EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Red n° 7

COLIPA n° B36

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 5th plenary meeting
of 8 December 2009

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 Safety (SCCS), 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).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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This opinion has been subject to a commenting period of four weeks after its initial publication. All comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged.

Keywords: SCCS, scientific opinion, hair dye, B36, HC Red n° 7, directive 76/768/ECC, CAS 24905-87-1, EINECS 246-521-9

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on HC Red n° 7, 8 December 2009

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1. BACKGROUND

Submission I for HC Red No 7 with the chemical name 1-Amino-2-nitro-4-(ß-hydroxyethyl)-aminobenzene was submitted in January 1996 by COLIPA [1, 2].

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP)) adopted at its 24th plenary meeting on 24-25 June 2003 the opinion (SCCNFP/0678/03, final) with the conclusion that:

"The SCCNFP is of the opinion that the information submitted is inadequate to assess the safe use of the substance. Before further consideration, the following information is required:

- *quantitative data on solubility in e.g. water and ethanol; determination of the purity of all batches used in the toxicity studies; determination of the impurities in these batches and their related health hazards; experimental data on the stability of the test substance;*
- *analytical data on the nitrosamine content in more than one sample as well as in hair dye formulations;*
- *the effects on the spleen in the sub-chronic toxicity study have to be re-evaluated*
- *data on genotoxicity/mutagenicity following the relevant SCCNFP opinions and in accordance with the Notes of Guidance."*

Submission II for HC Red No. 7 was submitted by COLIPA in July 2005. According to this submission HC Red No. 7 is used in semi-permanent hair dye formulations at a maximum concentration of 1.0%.

Submission II 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 Safety (SCCS) consider HC Red No. 7 safe for use as a non-oxidative hair dye with a concentration of maximum 1.0 % taken into account the scientific data provided?*

2. *Does the SCCS recommend any further restrictions with regard to the use of HC Red No. 7 in any non-oxidative hair dye formulations?*

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

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Red n° 7 (INCI name)

3.1.1.2. Chemical names

1-Amino-2-nitro-4-(β-hydroxyethyl)-aminobenzene
2-Amino-5-(2-hydroxyethylamino)nitro-benzene
2-(4-Amino-3-nitroanilino)ethanol
2-[(4-Amino-3-nitrophenyl)amino]ethanol
Ethanol, 2-(4-Amino-3-nitroanilino)-
Ethanol, 2-[(4-Amino-3-nitrophenyl)amino]-
2-Nitro-4-(β-hydroxyethylamino)aniline

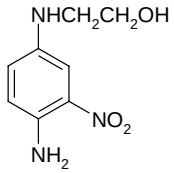
3.1.1.3. Trade names and abbreviations

Imexine FZ
COLIPA B36

3.1.1.4. CAS / EC number

CAS: 24905-87-1
EC: 246-521-9

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_8H_{11}N_3O_3$

3.1.2. Physical form

Dark brown powder

3.1.3. Molecular weight

Molecular weight: 197.19 g/mol

3.1.4. Purity, composition and substance codes

Comparison of batches of HC Red 7

Batch number	Op. 57	0510149	T81	0503334
Titre by potentiometry (g/100g, based upon alkalinity)	98.9	99.3	99.8	99.1
Water content (g/100g)	0.13	0.05	/	/
HPLC profile *	> 99%	> 99.5%	/	/
Impurity content (g/100 g) (HPLC)				
Impurity A	0.13	D < 0.1	0.04	/
Impurity B	D < 0.1	D < 0.1	/	/
Impurity C	ND < 0.1	ND < 0.1	/	/
Residual solvents (µg/g) ** (GC)				
Isopropyl acetate	ND < 10	/	/	/
Ethanol	/	D < 100	/	/
DMF	/	ND < 400	/	/
Visible spectrum	The visible spectra are comparable			/
Infra-red spectrum	In conformance with the proposed structure		/	In conformance with the proposed structure
^{1}H and ^{13}C NMR spectra	In accordance with the proposed structure		/	/
Mass spectrometry	Compatible with the proposed structure		/	/

* UV detection- UV purity – area% without response factor. Irrespective of residual solvents, salts and other non-detectable products

** solvents used during the manufacturing process were different: isopropyl acetate for batch Op. 57, ethanol and DMF for batch 0510149

Impurity A: 2-Nitro-benzene-1,4-diamine / 2-nitro-p-phenylenediamine
Impurity B: 3-(4-Amino-3-nitro-phenyl)-oxazolin-2-one
Impurity C: 2-chloroethyl-4-amino-3-nitrophenylcarbamate
ND: not detected
D: detected
/: not analysed

Impurities:
Ash content: < 0.1 g/100 g
Heavy metal
As, Sb, Hg: < 5 mg/kg
Cd: < 10 mg/kg
Pb: < 20 mg/kg

Comments
- In addition to the described impurities, traces of 5 other impurities were visible in the HPLC chromatogram of HC Red n° 7
- 2-Nitro-p-phenylenediamine and its salts are banned for use in hair dye formulations according to the cosmetics directive. (Annex II, entry n° 1319). However, the SCCS considers that this impurity at the levels present does not constitute a safety concern.

