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

CONCERNING

HC RED Nº 7

COLIPA n° B36

adopted by the SCCNFP during the 24th plenary meeting
of 24-25 June 2003

1. Terms of Reference

1.1 Context of the question

The adaptation to technical progress of the Annexes to Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products.

1.2 Request to the SCCNFP

The SCCNFP is requested to answer the following questions :

* Is HC Red n° 7 safe for use in cosmetic products?

* Does the SCCNFP propose any restrictions or conditions for its use in cosmetic products?

1.3 Statement on the toxicological evaluation

The SCCNFP is the scientific advisory body to the European Commission in matters of consumer protection with respect to cosmetics and non-food products intended for consumers.

The Commission's general policy regarding research on animals supports the development of alternative methods to replace or to reduce animal testing when possible. In this context, the SCCNFP has a specific working group on alternatives to animal testing which, in co-operation with other Commission services such as ECVAM (European Centre for Validation of Alternative Methods), evaluates these methods.
The extent to which these validated methods are applicable to cosmetic products and its ingredients is a matter of the SCCNFP.

SCCNFP opinions include evaluations of experiments using laboratory animals; such tests are conducted in accordance with all legal provisions and preferably under chemical law regulations. Only in cases where no alternative method is available will such tests be evaluated and the resulting data accepted, in order to meet the fundamental requirements of the protection of consumer health.

2. Toxicological Evaluation and Characterisation

2.1. General

2.1.1. Primary name

HC Red n° 7 (INCI name)

2.1.2. Chemical names

Chemical name	:	1-Amino-2-nitro-4-[(β-hydroxyethyl)-aminobenzene]
CAS name	:	Ethanol, 2-[(4-amino-3-nitrophenyl)amino]-
Synonyms	:	Ethanol, 2-[(4-amino-3-nitroanilino]-
	:	2-nitro-4-(β-hydroxyethylamino)aniline
	:	2-(4-amino-3-nitroanilino)ethanol; 2-[(4-amino-3- nitrophenyl) amino] ethanol

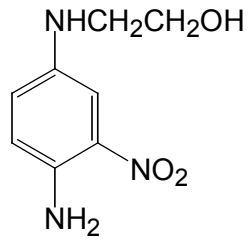
2.1.3. Trade names and abbreviations

Trade name	:	IMEXINE® FZ (Chimex)
COLIPA n°	:	B36

2.1.4. CAS/EINECS no.

CAS no	:	24905-87-1
EINECS	:	246-521-9

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula	:	$C_8H_{11}N_3O_3$
Mol weight	:	197.19

2.1.7. Purity, composition and substance codes

All analytical data relate to batch op.57.

Purity	:	98.9% (titre as determined by HPLC)
Water content	:	0.13%
Heavy metals	:	< 10 ppm

Potential impurities

- Reagents and intermediate reaction products

2-Nitrobenzene-1,4-diamine	:	0.127%
3-(4-Amino-3-nitro-phenyl)-oxazolidin-2-one	:	0.027%
4-(Amino-3-nitro-phenyl)-carbamic acid 3-chloro-propyl ester	:	<0.025%

- Solvent residues

Isopropyl acetate	:	< 10 ppm
Chloride ions	:	< 0.1%

2.1.8. Physical properties

Appearance	:	Brick red powder
Melting point	:	95-96 °C
Boiling point	:	/
Density	:	0.3 g/cm³
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	0.8 (calculated), 0.51 (measured)

2.1.9. Solubility

Water[(1)]	:	/
Ethanol 95%	:	10 %
Receptor fluid[(2)]	:	120 µg/ml at 32 °C

(1) The reported expression "insoluble in water at 1%" is meaningless. Most available data indicate that HC Red n° 7 is insoluble or almost insoluble in water.

(2) receptor fluid used in percutaneous absorption study : Instamed® PBS buffer w/o Ca^{2+}, Mg^{2+} 9.55g/l containing 0.25% of Tween 80

General comments on analytical and physico-chemical characterisation

* With reference to Submission I, the SCCNFP asked for appropriate information on solubility : no additional data available from Submission II, i.e. no quantitative data given for solubility in water and in solvents used in some toxicological studies and analytical

determinations (methanol, aqueous 0.5 % carboxymethylcellulose, ethylene glycol, polyethylene glycol 400).

* Chemical purity not stated in a number of toxicity study reports.

* Purity of the chemical assessed reliably (HPLC) and reported for only one batch: it would be advisable to have an statement of the range of impurities that be may be present, based on the analysis of more than one batch.

