used as a non-reactive hair colouring agent ("Direct Dye") in semi-permanent hair dye formulations at a maximum on-head concentration of 2.5 %.

b) Oxidative Hair Colorants

HC Red n° 13 is used in oxidation hair dye formulations at a maximum concentration of 2.5%, which after mixing typically in 1:1 ratio with hydrogen peroxide prior to use, corresponds to a final on head concentration of 1.25% upon application.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from opinion n° XXIV/1287/97 of 20 May 1998

Guideline:	/
Species/strain:	rat, CFY
Group size:	5 per sex and dose
Test substance:	HC Red n° 13
Batch:	not specified
Purity:	not specified
Route:	oral, gavage
Vehicle:	10% suspension in aqueous gum tragacanth (0.5%) containing sodium sulphite (0.05%), pH 7.0
Observation period:	14 days
GLP:	/

The test substance was administered once as a 10% suspension in aqueous gum tragacanth (0.5%) by gavage at doses 0 (control), 1000, 1600, 2500 and 4000 mg/kg bw to groups of 10 (5/sex) CFY rats (weight range was 100-120 g). During the following observation period of 14 days a record was kept of all mortalities and signs of toxicity. Autopsy of death was carried out for all rats that died. At the end of the observation period all surviving animals were sacrificed and gross necropsies performed.

Results
As substance-related effects lethargy, piloerection, diuresis and purple staining of the urine in all exposed groups were observed shortly after dosing, accompanied by ataxia in rats treated with 1000, 2500, or 4000 mg/kg bw and by increased lacrimation, decreased respiratory rate and purple staining of external extremities in rats treated with 2500 and 4000 mg/kg bw. Fine body tremors were observed within two hours of treatment in male rats at 2500 mg/kg bw. During the first week of observation depressed body weight gains were noted in the surviving rats at 2500 mg/kg bw.

Necropsy of mortalities revealed slight haemorrhage of the lungs and purple staining of all internal organs except the lungs. Terminal autopsy findings of survivors showed no extraordinary results. LD_{50} was calculated to be 2120 (1810 - 2480) mg/kg bw.

Ref.: 15

Comment
Despite the deficiencies of this study (purity and batch number unknown, not according to a guideline), it is useful for evaluation.

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

Rabbits

Guideline: /
Species/strain: albino rabbit
Group size: 3, sex not indicated
Test substance: HC Red n° 13
Batch: /
Purity: /
Dermal: Dermal
Dose: 0.5 ml, 2.5% aqueous solution applied to abraded and intact skin, 24 h, occlusive
GLP: /

0.5 ml of a 2.5% aqueous solution of HC Red n° 13 in distilled water containing 0.05% sodium sulphite with pH 7.0 was applied to intact and abraded dorsum skin (6.5 cm^2) clipped free of hair of 3 rabbits and kept in contact for 24 hours under occlusive conditions. Reactions were scored after patch removal as well as 24 and 48 hours later

Results
Under the conditions of the experiment, whereby only a 2.5% aqueous solution was applied, there was no irritant effect throughout the 72 hours observation period.

Ref.: 16

Comment
For hazard identification, the test concentration used was too low.

3.3.2.2. Mucous membrane irritation

Rabbits

Guideline: /
Species/strain: albino rabbits
Group size: 3, sex not indicated
Test substance: HC Red n° 13
Batch: /
Purity: /
Route: Ocular
Dose: 0.1 ml 2.5% solution of HC Red n° 13 in aqueous solution containing 0.05 % sodium sulphite (pH: 7.0)
GLP: /

0.1 ml of a 2.5% aqueous solution of HC Red n° 13 in distilled water containing 0.05% sodium sulphite with pH 7.0 was instilled into one eye of each of three albino rabbits, the other eye served as control. The treated eye was irrigated with 20 ml distilled water 10 seconds after instillation to remove the test item. The grade of ocular reaction was recorded at a 7-day observation period.

Results
Under the conditions of the experiment, whereby only a 2.5% aqueous solution was applied, there was no irritant effect throughout the 7 days observation.

Ref.: 17

Comment
For hazard identification, the test concentration used was too low. The diluted preparation was washed out 10 seconds after instillation.

3.3.3. Skin sensitisation

Guinea pig

Taken from opinion n° XXIV/1287/97 of 20 May 1998

Landsteiner and Draize protocol

Guideline: /
Species/strain: Guinea pig, Pirbright
Group size: 15 females, control group 10 females
Test substance: C.P. Kardinalrot
Batch: /
Purity: /
Concentrations: 1% induction, 1% challenge, in Ringer solution
GLP: /

Inducing procedure was performed by intracutaneous application of 0.1 ml of a 1% test substance dilution (in Ringer solution) into the shaven shoulder areas of 15 female Pirbright guinea pigs, 3 times daily on 5 consecutive days (10 animals served as control). Four weeks later test and control animals were challenged by an intracutaneous injection of 0.1 ml of the test solution (1%) into the untreated flanks.

Results
No allergic reaction was observed.

Ref.: 4 (XXIV/1287/97)

Comment
The concentrations used were far too low. The study can not be used in the evaluation.

Taken from opinion n° XXIV/1287/97 of 20 May 1998

Magnusson/Kligman protocol

Guideline: /
Species/strain: Guinea pig, Pirbright, Hoe:DHPK (SPF-LAC)/Boe
Group size: 20 females
Test substance: HC Red n° 13
Batch: /
Purity: 99%
Concentrations: 3% induction; 1, 2 and 3% challenge
GLP: /

Induction was performed by pair wise intra-cutaneous injections on the clipped shoulder region of 20 female Pirbright guinea pigs in the following sequence:
- 2 x 0.05 ml of Freund's Complete Adjuvant (FCA) (1:1 in aqua deion.)
- 2 x 0.05 ml of the test substance at 3% in aqua deion.

10 animals treated with 1-Chlor-2,4-dinitrobenzene (DNCB) served as positive control. They received 4 pair wise intra-cutaneous injections in the following order:
- 2 x 0.05 ml FCA (1:1 in aqua deion.)

- 2 x 0.05 ml 0.005% DNCB diluted in aqua deion.

Negative control consisted of a group of 10 animals:
- 2 x 0.05 ml FCA (1:1 in aqua deion)
- 2 x 0.05 ml aqua deion.

On the next day and 6-8 hours before the first dermal treatment all animals were pre-treated with sodium lauryl sulfate (10% in white Vaseline). Induction by percutaneous route was carried out by application of 0.5 ml of the test substance at a concentration of 3% in white Vaseline (24 h closed patch). Positive controls were treated with 0.025% DNCB (in white Vaseline) (0.5 ml), negative controls with 0.5 ml 3% aqua deion. (in white vaseline).

The second intradermal treatment was carried out 48 hours after the first one:
Test group: - 2 x 0.05 ml test substance 3% in FCA (diluted in arachidis oil 1:1)
Positive control: - 2 x 0.05 ml DNCB 0.005%
Negative control: - 2 x 0.05 ml aqua deionised

14 days after the last exposure test and control animals were challenged by a cutaneous application of (24 h closed patch):
Test group: - 0.5 ml each of 3, 2 and 1% test substance in FCA (diluted in arachidis oil 1:1)
Positive control: - 0.5 ml each of 1, 0.5 and 0.01% DNCB in FCA (diluted in oil arachidis 1:1)
Negative control: - 0.5 ml aqua deion.