Declaration by the applicant

Raw material *(marketed product)*

Purity: > 98.5 area% (HPLC)

Impurities:
2-Nitro-benzene-1,4-diamine / 2-nitro-p-phenylenediamine: < 0.2 g/100 g

3-(4-Amino-3-nitro-phenyl)-oxazolin-2-one:	*< 0.1 g/100 g*
2-chloroethyl-4-amino-3-nitrophenylcarbamate:	*< 0.1 g/100 g*

Comments
According to the HPLC profile and titre by potentiometry of HC Red 7, the purity of the raw material (commercial ingredient) should be described as at least 98.9% (HPLC area% at 490 ± 5 mn).

3.1.5. Impurities / accompanying contaminants

See 3.1.4

3.1.6. Solubility

Water: < 1 g/100 ml at 22 °C after 24h
Ethanol: < 1 g/100 ml at 22 °C after 24h
DMSO: ≥ 20 g/100 ml at 22 °C after 24h

Water solubility according to EEC method A6: 3.74 g/L at 20 °C *

Comment
* An analytical file is not provided

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}:	0.80	(calculated)
	0.38 at 25 °C at ph 7.11	(experimental, according to EEC A8 - HPLC) *

Comment
* An analytical file is not provided

3.1.8. Additional physical and chemical specifications

Melting point:	90 – 96 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	λmax at 249.8 nm and 494 nm

3.1.9. Homogeneity and Stability

Homogeneity
The results of the analyses demonstrated that the HC Red n° 7 formulations (10 and 100 mg/mL) in aqueous 0.5% CMC were homogeneous (maximum CV =3%) on the day of preparation, protected from light and under inert gas atmosphere.

Stability
The results of the analyses demonstrated the solutions of HC Red n° 7, as described below, were stable (within ± 11% of the nominal value) after 2- and 4-hour storage at room temperature, protected from light and under inert gas atmosphere:

- 10 mg/mL in 0.5% CMC,
- 100 mg/mL in 0.5% CMC,
- 0.1 mg/mL in DMSO,
- 250 mg/mL in DMSO,
- 5 mg/mL in acetone/olive oil,
- 100 mg/mL in acetone/olive oil.

General Comments to physico-chemical characterisation

- According HPLC profile and titre by potentiometry of HC Red n° 7, the purity of raw material (commercial ingredient) should be described as at least 98.9% (HPLC area% at 490 ± 5 nm)
- The impurity 2-nitrobenzene-1,4-diamine (or 2-nitro-p-phenylenediamine) is classified by the German MAK Commission as a carcinogen, category 3B and is banned according to the cosmetics directive. (Annex II, entry n° 1319). The SCCS considers that this impurity at the levels present does not constitute a safety concern.
- HC Red n° 7 is a secondary amine, and thus it is prone to nitrosation. The nitrosamine content of HC Red n° 7 is not reported.
- The stability of HC Red n° 7 in typical hair dye formulations is not reported.

3.2. Function and uses

HC Red n° 7 is used in semi-permanent hair dye formulations at a maximum concentration of 1.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 420 (2001)
Species/strain:	rat, Sprague-Dawley Rj:SD (IOPS Han)
Group size:	2 groups of 5 females (nulliparous and non-pregnant)
Test substance:	HC Red n° 7
Batch:	0510149
Purity:	99.3%
Vehicle:	0.5% carboxymethylcellulose in purified water
Dose:	300 and 2000 mg/kg bw
Dose volume:	20 ml/kg bw
Route:	oral gavage
GLP statement:	in compliance
Study period:	9 November – 24 December 2004

Hypoactivity, dyspnea and piloerection were observed within 4 hours of treatment in the animals given 2000 mg/kg. At 2000 mg/kg, the mortality was 3/5 (60%).
Hypoactivity, then sedation, piloerection, dyspnea, purple urine and purple to purplish red coloration of the extremities (tail, ears, eyes, legs and nose) were observed prior to death.
In the surviving female, hypoactivity or sedation, piloerection, dyspnea, purple urine and light red coloration of the extremities (ears, eyes, legs and nose) were noted on day 1 only.
At 300 mg/kg no mortality was observed. Purple urine was noted in 1/5 females on day 1 only.
The overall body weight gain was not affected by treatment with the test item. At necropsy, no apparent abnormalities were observed.

Under the experimental conditions, authors indicated a maximal non-lethal dose of HC Red No. 7 (B036) (batch No. 0510149) of 300 mg/kg by the oral route in rats; 2000 mg/kg as the minimal lethal dose.