* The impurity 2-nitrobenzene-1,4-diamine (or 2-nitro-p-phenylenediamine) is classified by the German MAK Commission as carcinogen, category 3B.

* No experimental data on stability provided.

* HC Red No.7 is a secondary alkanolamine, and thus it is prone to nitrosation. No data is provided on the nitrosamine content of the dye or in the hair dye formulations.

2.2. Function and uses

HC Red n° 7 will be incorporated in semi-permanent and temporary hair dyeing lotions at a maximum concentration of 1.5%. It is common practice for 35ml of undiluted formulation to be applied for a period of 30 minutes before washing. Application may be repeated at weekly intervals.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	/
Species/strain	:	Sprague Dawley rat
Group size	:	5 males + 5 females
Test substance	:	HC Red n° 7 in 1% carboxymethylcellulose
Batch no	:	16.3.77 (purity not stated)
Dose	:	5, 7 and 9 g/kg bw. in a volume of 30 ml/kg
Observ. Period	:	14 days
GLP	:	pre-dates GLP

Groups of 5 male and 5 female rats received a single dose of test substance by gastric gavage. The doses were based on a preliminary study in which mortalities occurred at 10 g/kg, but not at 3.16 g/kg. The animals were observed for mortalities, clinical signs and bodyweights for 14 days. No autopsy details are provided.

Results

Mortality rates were 40, 40 and 70% at 5, 7 and 9 g/kg bw, respectively, with all deaths occurring on the day of dosing. The proportion of deaths was higher for females than for males.

Clinical signs included hypoactivity, tremor and piloerection. All animals appeared normal after the first day and weight gain of surviving animals did not differ between groups. The oral LD50 was reported to be 6.8 g/kg bw.
This study does not meet currently accepted standards. The dosing volume was too high, and it would not now be considered appropriate to use such high dose levels.

Ref. : 1

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

No data

2.3.5. Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

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 no	:	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. : 5

2.3.8. Sub-chronic dermal toxicity

No data

2.3.9. Sub-chronic inhalation toxicity

No data

2.3.10. Chronic toxicity

No data

2.4. Irritation & corrosivity

2.4.1. Irritation (skin)

Guideline	:	/
Species/strain	:	New Zealand albino rabbit
Group size	:	3 males at each time point
Test substance	:	HC Red n° 7, 4% in propylene glycol
Batch no	:	16.3.77 (purity not stated)
Dose	:	0.5 ml
GLP	:	pre-dates GLP

The study pre-dates the OECD guideline and was conducted under more extreme conditions than now required. 0.5 ml of the test substance was applied to 4 cm^2 areas of intact and scarified skin of 6 male rabbits. Occlusive patches were applied and left in place for 23 hours on 3 animals. The remaining 3 animals were examined 72 hours after application, but the study report does not describe whether the patches remained on the skin for the entire period. Skin biopsies were performed and examined for cutaneous reactions.

Results
Biopsies appeared normal after the 23 hours of application. Moderate reactions were observed in the 72 hour biopsies of scarified application sites of two animals, but not in the intact application sites of any of the animals.
The substance was non-irritating to intact skin.

Ref. : 3

2.4.2. Irritation (mucous membranes)

Guideline	:	/
Species/strain	:	New Zealand albino rabbit
Group size	:	6 males
Test substance	:	HC Red n° 7, 4 % in propylene glycol
Batch no	:	16.3.77 (purity not stated)
Dose	:	0.1 ml
GLP	:	pre-dates GLP

The study pre-dates the OECD guideline, but appears to conform to its requirements. 0.1 ml was applied once to the left eye of 6 male rabbits, without rinsing. The right eye served as control and was untreated. Ocular reactions were recorded at 1 hour and 1, 2, 3, 4, and 7 days after instillation.

Results
Mild reactions were observed in the conjunctivae of all rabbits and in the iris of 3 rabbits, returning to normal by day 3. The mean ocular irritation index was reported to be 6.17 out of 110 after one hour, and 1.67 on days 1 and 2.
The test substance was slightly irritant to the rabbit eye at a concentration of 4% in propylene glycol.