Challenge sites were evaluated for cutaneous reactions 24 and 48 hours p.a.

Results
No primary skin irritations and no allergic reactions were observed, thus the substance was classified as non sensitizer, in spite the used challenge concentrations were relatively low (3%).

Ref.: 5 (XXIV/1287/97)

Comment
Both the induction and challenge concentration were too low. The study is considered inadequate.

Mice

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species/strain:	Mouse, strain CBA/J
Group size:	5 females
Test substance:	HC Red No. 13
Batch:	4/55/6-1 Fass 3
Purity:	98.4% (HPLC at 254 nm)
Concentrations:	0.5, 1.5, 5.0, and 10.0% (w/v) in DMSO and in aqua/acetone (1:1) mixed with olive oil (3:1)
GLP:	In compliance

25 µl of 0 (vehicles only), 0.5, 1.5, 5 and 10 % HC Red No. 13 in DMSO or in a mixture of aqua/acetone (1:1) with olive oil (3:1) (equal to the maximum solubility) were applied to the surface of the ear of five female mice per group for three consecutive days. As a positive control, p-phenylenediamine (PPD) at 1% in DMSO was investigated in parallel under identical test conditions.

Observation for clinical signs was done daily before and at least once after dosing and once daily thereafter. Body weigh was determined on day –1 and on day 5.

At day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 23.7 µCi of [H^3] methyl thymidine. Approximately five hours later, the mice were killed, and the draining auricular lymph nodes were removed and collected in PBS. After preparing a single cell suspension for 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).

Results
The stimulation indices in the groups with DMSO used as vehicle were:

1.5 (0.5% test group)
1.8 (1.5% test group)
2.1 (5.0% test group)
3.5 (10.0% test group)

From treatment with HC Red No. 13 in DMSO an EC3 was estimated by extrapolation to be 8.2%.

The stimulation indices in the groups with aqua/acetone/olive oil used as vehicle were:

1.3 (0.5% test group)
2.2 (1.5% test group)
1.2 (5.0% test group)
1.4 (10.0% test group)

From treatment with HC Red No. 13 in aqua/acetone/olive oil an EC3 could not be calculated as all values were below 3.

The sensitivity of the test system was demonstrated by the reaction of the positive control p-phenylenediamine (1%) in DMSO which exhibited a stimulation index of 12.5.

Ref.: 19

Comment
The substance is considered to be a moderate skin sensitiser.

3.3.4. Dermal / percutaneous absorption

Non-oxidative hair dye formulation

Guideline:	OECD 428 (2004)
Tissue:	Porcine skin (frozen/thawed; thickness: $\leq$ 1000 µm)
Test substance:	HC Red No. 13
Batch:	4/5/6-1 Fass 3
Purity:	98.49% (HPLC at 254 nm)
Dose levels:	2.5 mg/cm^2, (400 mg formulation containing 2.5% dye, applied to 4 cm^2) tested as part of non-oxidative hair dye formulation
No. of chambers:	6 (five for the formulation containing the test item and one for the blank formulation)
Receptor solution:	physiological phosphate buffer containing NaCl and antibiotics
GLP:	in compliance

The skin absorption of HC Red n° 13 was investigated with pig skin (approximately 940 µm thick) in non-oxidative formulation.

Diffusion chambers were used. The receptor solution was pumped through the receptor chamber at a rate of 5 ml/h. The integrity of the skin was monitored at the beginning of the experiment using tritiated water. Penetration rates of 0.94 to 1.9% of the applied dose were obtained. Two out of 6 skin samples were slightly outside the limit of acceptance (= 1.5%).

Sixty minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, then once with 4 ml of a shampoo-formulation (diluted to approximately 14%), and again twice with water. The rinsing solutions were combined and the amount of dye was determined by HPLC.

Fractions of the receptor fluid were collected after 16, 24, 40, 48, 64 and 72 hours. 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
The limit of quantification of the applied method was 10 ng/HPLC-injection. The mean recovery of the test item was 97.55% with the major part of the test item (96.1 ± 0.6% (2.4 ± 0.01 mg/cm^2) being not absorbed into the skin during the application period as it was determined in the combined washing solutions.

72 hours cutaneous absorption of 2.5% HC Red No 13 in a typical hair dye formulation

Skin no	Integrity Test (% dose)	Receptor fluid 72 hour µg/cm^2 (% dose)	Epidermis 72 hour µg/cm^2 (% dose)	Upper dermis 72 hour µg/cm^2 (% dose)	Dermal absorption 72 hour µg/cm^2 (% dose)	Rinsing solution 60 min µg/cm^2 (% dose)
2	0.94	0.1 (0.0)	1.3 (0.1)	1.0 (0.0)	2.4 (0.2)	2400.7 (96.1)
4	1.74	1.4 (0.1)	1.6 (0.1)	1.4 (0.1)	4.4 (0.3)	2411.4 (96.5)
6	1.49	0.5 (0.0)	1.5 (0.1)	1.0 (0.0)	3.0 (0.2)	2380.1 (95.2)
8	0.97	0.6 (0.0)	2.3 (0.1)	0.5 (0.0)	3.4 (0.2)	2396.5 (95.9)
10	1.88	0.2 (0.0)	2.5 (0.1)	0.5 (0.0)	3.2 (0.2)	2418.8 (96.8)
12	1.09	-	-	-	-	-
Mean ±S.D	1.35 0.41	0.6 0.5	1.9 0.5	0.9 0.4	3.28 0.73	

After 72 hours, 0.6 ± 0.5 µg/cm^2 (highest value 1.4 µg/cm^2) of the test item was found in the receptor fluid. In the upper skin, 0.9 ± 0.4 µg/cm^2 (highest value 1.6 µg/cm^2), and in the lower skin, 1.9 ± 0.5 µg/cm^2 (highest value 2.5 µg/cm^2). By combining the amounts found in the receptor fluid and in the upper and lower skin, a skin penetration of 3.3 ± 0.7 µg/cm^2 (0.136%) was obtained. The highest value measured was 4.4 µg/cm^2.

Ref.: 20

Comment
The highest value measured was 4.4 µg/cm^2. The mean + 2x standard deviation was 4.7 µg/cm^2.

Oxidative hair dye formulation

Guideline: OECD 428 (2004)
Tissue: Porcine skin (frozen/thawed; thickness: ≤ 1000 µm)
Test substance: HC Red No. 13
Batch: 4/5/6-1 Fass 3
Purity: 98.49% (HPLC at 254 nm)
Dose levels: 1.25 mg/cm^2 (400 mg formulation containing 1.25% dye, applied to 4 cm^2) tested as part of an oxidative hair dye formulation (in the presence of hydrogen peroxide)
No. of chambers: 6 (five for the formulation containing the test item and one for the blank formulation)
Receptor solution: physiological phosphate buffer containing NaCl and antibiotics
GLP: in compliance

The skin absorption of HC Red n° 13 was investigated with pig skin (approximately 940 µm thick) in an oxidative formulation in the presence of hydrogen peroxide.

Diffusion chambers were used. The receptor solution was pumped through the receptor chamber at a rate of 5 ml/h. The integrity of the skin was monitored at the beginning of the experiment using tritiated water. Penetration rates of 1.2 to 2.6% of the applied dose were obtained. Two out of 6 skin samples were slightly outside the limit of acceptance (=1.5%).