Ref.: 1

Comments
20 ml/kg is a high volume to be administered, especially of a solution containing carboxymethylcellulose. Nevertheless, the substance is minimally toxic by ingestion of a single dose.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404
Species/strain:	New Zealand albino rabbit
Group size:	3 males
Test substance:	IMEXINE FZ
Batch:	T81
Purity:	99.8%
Vehicle:	water
Dose volume:	0.5 g test substance moistened with water
GLP:	in compliance
Study period:	13 – 17 November 1995

Results
Under the experimental conditions IMEXINE FZ Batch T81 applied for 4 hours to the skin does not produce any dermal reaction. The evaluation of erythema formation could not be performed on account of the purple coloration induced by the test material.
The study authors concluded that the product did not induce any dermal irritation reaction, without oedema.

Ref.: 2

Comment
The study is of limited value because of the purple coloration induced by the test material that interfered with the evaluation.

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405
Species/strain:	New Zealand albino rabbit
Group size:	3 (2 males and 1 female)
Test substance:	IMEXINE FZ
Batch:	T81
Purity:	99.8%
Vehicle:	/
Dosing volume:	0.1 g neat substance
GLP:	in compliance

Study period: 13 – 16 November 1995

Results
One hour after instillation of the substance, irritation reactions are noticed in all animals at the level of the conjunctivae in the three rabbits (discharge and very slight chemosis). These reactions disappear totally in less than 72 hours. An iris congestion quickly reversible (24 hours) is also noticed in one rabbit. 24 hours after instillation a corneal partial opacity is observed in one animal then disappears in 24 hours.

Conclusion
According to the study authors, the test substance is not irritating to rabbit eyes whereas the applicant considered the test substance to be slightly irritant.

Ref.: 3

Comment
Based on the effects seen in this assay, the SCCS considers the test substance to be slightly irritant to the eyes.

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline: OECD 429 (2002)
Species/strain: mouse, CBA/J (nulliparous and non-pregnant)
Group size: 28 (7 groups of 4 females)
Test substance: HC Red n° 7
Batch: 0510149
Purity: 99.3%
Vehicle: acetone:olive oil (4:1, v/v)
Concentration: 0, 0.5, 1, 2.5, 5 and 10%
Positive control: α-hexylcinnamaldehyde
GLP: in compliance
Study period: 3 – 8 November 2004

Method
The skin sensitising potential of HC Red N°7 was evaluated in a Local Lymph Node Assay (LLNA) in mice. It was tested at concentrations ranging from 0.5 to 10% in a mixture of acetone/olive oil, 10% being the maximum practicable concentration due to limited solubility.

Results

Concentration	Stimulation Index
Test item	
0.5%	1.83
1%	2.54
2.5%	6.02
5%	3.19
10%	3.90
α-hexylcinnamaldehyde	
25%	11.98

The EC3 for HC nº 7 is equal to 1.2%

Conclusion
The study authors concluded that, on the basis of the results of this murine LLNA, HC Red N°7 was a moderate skin sensitizer.

Ref.: 4

Comment
According to the classification scheme applied by the SCCS (SCCP/0919/05), the substance is a strong sensitizer.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (2000)
Test substance:	HC Red n° 7
Batch:	0503334
Tissue:	Human abdominal (kept frozen at - 20°C) dermatomed skin
Skin integrity:	TEWL measurement
Method:	Static diffusion cell 2 cm² / receptor compartment 3 ml
Receptor fluid:	Instamed® PBS buffer w/o Ca^{2+} Mg^{2+} 9.55 g/l with 0.25% Tween 80
Formulation tested:	typical commercial formula
Dose of formulation applied:	20 mg/cm²
Concentration of ingredient:	0.86 % (amount applied 173.6 ± 1.56 µg/cm²)
Replicate cells:	4 skin donors, 2 cells/donor, 8 cells mounted and interpreted
Duration of the contact:	30 minutes
Duration of the diffusion:	24 hours
Analytical method:	HPLC with visible detection
Validation:	limit of detection and limit of quantitation measured in the receptor fluid and in the extraction solvent of the tissue samples
Solubility in the receptor:	verified at 32°C > 0.12 mg/ml
Stability of the ingredient:	no information
GLP:	in compliance

The skin penetration of HC Red n° 7 was evaluated in a static Franz diffusion cell system. Human abdominal skin previously frozen was dermatomed to a constant thickness (566 ± 100 µm). The integrity of the skin was evaluated by the measurement of the TEWL, the skin surface temperature was monitored (31.7 ± 0.3 °C). The solubility of HC Red n° 7 in the receptor fluid (PBS buffer with 0.25% of Tween 80 as a solubilizer) was checked in the range of the concentration used. The test substance was prepared at a concentration of 0.86% in a "commercial type" formulation. Approximately 20 mg/cm² of the formulation (exactly measured) were applied to 2 cm² for 30 minutes. The excess from the skin surface was rinsed first with water, followed by a wash with 2% sodium lauryl sulphate aqueous solution, again rinsed with water and finally dried with a cotton swab. 24 hours after the application the substance was measured using HPLC in the receptor fluid, in the horny layer collected by tape stripping (5 to 10 strips), in the epidermis and dermis altogether and in the remaining skin outside the application area. After assay of HC Red n° 7 in the washing material (skin excess) the mass balance of the study was calculated (96.57 ± 2.26% of the applied dose).