Ref. : 2

2.5. Sensitisation

Epicutaneous maximisation test

Guideline	:	/
Species/strain	:	Hartley albino guinea pig
Group size	:	10 males + 10 females (no control group)
Test substance	:	IMEXINE FZ
Batch no	:	13.12.78 (purity not stated in study report)
Concentrations	:	intradermal induction : 0.1 ml 50% Freund's complete adjuvant (FCA) topical induction : 10 applications of neat test substance over 22 d. challenge : 0.3 g of test substance for 48 hours
GLP	:	Pre-dates GLP

The protocol was as described by Brulos *et al*., (J. Soc. Cosmet. Chem. 28, 357-365, 1977). The study did not include a control group.
Induction consisted of two injections of FCA on days 1 and 10, and topical application of the neat test substance (0.3 g) on days 1, 3, 5, 8, 10, 12, 15, 17, 19 and 22. Application sites were occluded for 48 hours after application. Following removal of the final patch (day 24), a period of 12 days was allowed before challenging with 0.3 g of neat test substance under occluded patch for 48 hours on a previously untreated area of skin. The skin was examined at 1, 6, 24 and 48 hours after removal of the patches.

Results
Red staining of the skin, due to the test substance, made macroscopic observation of possible erythema difficult. Histological examinations indicated that inflammation occurred in 6 of 19 guinea pigs. One animal died in the course of the study, but the report does not provide information on the cause of death.

Ref. : 4

2.6. Teratogenicity

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 no	:	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 was 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 14^{th} 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 14^{th} 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. : 11

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.1 Percutaneous Absorption *in vitro*

Study 1

Guideline	:	/
Tissue	:	Human abdominal epidermis, heat-separated
Method	:	Franz diffusion cell (static)
Test substance	:	IMEXINE FZ, 1.28% in formulation
Batch no	:	op.21 (purity not stated in study report)
Dose levels	:	c. 40mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	8 cells with hair and 7 cells without hair
GLP	:	not in compliance

The skin penetration of HC Red n° 7 was evaluated in a static Franz diffusion cell system. Human epidermis was prepared by heat-separation from previously frozen breast skin. The test substance was prepared at a concentration of 1.28% in a formulation. Approximately 40 mg of the mixture was applied to 2cm^2 of epidermal membrane with and without addition of 10 mg finely chopped bleached hair for 30 minutes and then excess washed off with 2% sodium lauryl sulphate solution and dried. Four hours later the levels of substance were measured in the receptor fluid (physiological saline) using HPLC. Integrity of the epidermal membrane was checked by microscopy before the study, and by means of addition of Chinese ink at the end of the study. Any cells showing penetration of the ink were eliminated from the analysis.

Results
The quantity of test substance penetrating through the epidermis to the receptor fluid corresponded to 0.009% of applied dose in the presence of hair and 0.019% in the absence of hair.
This study did not include determination of recovery of the test substance. Physiological saline was used as the receptor fluid, which may not be adequate for a relatively lipophilic substance and insufficient time was allowed for permeation from the epidermal membrane into the receptor fluid. Therefore, the study is considered inadequate.

Ref. : 12

Study 2

Guideline	:	OECD 428 (2000)
Test substance	:	HC Red n° 7
Batch no	:	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: 14

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline	:	OECD 471 (1983)
Species/strain	:	*S. typhimurium,* TA98, TA100, TA1535, TA1537; *E. coli,* WP2uvrA
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	IMEXINE FZ in DMSO solution
Batch no	:	op T81 (purity 99.8%)
Concentrations	:	6 concentrations covering two logarithmic decades from 33.3-5000 µg/plate with and without metabolic activation
GLP	:	in compliance

HC Red n° 7 has been investigated for gene mutation in *S. typhimurium* and *E. coli,* using the direct plate incorporation method both with or without S9 mix. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guideline. Because of the lack of toxicity in the preliminary dose range finding assay, the concentration range of 33.3 - 5000 μg/plate was selected

Results
- without S9 mix : no dose related or biologically relevant increase in revertant numbers was observed, in any of the tester strains (*S. typhimurium* and *E. coli*), in any of the experiments performed.

- with S9 mix : a significant and reproducible increase in revertant numbers was observed only in two frameshift tester strains *Salmonella* TA98 and *Salmonella* TA1537. These increases exceeded largely the 2 fold criteria for positivity.

Test # 1 TA 98 (1.1– 13.2 x), *Salmonella* TA1537 (1.0– 11.1 x);
Test # 2 TA 98 (1.4– 20.1 x), *Salmonella* TA1537 (1.2– 5.0 x).

In addition, while non statistically significant, a trend for concentration-related increases was observed in one of the two experiments with TA100 and TA1535. No increases were seen with the *E. coli* tester strain.