Sixty minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, then once with 4 ml of a shampoo-formulation (diluted to approximately 14%), and again twice with water. The rinsing solutions were combined and the amount of dye was determined by HPLC.

Fractions of the receptor fluid were collected after 16, 24, 40, 48, 64 and 72 hours. 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
The limit of quantification of the applied method was 10 ng/HPLC-injection. The mean recovery of the test item was 97.6 ± 0.9% with the major part of the test item (97.4% being not absorbed into the skin during the application period as it was determined in the combined washing solutions.

72 hours cutaneous absorption of 1.25% HC Red No 13 in a typical hair dye formulation with 3% hydrogen peroxide

Skin no	Integrity Test (% dose)	Receptor fluid 72 hour µg/cm^2 (% dose)	Epidermis 72 hour µg/cm^2 (% dose)	Upper dermis 72 hour µg/cm^2 (% dose)	Dermal absorption 72 hour µg/cm^2 (% dose)	Rinsing solution 60 min µg/cm^2 (% dose)
2	1.6	0.11 (0.01)	0.68 (0.05)	0.24 (0.02)	1.03 (0.08)	1219 (97.5)
4	1.2	0.11 (0.01)	0.81 (0.07)	0.14 (0.01)	1.06 (0.08)	1203.4 (96.3)
6	1.3	0.13 (0.01)	0.87 (0.07)	0.09 (0.01)	1.09 (0.08)	1231.8 (98.5)
8	1.3	0.16 (0.01)	1.67 (0.13)	0.07 (0.01)	1.90 (0.15)	1211.9 (97.0)
10	2.6	0.24 (0.02)	1.63 (0.13)	0.06 (0.01)	1.93 (015)	1223.8 (97.9)
12	1.5	-	-	-	-	-

Skin no	Integrity Test (% dose)	Receptor fluid 72 hour µg/cm² (% dose)	Epidermis 72 hour µg/cm² (% dose)	Upper dermis 72 hour µg/cm² (% dose)	Dermal absorption 72 hour µg/cm² (% dose)	Rinsing solution 60 min µg/cm² (% dose)
Mean +S.D	1.6 0.5	0.15 0.05	1.13 0.48	0.12 0.07	1.40 (0.47)	

After 72 hours, 0.15 ± 0.05 µg/cm² (highest value 0.24 µg/cm²) of the test item was found in the receptor fluid. In the upper skin, 0.12 ± 0.07 µg/cm² (highest value 0.24 µg/cm²). In the lower skin, 1.13 ± 0.48 µg/cm² (highest value 1.67µg/cm²). By combining the amounts found in the receptor fluid and in the upper and lower skin, a skin penetration rate of 1.4 ± 0.5 µg/cm² was obtained. The highest value measured was 1.93 µg/cm².

Ref.: 21

Comment
The highest value measured was 1.93 µg/cm². The mean + 2x standard deviation was 2.3 µg/cm².

General comment on percutaneous absorption studies *in vitro*
These *in vitro* studies are not acceptable because:
- only five skin samples from one donor were evaluated;
- a dose of 100 mg formulation/cm² was used instead of 20 mg/cm².

Percutaneous absorption *in vivo*

Taken from opinion n° XXIV/1287/97 of 20 May 1998

Humans

A hair dye formulation at an average amount of 43.16 g containing 2.3% of the test substance (= dose of 15.13 mg/kg bw) was applied to 5 healthy female volunteer's washed hair for 15 minutes. Blood samples were taken 0, 10, 20, 30, 45 minutes and 1, 2, 3 and 24 hours after the application. Blood and urine samples were examined via HPLC (Detection limit 20 ng/ml in serum, 6 ng/ml in urine).

Results
The test substance could not be detected either in the serum or in the urine of the test persons. Therefore it was concluded that the amount of test substance absorbed was nil or, at any rate, less than 0.13% of the amount applied (less than 0.0195 mg/kg bw).

Ref.: 6 (XXIV/1287/97)

Study in rats

Guideline: /
Species/strain: Rat, Sprague Dawley Him:OFA (SPF)
Group size: 3 per sex and dose
Test substance: ^{14}C-HC Red No. 13 (ring-labelled)
Batch: /
Purity: > 97% (Radiochemical)
Dose levels: **A** Formulation without H_2O_2: 2%; 2.03 mg/cm²
B Formulation with H_2O_2: 2%; 2.21 mg/cm²
C Solution in water/DMSO (1:2); 6.66%; 2.27 mg/cm²
D Solution in water/DMSO (1:2); 2%; 21 mg/kg bw
E Solution in water/DMSO (1:2); 2%; 20.4 mg/kg bw

Route:	**A**, **B**, **C**; dermal **D**, **E**; oral
Treatment conditions:	**A**, **B**, **C**; Single dermal application for 30 min **D**, **E**; Single oral administration (gavage)
GLP:	In compliance

*Dermal application (experiment **A**, **B**, and **C**)*
^{14}C-HC Red No. 13 was applied dermally to groups of 3 male and 3 female Sprague Dawley rats (body weight about 200 g). The application area was 9 cm^2 and the test substance was applied at concentrations of 6.66% in solution (water/DMSO 1:2, **C**) and of 2% in the formulation without (experiment **A**) and with hydrogen peroxide (**B**) for 30 min each. The mean amount of dye applied was 2.03, 2.21 and 2.27 mg/cm^2, respectively.
After treatment, the remaining test substance was scraped off and the skin rinsed with a shampoo formulation followed by water until the rinsing water was free of colour. After rinsing, the area was covered with gauze during the 72 h in metabolism cages.

*Oral administration (experiment **D** and **E**)*
Doses of 21 mg/kg bw (**D**) and 20.4 mg/kg bw (**E**) ^{14}C-HC Red No. 13 were administered as a 2% solution in water/DMSO by gavage to two groups of 3 male and 3 female rats which were fasted for 16 hours before treatment. In one group (**D**), animals were placed in metabolism cages for 72 h. In the other group (**E**), blood was taken at several time points after administration until the animals were killed after 24 h.

Results
Total recovery of the applied radioactivity for the individual animals ranged from 96.9 to 99.0% of the applied doses.

Dermal application
Seventy two hours after application, the amount of radioactivity remaining at the application site (skin) was less than 1.2%. The majority of the dye (96.3 to 98.0%) was removed by the rinsing 30 min after application. 0.042, 0.047 and 0.049 % of the applied doses were eliminated via urine and faeces within 72 h in experiment **A**, **B**, and **C**, respectively. Elimination was fast: 78 – 90% was excreted within the first 24 h. 57 – 62% was excreted via urine. 0.2 to 2.1% of the absorbed dose remained in the carcass after 72h.
Based on the results, a cutaneous absorption of 0.86 μg/cm^2 was calculated for the formulation without hydrogen peroxide and 1.05 μg/cm^2 for the formulation with hydrogen peroxide and 1.13 μg/cm^2 for the pure dye in water/DMSO.

Oral administration
HC Red No. 13 was eliminated to an almost equal extent via urine (46%) and faces (54%). Elimination was fast as 92% was excreted within the first 24 h. The radioactivity found in tissues 72 h after administration was less than 0.015% of the applied dose per g organ. The blood level (**E**) reached a peak at 35 min after application and declined with an initial half-life of 2.3 h.