Results
Most of the hair dye applied was recovered at the skin surface in the washing liquids (92.27 ± 2.37%). The quantity of test substance penetrating through the skin to the receptor fluid was 0.091 ± 0.064% of the applied dose (0.159 ± 0.113 µg/cm²). The amount recovered in the horny layer was 0.13 ± 0.06% (0.220 ± 0.106 µg/cm²), it was not considered to be percutaneously absorbed. The epidermis and the dermis content was 0.01 ± 0.01% of the applied dose (0.019 ± 0.015 µg/cm²). The absorbed amounts of HC Red n° 7 (epidermis + dermis + receptor fluid) represents 0.10 ± 0.07% of the applied dose (0.178 ± 0.124 µg/cm²) at the end of 24 hours of diffusion after a contact with the skin of 30 minutes.

Ref.: 13

Comment
Although the number of donors and chambers was adequate, the used concentration was too low. In addition, there was a high variability of the data. Accordingly, the mean + 2 SD (0.178 + 2 x 0.124 = 0.426 µg/cm²) is used for calculating the MOS.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral / dermal / inhalation toxicity

No data submitted

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

Taken from SCCNFP/0678/03

Guideline:	OECD 408 (1981)
Species/strain:	Sprague Dawley rats. Crl: CD (SD) BR
Group size:	10 males + 10 females
Test substance:	IMEXINE FZ suspended in 0.5% aqueous carboxymethylcellulose
Batch:	op.57 (purity not stated)
Dose levels:	0, 50, 150 and 500 mg/kg bw/day, 7 days/week by gavage
Exposure period:	13 weeks
GLP:	in compliance

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 50, 150 and 500 mg/kg bw/day, 7 days/week for 13 weeks. The dosing solutions were analysed during weeks 1 and 13 for stability and verification of homogeneity and concentration. During the study, the animals were observed daily for clinical signs and mortality, and weekly for bodyweight and food consumption. During weeks 4 and 13 urine was collected overnight for urinalysis, and blood was sampled from the lateral tail vein for haematology and blood biochemistry. At the end of the treatment periods, a full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs. Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on control and high dose animals.

Results
Two mortalities on day 2 (one male and one female dosed at 500 mg/kg bw) were attributed to dosing error and the animals were replaced. There were no treatment-related mortalities or clinical signs of toxicity. Hairloss and scabbing were noted in some animals of all dose groups, including controls. Purple fur and tail staining was reported in animals dosed at 150 and 500 mg/kg bw/day from day 2, and in low dose animals from day 6 onwards. These were considered to be due to the colour of the test substance and not of toxicological significance. Bodyweight gain and food consumption were similar for all dose groups. Opthalmological examinations revealed no differences between control and high-dose animals.
There was an apparent dose-related decrease in red blood cell count, particularly in females in week 13 where there was statistical significance compared with controls in all treatment groups (changes within 10% of control). There were other associated minor changes in haematological parameters but all values were within the range of historical controls and the author concluded that the changes were of uncertain significance. Alanine aminotransferase showed a dose-related increase in males, which was statistically significant at 500 mg/kg bw/day in both sampling weeks. In week 13, the value was above the historical control range (c. 150% of concurrent control). Smaller increases were seen in females, which were not dose-related and all within the historical control range. Other minor changes were reported, the most consistent being elevation of cholesterol in week 13, which was statistically significant in all female dose groups and in the high dose males. However,

with the exception of the blood cholesterol in the male high dose group, all values were within the historical control range.
Interpretation of urinalysis was made difficult by dark purple discoloration in the test groups and no treatment-related effects were apparent.
Absolute and relative liver weights showed dose-related increases in both sexes. The increases were statistically significant in both sexes at 150 and 500 mg/kg bw/day (male: 113-142% of control; female: 112-132% of control). In addition, the relative liver weight of the 50 mg/kg bw/day males was significantly elevated (110% of control). Significant increases in relative kidney weight were seen in males at all dose levels, but not in a dose-related manner and in females at the high dose. Spleen and ovarian weights were increased in high dose females and thyroid weights were reduced in all female treated groups. These changes were within the normal range for the age and strain of rat, and not considered to be of toxicological significance.
The only abnormalities observed at autopsy were related to the staining properties of the substance. Histopathological examination revealed increased haemosiderin deposits in the spleen of 6/10 males and 9/10 females dosed at 500 mg/kg bw/day. Only 2 of 10 slides were examined for the lower dose groups, which appeared normal. There were a small number of other observations which were considered to be within the normal range for the age and strain of rat.

The author concluded that the NOAEL was 50 mg/kg bw/day and failed to mention the significant increase in relative liver weight in male animals treated at 50 mg/kg bw/day. This dose should be viewed as a LOAEL, although it cannot be definitely concluded that a 10% increase in liver weight is adverse. Effects on the spleen were observed in the high dose group, but it is not possible to draw conclusions on possible effects at 50 and 150 mg/kg bw/day, since insufficient slides were examined.