The test is acceptable for evaluation. Based on the reversion rate, and under the conditions of the assays performed, it is concluded that the test agent IMEXINE FZ is positive in the *S. typhimurium* TA 1537 and TA 98 frameshift tester strains in the presence of S9 mix.

Ref. : 6

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

Guideline	:	OECD 476 (1984)
Cells	:	L5178Y ($TK^{+/-}$) mouse lymphoma cells
Replicates	:	2 independent tests
Test substance	:	IMEXINE FZ in DMSO solution
Batch no	:	op 57 (purity not stated in the this study report)
Concentrations	:	150, 300, 750 and 1500 μg/ml with and without metabolic activation
GLP	:	in compliance

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 1000μg/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.

Cytotoxicity
No raw data regarding the cloning efficiency (CE) is presented.

Mutant frequency
Without S9 mix : A statistical and biological 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.

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. : 7

***In vitro* Mammalian Chromosomal Aberration Test**

Guideline	:	/
Species/strain	:	Chinese Hamster Ovary Cells
Replicates	:	Duplicate cultures
Test substance	:	IMEXINE FZ in DMSO
Batch no	:	op 2 Brut (purity 95.4%) op 2, recrystallised (purity 99.6%)
Concentrations	:	19, 60 and 190 µg/ml op 2 Brut without metabolic activation 190, 600 and 1900 µg/ml op 2 Brut with metabolic activation 182 µg/ml op 2, recrystallised without metabolic activation 1820 µg/ml op 2, recrystallised with metabolic activation
GLP	:	in compliance

HC Red n° 7 has been investigated for induction of chromosomal aberrations in CHO cells. Liver S9 fraction from rats induced with β-naphthoflavone/phenobarbitone was used as the exogenous metabolic activation system. The test concentrations were established from a preliminary toxicity study.
In the absence of S9 Mix, at 250 µg/ml, a reduction of 57 % of the mitotic index as compared to the corresponding solvent control was noted. In the presence of S9 Mix, a reduction of 57 % of the mitotic index was seen at 2500 µg/ml.

Exposure and concentrations
- without S9, exposure was for 24 hours and the cells were then harvested immediately.
- with S9, exposure was for 3 hours, with harvest times of 12 and 24 hours.
A recrystallised sample of the test substance was tested at a single concentration in a second experiment.
Negative and positive controls were in accordance with the OECD guideline.
Four distinct experiments have been conducted in order to provide adequate data,
However, these assays cannot be considered as a real independent repeat experiment.

Results
Toxicity
No relevant toxic effect as evidenced by a decrease in Mitotic Index (MI) was observed in the absence or in the presence of S9 mix in the range of concentrations tested.

Structural chromosome aberrations
- without S9 mix : IMEXINE FZ (batch No op 2 ; purity 95.4%). No statistically and biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control.
IMEXINE FZ (batch No op 2, recrystallised ;purity 99.6%). With the purified preparation, there was a significant increase in cells displaying chromosomal aberrations.
- with S9 mix : IMEXINE FZ (batch No op 2 ;purity 95.4%). An increase of the frequency of cells displaying structural chromosome aberrations was observed at 600 µg/ml.
IMEXINE FZ (batch No op 2, recrystallised ;purity 99.6%). With the purified preparation, no significant increase in cells displaying chromosomal aberrations was observed for the single dose tested (1820 mg/ml).

Polyploidy
No data given.

There are some indication of clastogenicity and the test, as conducted, should therefore be considered equivocal. The assay is unsuitable for evaluation (there is no independent repeat study, the dose range is inadequately selected, test substances are not identical).

Ref. : 8

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline	:	/
Species/strain	:	Mouse, Swiss CD1
Group size	:	5 male + 5 female
Test substance	:	IMEXINE FZ suspended in 0.5% aqueous carboxymethylcellulose
Batch no	:	op 2 Brut (purity 95.4%)
Dose levels	:	0, 1120 and 2230 mg/kg bw for males; 0, 709 and 1420 mg/kg bw for females
Sacrifice times	:	12, 48 and 72 hours
GLP	:	in compliance

HC Red n° 7 has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined on the basis of the results of an acute oral toxicity study in mice. Dose selected were 40 % and 80 % of the LD 50 of that study.
Negative and positive controls were inadequate.