Conclusion
After dermal application, only minor amounts of HC Red No. 13 are absorbed through the skin and become systemically available. If applied in a hair dye formulation as vehicle less than 1.1 μg/cm^2 of the dye formulation penetrated the skin. The majority of the penetrated dye is excreted within 24 h after administration.
HC Red No. 13 given orally is quickly absorbed and nearly completely excreted within 72 h, with the majority eliminated within 24 h after application. Excretion takes place via urine and faces to almost equal amounts.
Low tissue residue levels were noted for both routes of exposure indicating that bioaccumulation is not expected. There were no significant sex differences in absorption, tissue distribution and excretion pattern.

Ref.: 35

3.3.5. Repeated dose toxicity

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

No data submitted

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

Taken from opinion n° XXIV/1287/97 of 20 May 1998

Study 1

Guideline:	OECD 408 (1981)
Species/strain:	Wistar rats, strain Bor.Wis.W. (SPF)
Group size:	15 animals per sex and group
Test substance:	Kardinalrot
Batch number:	/
Purity:	82 %, 17 % water
Dose levels:	0, 75, 150 and 300 mg/kg bw/day in de-ionised water by gavage
Exposure period:	13 weeks, 4 weeks recovery for satellite groups (control and high dose)
GLP:	in compliance

The test substance (dissolved in water) was administered by oral gavage once daily to groups of 30 Wistar rats (15/sex) (Bor: (Wi)W, SPF) at doses 0 (group I), 75 (group II), 150 (group III) and 300 (group IV) mg/kg/day for 90 days. 19 rats (10 f, 9 m) of the control group and additional 30 rats (15 f, 15 m), which were treated at the highest dose for the same period were examined for signs of reversibility after 4 weeks without treatment. The age at the start of the study was approximately 7-8 weeks and the average body weight was 134.4 g for females and 154.8 g for males. Food and water was provided *ad libitum*. After 13 weeks (group V: 16 weeks) the animals were sacrificed.

Examinations:

- Clinical signs and mortality daily.
- Ophthalmoscopic examination of groups I, IV and V at the start and at the end of the study.
- Body weights, food and water consumption weekly.
- Haematology (RBC, WBC, Diff., Hb, Hct, MCV, MCH, MCHC)
- Clinical Chemistry: (bil, glu, total protein, SGOT, SGPT, LDH, AP, Fe, Ca, urea, uric acid, creat, chol, triglyceride) at the start of the study and after 6 and 12 weeks (group I-V) and after 16 weeks (group I and V).
- Urinalysis (nitrite, leuco, pH, prot, gluc, ketones, urobil, bil, blood, sed) at the start of the study and after 5 and 11 weeks (group I-IV) or after 6 and 12 weeks (group V) and after 15 weeks (group I and V).
- Relative and absolute organ weights, gross pathology and histopathology of 10 animals/group (5/sex).

Results
- Two animals dosed at 300 mg/kg/day died.
- Haematology: Hb-values were significantly reduced in the females of groups II, IV and V after 12 weeks of treatment. After 6 weeks, eosinoph. granulocytes were reduced in the females in groups II-V, and after 12 weeks leukocytes were reduced significantly in the same groups. In males lymphocytes were significantly reduced in groups II, IV and V after 6 weeks of treatment and also in group V after 4 weeks of recovery.

- Clinical Chemistry: Alkaline Phosphatase was significantly reduced in the females after 6 weeks (group II, IV, V) and after 12 weeks of treatment (groups II and III). Ca-values were reduced in the males and in the females of groups III-V after 6 and after 12 weeks of treatment, as well as in the females of group V (after 4 weeks of recovery). After 12 weeks of treatment Fe was reduced significantly in groups IV and V in males as well as in females. After 4 weeks of recovery in the high dose females (Group V) significantly increased GOT-values were observed.
- Urinalysis: The urines of the groups treated at 150 and 300 mg/kg/day and some animals of group II were discoloured since the 3rd or 4th week of treatment. The discolouration disappeared after the reversibility period.
- Organ weights: Increased organ weights were noted for spleen (Group III, females (abs.) and II, III, IV, females (rel.)) and kidneys (Group IV, males, (rel.) and IV, females (rel.)). Brain weights were reduced significantly in the females of groups II-IV (V?). [Statistical comparison of relative and absolute organ weights after 4 weeks of recovery (group I and V) was not performed.]
- Gross pathology: Gross pathology revealed a dark discoloration of the thyroids in all groups except the control group. The number of affected animals increased with the dose level (1 in group II, 7 in group III, 16 in group IV; 9 in group V after recovery period.).
- Histopathology: The histopathological examination showed an activation of thyroid epithelium at doses of 150 and 300 mg/kg/day with increased intensity, accompanied by an enlargement of epithelium cell nuclei at the higher dose. This substance-related effect was restricted to males (4 in group III, 5 in group IV and 4 in group V). Furthermore a liver cell hypertrophy was observed in animals treated with 300 mg/kg/day (8 in group IV and 5 in group V after recovery period). In group V an increase of lipocytes in bone marrows was noted in 5/10 animals.

Conclusion
No NOAEL could be established.

Ref.: 22

Study 2

Guideline:	OECD 408 (1981)
Species/strain:	Wistar rats, strain Bor.Wis.W. (SPF)
Group size:	12 animals per sex and group
Test substance:	Kardinalrot
Batch number:	/
Purity:	82%, 17% water
Dose levels:	0, 10 mg/kg bw/day in de-ionised water by gavage
Exposure period:	13 weeks
GLP:	in compliance

The test substance (dissolved in water) was administered by oral gavage once daily to a group of 24 Wistar rats (Bor (Wi)W, SPF) (12/sex) at 10 mg/kg/day (group II) for 90 days, 24 animals served as control (treated with the vehicle alone) (group I). The age at the start of the study was approximately 6-7 weeks and the average body weight was 113 ± 6 g for females and 115 ± 5 g for males. Food and water was provided *ad libitum*. After 14 weeks the animals were sacrificed.

Examinations
Clinical signs and mortality daily. Ophthalmoscopic examination at the start and at the end of the study. Body weights weekly.
Haematology (RBC, WBC, Diff., Hb, Hct, MCV, MCH, MCHC) and clinical chemistry (bil, glu, total protein, SGOT, SGPT, LDH, AP, Fe, Ca, urea, uric acid, creat, chol, triglyceride) at the start of the study and after 8 and 14 weeks. Urinalysis (nitrite, leuco, pH, prot, glu,

ketones, urobil, bil, blood, sed) at start of the study and after 6 and 12 weeks. Relative and absolute organ weights, gross pathology and histopathology.

Results
- Haematology: After 7 weeks of treatment erythrocytes, haematocrit and MCV, MCH and MCHC were significantly reduced in the females of the test group. At the same time the haematocrit and the MCV were significantly reduced in the male rats of the test group as well. After 13 weeks leukocyte values in the test group females were significantly increased.
- Clinical chemistry: Fe-values were reduced significantly in the males of the test group after 7 weeks of treatment.
- Urinalysis: The urine of the test group animals was discoloured.
- Organ weights: Absolute and relative organ weight of the spleen was reduced in the females of the test group, whereas the relative spleen weight and the relative weight of the kidney were increased significantly in the corresponding males.
- Histopathology: The histomorphological examination revealed a slight activation of the thyroids in 10 male and 1 female rat of the test group. As another substance-related effect lymphatic enteritis was observed in 10 animals of group II

Conclusion
No NOAEL could be established.