Ref.: 5a

Following the previous opinion SCCNFP/0679/03, the applicant performed additional microscopic examination, reconsidered the data obtained in the study and undertook a peer-review of this study by an independent expert toxicologist. The review focussed on haematology, clinical chemistry and organ weight data, to interpret them in relation to the macroscopic and microscopic data reported.

Decreased red blood cell parameters, increased bilirubin, increased spleen weights, and increased splenic haemosiderin observed during the study were considered toxicologically significant findings. Organ weight and microscopic findings were more pronounced in female rats, and changes were more pronounced at 150 and/or 500 mg/kg bw/d. Changes in haematological parameters at all dose levels were < 10% of concurrent control values. Though fewer parameters were affected at 50 mg/kg bw/d, the magnitude of the changes observed at this dose level was to be classified as toxicologically significant and a compensatory erythropoetic response could not be ruled out.
The decreases in absolute and relative thyroid weights in females at 150 and 500 mg/kg bw/d were considered also to be toxicologically significant, while in the absence of a correlating decrease in relative weights, the decrease in absolute thyroid weights at 50 mg/kg bw/d was not considered to be so. The compound-related increase in relative liver weights was considered to be an adaptive response, and as such not considered adverse. The toxicological significance of the total protein and kidney weight changes as well as the increased cholesterol in females at all dose levels was considered uncertain.

Ref.: 5b, 5c

Comment
Based on the review of the data presented in the study report, the No Observed Adverse Effect Level (NOAEL) for this study is considered to be below 50 mg/kg bw/d due to haematotoxicity seen in all dose groups. 50 mg/kg bw/d is considered as LOAEL.

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 Assay

Guideline:	OECD 471 (1997)	
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537	
Replicates:	triplicates in 2 individual experiments	
Test substance:	HC Red n° 7	
Batch:	0510149	
Purity:	> 99.5%	
Vehicle:	DMSO	
Concentration:	Experiment 1:	1.6, 8, 40, 200, 1000 and 5000 µg/plate, without and with S9-mix
	Experiment 2:	156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate, without and with S9-mix
	Experiment 3:	156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate, without (TA102, TA1535) and with S9-mix (TA1535)
Treatment:	direct plate incorporation with 72 h incubation pre-incubation method was used in second experiment with S9-mix	
GLP:	in compliance	
Study period:	27 July – 7 September 2004	

HC Red n° 7 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 toxicity range-finding experiment with strain TA100 both without and with S9 mix up to the prescribed maximum concentration of 5000 µg/plate. Toxicity was evaluated on the basis of a thinning of the bacterial background lawn and a reduction in the number of revertant colonies. The data obtained with TA100 in this range-finder experiment were incorporated in experiment 1. The experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the guideline.

Results
Toxic effects in the form of a thinning of the background bacterial lawn or precipitation of HC Red n° 7 were not observed in any of these experiments.
Statistically significant, reproducible and generally concentration dependent increases in the number of revertants were found in strains TA98 and TA100 in the absence and presence of S9 mix and in strain TA1537 in the presence of S9 mix.

Conclusion
Under the experimental conditions used HC Red n° 7 was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref. 6

***In vitro* Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1982)
Cells:	L5178Y ($tk^{+/-}$) mouse lymphoma cells
Replicates:	duplicates in 2 independent tests
Test substance:	IMEXINE FZ
Solvent:	DMSO

Batch:	op 57
Purity:	not stated in the this study report
Concentrations:	150, 300, 750 and 1500 µg/ml with and without metabolic activation
Treatment	3 h both without and with S9 mix; expression period 3 days and a selection period of 12 days.
GLP:	in compliance
Study period:	September 1994

IMEXINE FZ has been investigated for gene mutation at the *tk* locus in L5178Y ($tk^{+/-}$) mouse lymphoma cells. Liver S9 fraction from rats induced with β-naphthoflavone/phenobarbitone was used as the exogenous metabolic activation system.
Exponentially growing suspension cultures of L5178Y were treated with the test agent for 3 hours in the culture medium containing 20% horse serum in the presence and absence of S9 mix.
The concentration range 150, 300, 750 and 1000 µg/ml was selected on the basis of a preliminary toxicity study. Negative and positive controls were in accordance with the OECD guideline.

Results
No precipitate occurred. pH measurement of post-treatment medium was not performed. No raw data regarding the cloning efficiency (CE) is presented.
Without S9 mix a statistically and biologically significant increase in mutant frequency was observed over the concurrent solvent controls in 2 concentrations in test #1 (300 µg/ml: 12.4 x; 1500 µg/ml: 12.7 x), and in one concentration in test # 2 (750 µg/ml: 4.6 x). With S9 mix a statistical and reproducible significant increase in mutant frequency was observed over the concurrent solvent controls in the 2 assays.

Conclusion
From the results generated in 2 experiments it may be concluded that IMEXINE FZ shows reproducible positive results in these tests. Therefore, IMEXINE FZ is considered mutagenic in this test.