Ref.: 23

Study 3

Guideline:	OECD 408 (1981)
Species/strain:	Sprague-Dawley CFY rats
Group size:	10 animals per sex and group
Test substance:	Kardinalrot
Batch number:	/
Purity:	84% (13% 1-β-hydroxyethylamino-2-nitro-4-bis-(β-hydroxyethyl)amino- benzene and 3% 1-amino-2-nitro-4-(β-hydroxyethyl)aminobenzene
Dose levels:	0, 5 mg/kg bw/day in de-ionised water by gavage
Exposure period:	13 weeks
GLP:	in compliance

The test substance (dissolved in water) was administered by gavage once daily to a group of 20 Sprague-Dawley (CFY) rats (10/sex) at dose 5 mg/kg/day (group II) on 90 consecutive days, 20 animals served as control (Aqua dest.) (group I). The age at the start of the study was approximately 8 weeks and the body weight was 138-185 g for females and 144-190 g for males. Food and water was provided *ad libitum*. After 14 weeks the animals were sacrificed.

Examinations
Clinical signs and mortality daily. Ophthalmoscopic examination at the start and at the end of the study. Body weights and food weekly. Water consumption by visual inspection daily. Haematology (RBC, WBC, Diff., Hb, Hct, MCV, MCH, MCHC) and clinical chemistry (bil, gluc, total protein, albumin, γ-GT, AP, Na+, K+, Cl-, Ca2+, inorganic phosphorus, urea, creat, ALAT, ASAT, albumin/globulin ratio) during the last week of the study. Relative and absolute organ weights, gross pathology and histopathology.

Results
- Haematology: The MCV was decreased in both males and females of the test group, whereas the MCH was decreased only in males. Furthermore prothrombin time was increased in the treated males.

- Clinical chemistry: In the females of group II decreased glucose values, decreased albumin/globulin ratios and increased creatinine values were observed. Examination of the males of the test group revealed decreased Na+- and serum alanine aminotransferase values. All the values of these parameters were considered to fall into the normal range.
- Organ weight: No statistically significant absolute weight changes. Mean relative kidney weight (% of body weight) was elevated in treated males ($p < 0.05$), but none of the individual values was considered abnormal.
- Other signs: Purple-coloured urine was noted in approx. 60% of the females during the fist week and on isolated occasions up to 34 days after the start of the study.

Conclusion
Based on the effects seen (changes in haematological parameters and clinical chemistry, relative kidney weight), the marginal Effect level was therefore considered to be 5 mg/kg/day of Kardinalrot, corresponding to 4.2 mg/kg bw/day of HC Red n° 13.

Ref.: 24

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 gene mutation assay

Guideline:	OECD 471 (1983)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and TA1538.
Replicates:	triplicates in 2 individual experiments both in the presence and absence of S9-mix.
Test substance:	DA 261093 (1-amino-2-nitro-4-bis-(2-hydroxyethyl)-amino-benzene; HC Red n° 13)
Solvent:	DMSO
Batch:	199/027
Purity:	> 99%
Concentrations:	Experiment 1: 1 - 5000 µg/plate without and with S9-mix Experiment 2: 30 - 5000 µg/plate without and with S9-mix
Treatment:	Direct plate incorporation method in both experiments
GLP:	In compliance

HC Red n° 13 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. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and/or thinning of the bacterial background lawn. HC Red No. 13 was tested up to the prescribed maximum concentration of 5000 µg/plate. The experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Precipitation and toxicity of HC Red n° 13 were not reported. In experiment 1 without S9-mix a slight increase in the number of revertants was seen in TA1535 and TA 1538. This increase seems to be due to the low negative control value and was considered not biologically relevant because of the lack of a concentration-response relationship and the lack of confirmation in the second experiment. In experiment 2 an increase in the number of revertants was not observed.

Conclusion
Under the experimental conditions used HC Red n° 13 was not mutagenic in the gene mutation tests in bacteria.

Ref. 25

Comment
Historical control data were not reported.

In Vitro* Mouse Lymphoma gene mutation assay (*tk* locus*)

Guideline:	OECD 476 (1997)	
Cells:	L5178Y Mouse lymphoma cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	HC Red No 13 WR 23020	
Solvent:	Deionised water	
Batch:	4/5/6-1 Fass 3	
Purity:	95.3 weight % and approximately 1 weight % ethanol	
Treatment	Experiment I:	200 - 3200 µg/ml (without S9-mix)
		200 - 3200 µg/ml (with S9-mix)
	Experiment II:	200 - 800 µg/ml (without S9-mix)
Concentrations:	Experiment I1:	4 h both without and with S9-mix; expression period 72 h, selection growth 10-15 days.
	Experiment II:	24 h both without S9-mix; expression period 48 h, selection growth 10-15 days.
GLP:	In compliance	

HC Red No. 13 was assayed for mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9-mix metabolic activation. Test concentrations were based on the results of a cytotoxicity range-finding experiment measuring relative suspension growth. In the main test, cells were treated for 4 h (experiment I) or 24 h (experiment II) followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation. Liver S9-mix fraction from phenobarbital/ß-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was measured as percentage relative suspension growth of the treated cultures relative to the suspension growth of the solvent control cultures. Osmolarity and the pH value were determined in the solvent control and the maximum concentration in the pre-experiment with S9-mix. Negative and positive controls were in accordance with the OECD guideline.

Results
There was no relevant shift in osmolarity but a small shift of the pH value of the medium at the maximal concentration of the test item in the pre-experiment. Precipitation was not observed in both experiments. Except for culture II of experiment I without S9-mix, in both experiments in the absence and presence of S9-mix the appropriate level of toxicity was reached pointing to sufficient exposure of the cells.
In the first experiment both without and with S9-mix a substantial and reproducible biological relevant increase was not found. An isolated increase above the threshold of twice the solvent control was reached at the highest dose in culture II. Since this effect was not reproducible and within the historical control value, it was considered not biologically relevant. Without S9-mix a more or less dose dependent increase in mutation frequency was found both in culture I and II. As it was clearly within the historical control values the increase was considered not biologically relevant.
In the second experiment a reproducible and dose dependent increase in the number of mutant colonies was found. This increase was caused by an increase in the number of small colonies indicating to a primarily clastogenic effect of HC Red No. 13.

Conclusion
Under the experimental conditions used, HC Red No. 13 was considered mutagenic in the mouse lymphoma assay at the *tk* locus. Since the effect was due to an increase in the number of small colonies, HC Red No. 13 is considered clastogenic.

Ref. 26

Comments
Historical control data were only reported for the total number of mutant colonies; historical data for "small" and "large" colonies were not available.

In vitro micronucleus test

Guideline:	OECD draft 487 (2004)	
Cells:	human lymphocytes from 2 healthy, non-smoking, female volunteers	
Replicates:	duplicates in 2 independent experiments	
Test substance:	HC Red No 13 (WR 23020)	
Solvent:	purified water	
Batch:	4/5/6-1 Fass 3	
Purity:	89.5%	
Concentrations:	Experiment 1:	0, 400, 700 and 1000 µg/ml (without S9-mix)
		0, 1700, 2100, 2413 µg/ml (with S9-mix)
	Experiment 2:	0, 1089, 1322, 1556 µg/ml (without S9-mix)
		0, 2000, 2200, 2413 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	

HC Red No. 13 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. The suitable top concentrations for experiments 1 and 2 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 Red No. 13. 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 S9-mix 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 27-28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Liver S9-mix fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
Toxicity was determined by measuring the reduction in replication index (RI). Micronucleus preparations were stained and examined microscopically for RI and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Measurements on post-treatment media in the absence or presence of S9-mix indicated that HC Red No. 13 had no effect on osmolarity or pH as compared to concurrent vehicle controls.
Cytotoxicity levels, measured as RI, reached the (in the draft guideline) suggested reduction of 60% after treatment with the highest dose in the experiments with S9-mix but not in those without S9-mix. However, the highest dose tested is identical to the prescribed

maximum concentration of 10 mM (equivalent to 2413 µg/ml) from the draft and related OECD guidelines.
In experiment 1 without S9 metabolic stimulation HC Red No. 13 did not induce an increase in the frequency of micronuclei compared to concurrent vehicle controls. A statistically significant and more or less dose dependent increase in the MN-frequency was found in the presence of S9-mix. These results may be of questionable biological significance since they were just above the historical controls and not observed in both duplicate cultures.
In experiment 2 both in the absence and presence of S9-mix biologically relevant, dose dependent, and statistically significant increases in the MN-frequency were found compared to concurrent control values.

Conclusion
Under the experimental conditions used HC Red No. 13 induced micronuclei and, consequently, is genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

Ref: 27

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	/
Species/strain:	NMRI mice
Group size:	5 mice/sex/group
Test substance:	DA 261093 (1-amino-2-nitro-4-bis-(2-hydroxyethyl)-amino-benzene; HC Red No. 13)
Purity:	> 99%
Batch:	Ref 199/027
Dose level:	0, 375, 750 and 1500 mg/kg bw
Route:	gavage, once
Vehicle:	arachidis oil
Sacrifice times:	24 h for all dose levels, vehicle control and positive control, 48 h for the highest dose.
GLP:	In compliance

HC Red No. 13 has been investigated for the induction of micronuclei in bone marrow cells of mice. Mice were exposed by gavage. Test concentrations were based on an initial toxicity test. Bone marrow cells were collected 24 and 48 h (highest dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic to normochromatic erythrocytes (PCE/NCE ratio). Bone marrow preparations were stained and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were included.

Results
After dosing, the animals of all groups showed reduced motility and lethargy, which was most pronounced in the animals treated with the highest dose. The ratio PCE/NCE decreased dose dependently at 24 h and at 48 h as compared to the untreated controls, indicating that HC Red No. 13 did have cytotoxic properties in the bone marrow and consequently must have been biologically available.
Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found following treatment with HC Red No. 13 at any time point.

Conclusion
Under the experimental conditions used HC Red No. 13 did not induce micronuclei in bone marrow cells of treated mice and, consequently, HC Red No. 13 was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 28

Comment
It was not reported whether the micronucleus assay was performed according OECD guideline 474. The initial cytotoxicity test to determine the concentrations of HC Red No. 13 in the main tests, was not reported

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

Guideline:	/
Species/strain:	B6C3F$_1$ mice
Group size:	5 male mice/group
Test substance:	HC Red 13 [4-(Di(2-hydroxyethyl)amino)-2-nitro-aniline-monohydrochloride
Batch no:	4/5/6-1 Fass 3
Purity:	98%
Dose level:	0, 1000 and 2000 mg/kg bw
Route:	oral gavage, twice 20 ± 0.5 h apart.
Vehicle:	deionized water
Sacrifice times:	24 h after the first treatment (i. e. 4 h after the second treatment)
Organs studied:	blood, liver, duodenum and urinary bladder
GLP:	in compliance

HC Red No. 13 has been investigated for the induction of DNA damage in the alkaline single cell gel electrophoresis (comet) assay in various tissues of mice. Test concentrations were based on the results of an acute toxicity test to identify the maximum tolerated dose (MTD). As a result the mice were exposed by oral gavage to 1000 and 2000 mg/kg bw HC Red No. 13. Mice were treated twice, 20 h apart, and sacrificed 4 h after the last treatment.
Cytotoxicity was studied by low molecular weight (LMW) DNA diffusion analysis; cells with extensive DNA degradation associated with cell death exhibit a highly diffuse pattern of DNA compared to the condensed pattern associated with the high molecular weight DNA.
Per organ 100 nuclei were examined for Olive tail moment (the distance between the centre of gravity of the DNA distribution in the tail and the centre of gravity of the DNA distribution in the head magnified with the fraction of DNA in the tail). Ethyl methane sulphate (300 mg/kg bw) was used as positive control.

Results
Consistent with the toxicity test, in the main test mice showed clinical signs of toxicity such as squinting and decreased movement, at the dose levels used. In addition stained bedding and purple urine were observed indicating that HC Red No. 13 has affected the biological system and was available to all tissues tested.
The percentage of cells with LMW DNA was increased by HC Red No. 13 treatment at dose levels up to 2000 mg/kg bw in the urinary bladder, but not in the liver, duodenum and blood. The increase in LMW DNA in the urinary bladder indicates the presence of HC Red No. 13 at sufficient levels *in vivo* to induce a cytotoxic effect in this tissue.
The response induced by the positive control after 20 minutes of electrophoresis was not sufficiently robust for this positive control administered ip at 300 mg/kg bw. To increase the sensitivity of the assay all tissues evaluated for DNA damage were electrophoresed for 40 minutes. The Olive tail moment was not significantly increased in cells of the liver, duodenum, blood and urinary bladder indicating that HC Red No. 13 did not induce DNA damage in these tissues of the mice.

Conclusion
Under the experimental conditions used HC Red n° 13 induced significant increases in cell death in urinary bladder but did not induce DNA damage in urinary bladder, liver, duodenum and blood of mice and, consequently, HC Red No. 13 is not genotoxic in the comet assay with mice.

Ref.: 29

3.3.7. Carcinogenicity

Guideline: /
Species/strain: Mice, Han: NMRI
Group size: 75 per sex and dose group
Test substance: HC Red No. 13
Code: /
Purity: /
Dose levels: 0, 0.13, 1.0 and 2.0% in 0.05 ml/animal
Route: Dermal
Vehicle: Water and vehicle (water-based cosmetic formulation)
Dosing schedule: 18-months, three times per week
GLP: In compliance

The test material was applied dermally to groups of 75 male and 75 female NMRI mice (about 6 week old, body weight: males 16 – 33 g, females 15 – 30 g) for 18 months, three times per week, at dose levels of 0.013, 1.0, and 2.0% in a volume of 0.05 ml/animal dissolved in the vehicle (cosmetic formulation) The two control groups, 75 animals per sex, were dosed with the vehicle or water alone. The vehicle contained: 0.5% 1-amino-2-nitro-4-β-hydroxyethyl-amino-5-chlorobenzene (1.0% in the high dose, 0% in the vehicle control), 1.05% sodium laureth sulphate, 0.625% stearic acid diethanolamide, 0.312% copolymer of alkyl methaacrylate with methacrylic acid, 15% isopropanol and water (80 – 83%).

Mortality and clinical signs were noted twice daily during the entire study. Body weight was determined once weekly for the first 13 weeks and every two weeks thereafter until termination of the study. Food consumption was recorded at weekly intervals during the study. Drinking water intake was observed daily. In addition, a detailed clinical and skin analysis was performed once a week.