Ref.: 7a, 7b

Comment
The study was re-evaluated in June 2005 on behalf of the applicant by an expert on genetic toxicology. The conclusion was that the study is of limited value in assessing the genotoxic potential of IMEXINE FZ. The main reason is that at the time that the study was performed scientific practices and recommendations were accepted which are now superseded and not acceptable under the latest recommendations for this assay. The SCCS agrees with this conclusion.

***In vitro* Mammalian Cell Gene Mutation Test** (*hprt*-locus)

Guideline:	OECD 476 (1997)	
Species/strain:	L5178Y mouse lymphoma cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	HC Red n° 7	
Batch:	0510149	
Purity:	> 99.5%	
Vehicle:	DMSO	
Concentrations: µg/ml,	experiment 1:	250, 500, 750, 1000, 1250, 1500, 1750 and 1972 without and with S9-mix
	experiment 2:	100, 200, 400, 800, 1000, 1250, 1500, 1750 and 1972 µg/ml without and with S9-mix
Treatment	3 h both without and with S9 mix; expression period 7 days and a selection period of 11-12 days.	

GLP: in compliance
Study period: 29 July – 27 September 2004

HC Red n° 7 was assayed for mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. Test concentrations were based on the results of a cytotoxicity range-finding experiment measuring relative total growth. In the main test, cells were treated for 3 h followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. The highest concentration was the prescribed maximum concentration (1972 µg/ml ≈ 10 mM). Liver S9 fraction from Arachlor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as percentage relative survival of the treated cultures relative to the percentage relative survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the cytotoxicity range-finder experiment no significant changes in pH or osmolarity compared to concurrent controls were observed at the highest concentration tested. In both experiments in the absence and presence of S9 mix the appropriate level of toxicity (10-20% survival after the highest concentration) was reached.
No biologically or statistically relevant increases in mutant frequency were found following treatment with HC Red n° 7 at any concentration tested, both in the absence or presence of S9 mix in both experiments.

Conclusion
Under the experimental conditions used, HC Red n° 7 was considered not mutagenic in this *hprt* gene mutation assay in mouse lymphoma cells.

Ref. 8

***In vitro* Micronucleus Test**

Guideline:	OECD 487 (draft)
Species/strain:	human peripheral blood lymphocytes from 2 healthy non-smoking female volunteers (< 35 years)
Replicates:	duplicate cultures, two independent experiments
Test item:	HC Red n° 7
Batch:	0510149
Purity:	> 99.5%
Vehicle:	DMSO
Concentrations:	Experiment 1: 500, 850 and 1200 µg/ml, without S9-mix 1109, 1479 and 1972 µg/ml, with S9-mix Experiment 2: 1400, 1600 and 1972 µg/ml, without S9-mix 1109, 1479 and 1972 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
Study period:	30 August – 25 October 2004

HC Red n° 7 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 the experiments 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 n° 7. 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. The highest dose was the prescribed maximum concentration (1972 µg/ml ≈ 10 mM). 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 Red n° 7 had no effect on osmolarity or pH as compared to concurrent vehicle controls.
In experiments 1, biologically relevant increases in the number of micronucleated binucleate cells compared to concurrent control values were not found at any concentration tested both in the absence and in the presence of S9 mix.
In experiment 2, statistically significant increases in the number of micronucleated binucleate cells were observed at the three concentrations tested in the absence of metabolic activation. As the frequency of micronucleated binucleate cells fell outside the historical negative control range for most HC Red N°7-treated cultures, and as increased numbers of cells showing more than one micronucleus per micronucleated binucleate cell were observed, these increases were considered to be biologically significant. Slight increases in micronucleated binucleate cell frequency were obtained in the presence of S9 mix but these were considered to be devoid of biological significance. Most frequencies of micronucleated binucleate cells for cultures treated with HC Red N°7 indeed remained within the historical solvent negative control range, and no micronucleated binucleate cells showing more than one micronucleus were observed.

Conclusion
Under the experimental conditions used HC Red N°7 induced micronuclei and, consequently, is genotoxic (clastogenic and/or aneugenic) in cultured human peripheral lymphocytes *in vitro*.

Ref.: 9

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

In vivo Mammalian Erythrocytes Micronucleus Test

Guideline:	OECD 474 (1997)
Species/strain:	rat, Crl:CD (SD)
Group size:	5 rats/sex/dose group
Test substance:	HC Red n° 7
Batch:	0510149
Purity:	99.3%
Vehicle:	0.5% aqueous carboxymethylcellulose
Dose level:	0, 500, 1000 and 2000 mg/kg bw
Route:	oral gavage
Sacrifice times:	24 h and 48 h (control and high dose group only) after treatment.
GLP:	in compliance
Study period:	17 February – 20 June 2005