Clinical laboratory investigations (haematology and blood/clinical biochemistry) were performed after 12 and 18 months in 10 animals per sex and dose group.

All animals were subjected to detailed gross necropsy and a comprehensive histopathological evaluation of several tissues from all animals was carried out.

Results
The treatment caused no signs of toxicity and the survival rate was not affected by the treatment. A dose dependent reduced body weight gain in males for the first 3 months in all groups and over the entire study period for the high dose group was noted. Food consumption was not affected by the treatment. The clinical laboratory investigations revealed no treatment related effects. Lymphatic leucosis was observed in both control groups as well as in the test animals. It was pointed out that the frequency observed in the present study (10 – 19%) lies in the centre of the incidence given in the literature and is not considered to be related to the treatment. The histopathological evaluation showed no differences between treated and control groups with regard to the occurrence or frequency of tumours.

Ref.: 30

Comment
The concentrations used are considered to be too low to detect any carcinogenic effect. No toxic effects were detected.

3.3.8. Reproductive toxicity

3.3.8.1. One generation reproduction toxicity

Guideline:	OECD 415 (1983)
Species/strain:	Rat, strain HanBrI: WIST (SPF Quality)
Group size:	24 per sex and dose group
Test substance:	HC Red No. 13 in bi-distilled water
Code:	4/5/6-1 Fass 3
Purity:	98.4% (HPLC at 254 nm)
Dose levels:	30, 100 and 300 mg/kg bw/day
Route:	Oral, gavage
Dosing schedule:	Males: Once daily during the 70 days pre-mating period, during mating and until day 7 post partum of last litter (total: ≥ 100 days) Females: Once daily during the 14 day pre-mating period, during mating, gestation and lactation periods (total: ≥ 64 days) Offspring: Once daily for up to 21 days
GLP:	In compliance

The test substance was administered in a constant volume of 10 ml/kg bw once daily by oral gavage to groups of 24 male and 24 female HanBrI: WIST (SPF Quality) rats during the pre- mating (70 days for males, 14 days for females), mating, gestation and lactation (females only) period at doses of 10, 100 and 300 mg/kg bw/day. A concurrent control group received the vehicle (bi-distilled water) alone. Animals were paired one/one for a period of maximum of 21 days. Litters were raised until day 21 post partum. Animals were observed twice daily for clinical signs or mortality during the entire treatment period. Body weights were recorded daily until necropsy. Food consumption was measured on weekly intervals until delivery (except during mating). During lactation the food consumption was recorded on days 1, 7, and 14 post partum.
Successful mating was verified by vaginal smear analysis or when a copulation plug was observed during the 21 day mating period. A second mating period of 21 days with a different male was allowed in case mating was not successful. Females without litter or which lost their litters were killed and necropsied. After delivery, litters were examined for litter size, live birth, stillbirth and any gross anomalies. The sex ratio was noted at day 0, 4, and day 21 of lactation. Pup weights were noted on day 1, 4, 7, 14, and 21 of lactation. Pups were also observed daily for survival and behavioural abnormalities. Pup number was reduced to 4 males and 4 females per litter at post partum day 4. The remaining pups were macroscopically examined for all litters with more than 4 pups per sex. On day 21 post partum, all pups were examined internally and externally for abnormalities and, if indicated, skeletal development was examined after staining.
At necropsy of the parent animals, a macroscopical examination with special focus on the organs of the reproductive system was performed. Implantation sites were counted and the uterus was analysed for haemorrhagic alterations of implantation sites. Histopathology covered all gross lesions, ovaries, pituitary gland, prostate, seminal vesicles, testes with epididymides, thyroid gland, uterus and cervix, vagina and target organs.

Results
No treatment related mortality was noted. In the high dose groups (males and females) a sign of discomfort was noted right after the daily dosing. The discolouration of the bedding material noted in all dose groups was considered to be due to discolouration of urine. Food consumption and body weight in the parental generation were not affected at any time period by the treatment. Reproduction parameters including fertility and mating performance, duration of gestation, pre-coital times, implantation rate and post-implantation loss, litter size, postnatal loss and breeding loss were similar in all groups and not affected by the treatment. Litter data like sex ratio, pup weight or pup weight gain during lactation revealed no test-item related effects and no abnormalities were noted for

the treated pups. At necropsy, a dose-related increase in red discolouration of the thyroid was noted for both male and female parental animals. Histopathology revealed no adverse findings in either parental animals or pups. However, an increase in follicular cell hypertrophy in the thyroid was noted in parental animals of either sex (predominantly in males) in all dose groups. These effects in the parental animals were related to the treatment with the test substance.

Conclusion
At the highest test dose, clinical signs in terms of discomfort were noted in both males and females. Similar to the findings in the 90-day study an increase in follicular cell hypertrophy in the thyroid was noted (see Ref.: 22). HC Red n° 13 did not reveal any effect on any reproduction parameter. From this study, a NOAEL of 300 mg/kg bw for reproductive effects was deduced.

Ref.: 32

3.3.8.2. Teratogenicity

Old study, taken from opinion n° XXIV/1287/97 of 20 May 1998

The test substance (dissolved in water) was given daily from day 5-15 of gestation to groups of 24 pregnant Wistar rats, respectively, (Bor:Wisw-SPF TNO strain) by oral gavage of doses of 5 (group I), 15 (group II) and 30 (group III) mg/kg bw. 24 pregnant females treated with aqua deionised served as controls. Prior to treatment females were 14 weeks old and had a body weight range from 160-220 g. Food and drinking water *ad libitum*. According to sperm found in vaginal smear (day 0 of gestation), the females were sacrificed after 20 days *post conceptionem*.

Examinations
- Clinical observations daily. Bodyweights were taken at the beginning of the study and at day 5, 10, 15 and 20. Food consumption was measured for days 0-5, 5-15, 15-20 as well as for 0-20.
- Complete autopsy of the dams and a macroscopic evaluation of the organs were carried out on day 20.
- Determination of the number of dead and alive foetuses, distribution and site in the uterus, early and late resorptions, placentas, implantations, sex determination, corpora lutea.
- Determination of the weight of foetuses, placentas, graved uteri, uteri without foetuses.
- Externally visible deviations in foetuses, organic imperfections (in 1/3 of all foetuses) and skeletal defects (in 2/3 of all foetuses) were evaluated.

Results
No maternal abnormalities and no signs of maternal toxicity were observed. No abnormalities were found in the foetuses. Thus the NOAEL is 30 mg/kg.

New study

Guideline:	OECD 414 (1984)
Species/strain:	HanBri: WIST (SPF quality) rats
Group size:	22 mated females per dose group
Test substance:	HC Red No. 13 (WR 23020) in bi-distilled water
Batch number:	4/5/6-1 Fass 3 (Fa. Robinson)
Purity:	98.4 % (HPLC at 254 nm)
Dose levels:	0, 30, 100 and 300 mg /kg/day (oral, gavage)
Treatment period:	once daily, day 6 - 20 of gestation
GLP:	In compliance

The test substance was administered in a constant volume of 10 ml/kg bw once daily by oral gavage to groups of 22 pregnant HanBrI: WIST (SPF Quality) rats at doses of 10, 100 and 300 mg/kg bw/day from day 6 to day 20 of gestation. A concurrent control group received the vehicle (bi-distilled water) alone. Successful mating was verified by vaginal smear analysis or by the occurrence of a copulation plug. Animals were observed twice daily for clinical signs during the entire treatment period. Body weights were recorded daily. Food consumption was measured on 3-day intervals. At day 21 post coitum, all mated females were killed under CO_2-asphyxiation and a complete necropsy and a macroscopic examination of the organs was carried out. The uterus (prepared by caesarean section) was removed and the location of the foetuses in the uterus was examined. The number of implantation sites and of corpora lutea was also determined. Each live foetus was weighed, sexed and examined for gross external malformations. After appropriate processing, a skeletal and a visceral examination was performed for about 50 % of the foetuses each. In addition, placenta and uterus weights were recorded.

Results
No treatment-related effects in dams were noted with regard to clinical observations and post-mortem findings. Violet discolouration of fur and tail in the animals of the high dose group was linked to discolouration of urine and bedding material. Body weight and food consumption were not affected by the treatment. Reproduction parameters revealed no differences between treated and control groups. At gross necropsy no treatment related effects were observed. The uterus and placenta weights, the number of corpora lutea and implantations were similar to control values in all treated groups.
There were no treatment related effects with regard to litter size, foetal mortality, foetal body weight and sex ratio. The skeletal and visceral examination of the foetuses revealed no treatment related findings. Compared to the concurrent control, neither a statistically significant difference nor a dose-dependent increase in any type of malformation was noted. Further, there were no changes in variations which were regarded to be related to the treatment with HC Red No. 13. All observed variations represented common findings for the used rat strain and were within the spontaneous range for variations and/or revealed no dose-response relationship.

Conclusion
No treatment-related effects were noted in dams and foetuses in a rat teratogenicity study up to the highest test dose of 300 mg/kg bw/day. A NOAEL of 300 mg/kg bw for both maternotoxic and for embryo-foetal effects was deduced.

Ref.: 33

3.3.9. Toxicokinetics

3.3.9.1. Toxicokinetics *in vitro*

Biovailability study with human intestinal epithelial cells

Guideline:	/
Cells:	Human intestinal epithelial cell line TC-7
Test substance:	HC Red No. 13
Batch:	4/5/6-1 Fass 3
Purity:	98.4 % (HPLC at 254 nm)
Concentration:	≤ 50 µM in HBSS buffer containing 1 % DMSO
Incubation time:	60 min
Number of experiments:	Two independent experiments
GLP:	/

The bioavailability of HC Red No. 13 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 monolayer revealing a permeability of < 2.5 x 10^{-6} cm/sec were used. Propranolol, vinblastine and ranitidine were analysed concurrently to demonstrate the validity of the test system. According to the laboratory's classification system, a low permeability was 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 propanolol (90 % absorption in humans) was used as the high permeability reference compound.

Results
The total recovery for the reference substances and HC Red No. 13 ranged from 59 to 97 %. The figures for the reference substances propranolol (P_{app} = 25.9 x 10^{-6} cm/sec) and ranitidine (P_{app} = 0.2 x 10^{-6} sec) were well within the acceptable range for these compounds of 20 – 45 x 10^{-6} cm/sec and 0.2 – 2 x 10^{-6} cm/sec, respectively, and demonstrated the validity of the assay.
HC Red No. 13 revealed a P_{app} of 37.1 x 10^{-6} cm/sec and thus was classified to be of high permeability. The observed value indicates a good absorption from the gastro-intestinal tract.

Ref.: 34

3.3.9.2 Toxicokinetics *in vivo*

See point 3.3.4 Dermal / percutaneous absorption, reference 35

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

Not applicable

3.3.14. Discussion

Physico-chemical specifications
No data were submitted on the characterization of the substance by NMR and HPLC. HC Red n° 13 is 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 documentation was provided to support the data presented in the table "Batch comparison"
The stability of the test substance in marketed products was not reported.

General toxicity
LD_{50} was calculated to be 2120 (1810 - 2480) mg/kg bw.
Based on the effects seen in the 90-day study (changes in haematological parameters and clinical chemistry, relative kidney weight), the Lowest Observed Effect Level was considered to be 5 mg/kg bw/day of Kardinalrot, corresponding to 4.2 mg/kg bw/day of HC Red n° 13.
The NOAEL for reproductive effects was set at 300 mg/kg bw/day. A NOAEL of 300 mg/kg bw/day for both materno-toxic and for embryo-foetal effects was deduced.

Toxicokinetics
In vitro, HC Red n° 13 revealed a P_{app} of 37.1 x 10^{-6} cm/sec and thus was classified to be of high permeability. The observed value indicates a good absorption from the gastro-intestinal tract.
After dermal application in rats, only minor amounts of HC Red n° 13 are absorbed through the skin and become systemically available. If applied in a hair dye formulation as vehicle less than 1.1 $\mu g/cm^2$ of the dye formulation penetrated the skin. The majority of the penetrated dye is excreted within 24 h after administration.
After oral application, it is quickly absorbed and nearly completely excreted within 72 h, with the majority eliminated within 24 h after application. Excretion takes place via urine and faces to almost equal amounts.
Low tissue residue levels were noted for both routes of exposure indicating that bioaccumulation is not expected. There were no significant sex differences in absorption, tissue distribution and excretion pattern.

Irritation / sensitisation
Under the conditions of the experiment, there was no irritant effect to the rabbit skin. However, the test concentration used (2.5% aqueous solution) was too low for hazard identification.
There was no irritant effect to the rabbit eye throughout the 7 days observation. The test concentration used (2.5% aqueous solution) was too low for hazard identification. The diluted preparation was washed out 10 seconds after instillation.
The substance is considered to be a moderate skin sensitiser in a LLNA study.

Dermal absorption
The *in vitro* dermal absorption studies were not performed according to the SCCP Notes of Guidance. Unexpectedly, a lower value was observed in the *in vivo* toxicokinetic study in rats compared with the values derived from the *in vitro* dermal absorption studies.
Because of this, and due to the deficiencies of the *in vitro* studies, it is concluded that a new *in vitro* dermal absorption study conforming to the SCCP Notes of Guidance should be performed in order to give confidence to the calculation of the Margin of Safety.

Mutagenicity
Overall, the genotoxicity program on HC Red n° 13 investigated three endpoints of genotoxicity: gene mutations, structural chromosome aberrations and aneuploidy. HC Red No. 13 did not induce gene mutations in bacteria nor on the *tk* locus of mouse lymphoma

cells. However, in the mutation assay with mouse lymphoma cells indications for a clastogenic effect of HC Red No. 13 were found. The latter effect was confirmed by the positive results found in an *in vitro* micronucleus test with human lymphocytes. In an *in vivo* bone marrow micronucleus tests in mice, HC Red No. 13 did not produce an increased micronucleus frequency. Finally, the genotoxic effect was also not found in a Comet assay.
As the genotoxic effects found *in vitro* were not confirmed *in vivo*, HC Red n° 13 itself can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.
To reach a definitive conclusion, appropriate tests with HC Red No. 13 in combination with hydrogen peroxide have to be provided.

Carcinogenicity
The concentrations used are considered to be too low to detect any carcinogenic effect. No toxic effects were detected.

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, an *in vitro* percutaneous absorption study should be performed following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

HC Red n° 13 is a tertiary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

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

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