HC Red n° 7 has been investigated for the induction of micronuclei in bone marrow cells of rats. Test concentrations were based on the results of a dose range-finding study in male and female rats on toxic signs and mortality. In the main experiment rats were exposed by oral gavage to single doses of 0, 500, 1000 and 2000 mg/kg bw. Rats were examined approximately 1 h after dosing and at least daily for the duration of the experiment for signs

of clinical toxicity and mortality. Bone marrow cells were collected 24 h or 48 h (control and high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). A satellite group of 6 rats, treated with 2000 mg/kg bw, were included for determination of plasma concentrations of the test article. Blood was collected at 0.5 and 2 h after dosing. Bone marrow preparations were stained with acridine orange and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
Both in the preliminary study and the main experiment, HC Red n° 7 did not induce mortality at any concentration. Treatment with HC Red n° 7 did not result in substantially decreased PCE/NCE ratios compared to the untreated controls indicating that HC Red n° 7 did not have cytotoxic properties in the bone marrow. In contrast, clinical signs like purple urine and red brown genital discharge observed in all treated groups were considered to be evidence of systemic exposure. Moreover, at 1000 and 2000 mg/kg the rats showed hypoactivity and squinted eyes. The results of plasma analysis in animals given 2000 mg/kg confirmed systemic exposure (mean maximal plasma concentrations of 11.7 µg/mL (males) and 29.1 µg/mL (females) were obtained 0.5 hour after dosing).
Biologically relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment.

Conclusion
Under the experimental conditions used HC Red n° 7 did not induce an increase in the number of micronucleated PCEs in bone marrow cells of treated rats and, consequently, HC Red n° 7 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of rats.

Ref.: 10

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

Guideline:	OECD 486 draft guideline of 1991
Species/strain:	Wistar HanIbm: WIST rats
Group size:	4 males per test group
Test substance:	IMEXINE FZ
Batch:	op T81
Purity:	99.8%
Dose levels:	0, 150 and 1500 mg/kg bw
Route:	oral gavage
Vehicle:	polyethylene glycol 400
Sacrifice times:	15 hours: all dose groups; 2h: high dose group
GLP:	in compliance
Study period:	25 April 1995 – 29 August 1995

HC Red n° 7 has been investigated for induction of unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro* following *in vivo* dosing. Test concentrations were based on a preliminary study on acute toxicity at interval of 1 and 24 h. The maximum tolerated dose, in this study 1500 mg/kg bw, was determined to be the dose that caused toxic reactions without having major effects on survival within 24 h. Negative and positive controls were in accordance with the OECD guideline. Animals were sacrificed after 15 hours and for an additional high dose group after 2 hours. Hepatocytes were isolated and at least 3 cultures were established per animal. The hepatocytes were subsequently treated with ^{3}H-thymidine *in vitro*. The uptake of radio-labelled ^{3}H-thymidine was assessed by autoradiography.

Results

Treatment with HC Red n° 7 did not result in deaths. Animals given 1500 mg/kg showed red discolouration of urine which was considered to be evidence of systemic exposure following oral administration of HC Red N°7. In the final UDS assay, the viability of the hepatocytes was not substantially affected due to the treatment with HC Red n° 7 at any of the treatment periods or dosage groups.

HC Red N°7 did not produce any changes from controls in mean net nuclear grain counts at any dose level and sampling time. Additionally, there was no shift towards higher values in the percentage distribution of net nuclear grain counts.

Conclusion
Under the experimental conditions used HC Red N°7did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref: 11

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

Taken from SCCNFP/0678/03

Prenatal developmental study

Guideline:	OECD 414 (1981)
Species/strain:	Sprague-Dawley rat, Crl: CD (SD) BR
Group size:	24 females (mated)
Test substance:	IMEXINE FZ in 0.5% aqueous carboxymethylcellulose
Batch:	op 57 (purity 98.9%)
Dose levels:	0, 50, 200 and 800 mg/kg bw/day
Treatment period:	Days 6 to 15 of pregnancy, inclusive
GLP:	in compliance

Groups of 24 female rats were dosed with the test substance by gavage at 0, 50, 200 and 800 mg/kg bw/day on days 6 to 15 after mating. The dams were observed daily for clinical signs and mortality, bodyweight was recorded on days 0, 6-15 and 20 and food consumption on days 6, 9, 12, 15 and 20. They were sacrificed on day 20 of pregnancy, and examined for number of corpora lutea, number and distribution of live and dead foetuses, of early or late resorptions and of implantation sites, and for macroscopic observations. The foetuses were examined for bodyweight, sex and macroscopic external observations, and for skeletal and visceral abnormalities (half for each endpoint). The concentrations, homogeneity and stability of the dosing formulations were verified analytically.

Results
There were no premature deaths and no treatment-related clinical signs except for dose-related purple coloration of the urine, tail and fur. Hairloss and scabbing was reported in some animals of the control group and one high-dose animal. The high dose group animals exhibited reduced weight gain compared to controls throughout the dosing period, with actual weight-loss during the first two days. Bodyweight gain was also reduced in the 200

mg/kg bw/day group at the start of the dosing period. Weight gain of the low dose group was similar to controls throughout pregnancy. Food consumption was also decreased in a dose-dependent manner during dosing, and the decrease was statistically significant at 800 mg/kg bw/day.
The only macroscopic observations at autopsy related to the staining properties of the test substance, and this was dose-related. The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses, sex distribution and the mean foetal bodyweights were not significantly different for control and treated groups. However, numbers of corpora lutea, implantations and liver foetuses were slightly higher in the high-dose group. This was assumed to be a coincidental observation and large litter numbers were associated with decreased mean foetal weight in the high dose group, which was also not statistically significant.
The incidence of major skeletal and external/visceral abnormalities was 1, 4, 1, and 0 at 0, 50, 200 and 800 mg/kg bw/day respectively. The low incidence and absence of dose-response relationship indicated that the abnormalities were not treatment-related. The incidences of minor external and visceral abnormalities were in the normal range.
The incidence of minor skeletal abnormalities was higher in the 800 mg/kg bw/day group than in concurrent and historical controls. This was due to slightly higher incidences of foetuses with delayed ossification, 7 instead of 6 lumbar vertebrae and increased numbers of vestigial 14th ribs.
The difference was only statistically significant with respect to the incidence of non-ossification of the caudal neural arches. Incidences of minor abnormalities in the 50 and 200 mg/kg bw/day dose groups were similar to or lower than controls.
The test substance elicited dose-related maternal toxicity at 200 and 500 mg/kg bw/day. Delayed ossification was possibly related to lower foetal weight for larger litter sizes. However the incidence of 7 lumbar vertebrae and vestigial 14th ribs were considered to be indicative of an effect on foetal development, possibly resulting from the effect of treatment on maternal bodyweight. The NOAEL was 50 mg/kg bw/day for the dams and 200 mg/kg bw/day for the foetuses.

Ref.: 12

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

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 Red n° 7)

Absorption through the skin	**A (µg/cm^2)**	**=**	**0.426 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.247 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.004 mg/kg**
Lowest observed adverse effect level (90 day oral, rat)	**LOAEL**	**=**	**50 mg/kg**
Adjustment factor 3	**adjusted LOAEL**	**=**	**16.67 mg/kg**
Margin of Safety	**adj. LOAEL / SED**	**=**	**4167**

3.3.14. Discussion

Physico-chemical properties
HC Red n° 7 is used in semi-permanent hair dye formulations at a maximum concentration of 1.0%, in the absence of oxidative agents.
According HPLC profile and titre by potentiometry of HC Red n° 7, the purity of the raw material (marketed material) should be described as at least 98.9% (HPLC area% at 490 ± 5 nm). The impurity 2-nitrobenzene-1,4-diamine (or 2-nitro-p-phenylenediamine) is banned according to the cosmetics directive. (Annex II, entry n° 1319). The SCCS considers that this impurity at the levels present does not constitute a safety concern.
HC Red n° 7 is a secondary amine, and thus prone to nitrosation. The nitrosamine content of HC Red n° 7 is not reported but it should be < 50 ppb. HC Red n° 7 should not be used in the presence of nitrosating agents. The stability of HC Red n° 7 in typical hair dye formulations is not reported.

Toxicity
The substance is minimally toxic by ingestion of a single dose.
The No Observed Adverse Effect Level (NOAEL) for a 90-day study in rats was considered to be below 50 mg/kg/day. Due to the haematotoxicity seen in all dose groups, 50 mg/kg/day is considered as LOAEL. Because of the minor effects seen, an adjustment factor of 3 was considered adequate.
The NOAEL was set at 50 mg/kg bw/day for the maternal toxicity and at 200 mg/kg bw/day for foetotoxicity.

Skin/eye irritation and sensitisation
The evaluation of erythema formation could not be performed because of the purple coloration induced by the test material that interfered with the evaluation.
The test substance is slightly irritant to the eyes.
HC Red n° 7 is considered to be a strong sensitizer.

Percutaneous absorption

Although the number of donors and chambers was adequate, the used concentration was too low. In addition, there was a high variability of the data. Accordingly, the mean + 2 SD (0.178 + 2x 0.124 = 0.426 µg/cm²) is used for calculating the MOS.

Mutagenicity/genotoxicity
Overall, the genotoxicity of HC Red n° 7 is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. HC Red n° 7 did induce gene mutations in bacteria. In a gene mutation test in mammalian cells HC Red n° 7 induced an increase in mutant frequency. This study was considered of limited value in assessing the genotoxic potential of HC Red n° 7. A second gene mutation assay in mammalian cells was negative. In an *in vitro* micronucleus test an increase in micronucleated human peripheral blood lymphocytes was reported.
The positive *in vitro* findings with HC Red n° 7 could not be confirmed in *in vivo* assays. A rat bone marrow micronucleus tests and an *in vivo* UDS test were negative.
As the *in vitro* results were not confirmed in *in vivo* tests, HC Red n° 7 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data were submitted on carcinogenicity

4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of HC Red n° 7 as a non-oxidative hair dye with a maximum concentration on the head of 1.0 % does not pose a risk to the health of the consumer, apart from its strong sensitising potential.

HC Red n° 7 is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

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