IMEXINE FZ was administered by 1 single oral dose. Male mice : 1120 and 2230 mg/kg.
Female mice : 709 and 1420 mg/kg .
3 sacrifice times were selected : 12 h, 48 h and 72 h after oral administration.
Bone marrow smears were obtained from the positive control group 12 h, 48 h and 72 hours after dosing.

Number of cells scored : a total of at least 1000 erythrocytes were examined from each animal; the incidence of micronucleated erythrocytes and the ratio of polychromatic erythrocytes to normochromatic erythrocytes were calculated.

Results
Reactions to treatment
Except one male found death after 72 h at the top dose, no signs of clinical toxicity were observed.

Mean values of micronucleated PCE
No statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values were observed at any sampling times

PCE/NCE ratio
No significant reduction in the PCE/NCE ratio was observed in any of the dosage groups of mice treated with IMEXINE FZ (batch no op 2 brut). There is no demonstration of the exposure of the bone marrow target cells.

Ref. : 9

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

Guideline	:	OECD draft guideline of 1991
Species/strain	:	Wistar rat, HanIbm: WIST (SPF)
Group size	:	3 males
Test substance	:	IMEXINE FZ suspended in polyethylene glycol 400
Batch no	:	op T81 (purity 99.8%)
Dose levels	:	0, 150 and 1500 mg/kg bw, by gavage
Sacrifice times	:	15 hours: all dose groups; 2h: high dose group
GLP	:	in compliance

HC Red n° 7 has been investigated for induction of unscheduled DNA synthesis (UDS) in rat hepatocytes in vitro following *in vivo* dosing. A preliminary toxicity study showed signs of toxicity but no deaths at 1500 mg/kg bw and therefore that dose was selected as the Maximum Tolerated Dose, in accordance with the OECD draft guideline. 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}HTdR was assessed by autoradiography.

Results
In the final UDS assay, the viability of the hepatocytes was not substantially affected due to the *in vivo* pretreatment with HC Red n° 7 at any of the treatment periods or dosage groups.
Negative control animals gave a group mean NNG value of less than zero. Positive control animals gave a group mean positive NNG value.
Treatment with HC Red n° 7 at doses of 150 & 1500 mg/kg yielded group mean NNG values less than 2000 and caused no significant increases, as compared to control, in the mean nuclear grain counts. In addition, no significant shift to higher values was observed in the percentage distribution of the nuclear net grain counts.

The study is adequate.
Data indicate that single oral gavage treatment of male rats dosed once with 150 & 1500 mg/kg of HC Red n° 7 did not induce increased unscheduled DNA synthesis in hepatocytes isolated approximately 2 or 16 hours after dosing. Under the experimental conditions, it is concluded that HC Red n° 7 did not induce DNA-damage leading to increased repair synthesis detectable by this assay.

Ref. : 10

2.9. Carcinogenicity

No data

2.10. Special investigations

The COLIPA summary includes a published paper describing comparison of the *in vitro* toxicity to V79 cells with the LD50 obtained for intraperitoneal administration to mice, for 19 hair dyes. This study is not informative for the safety evaluation of HC Red n° 7 in hair dye formulations.

Ref. : 13

2.11. Safety evaluation

NOT APPLICABLE

2.12. Conclusions

The purity of HC Red n° 7 was determined in only one batch that was not the same batch used in most toxicological studies. No information is given about the stability of the dye.
HC Red n° 7 is a secondary alkanolamine, that may give rise to nitrosamine formation. Therefore, analytical data on the nitrosamine content on more than one sample as well as in hair dye formulations is considered essential.

The acute toxicity studies are old and not conducted to GLP or current guidelines. Nevertheless, the studies are sufficient to demonstrate that the substance is minimally toxic by ingestion of a single dose.

A 13-week oral repeat dose study in rats showed a dose-related increase in liver weight of male rats for which a NOAEL was not identified. The 10% increase seen at 50 mg/kg bw/day cannot be clearly defined as “adverse” in the absence of histopathological changes. Serum transaminases were significantly elevated at 500 mg/kg bw/day, but not at the lower doses. The other most notable observation was deposits of haemosiderin in the spleens of both sexes at 500 mg/kg bw/day. The spleens of animals of the intermediate dose groups were not all examined and therefore it is not possible to identify a NOAEL for this effect.
When administered during organogenesis, the substance adversely affected maternal food consumption and weight gain, with a NOAEL of 50 mg/kg bw/day. Foetotoxicity appeared to be a secondary effect of maternal toxicity, occurring at 800 mg/kg bw/day with a NOAEL of 200 mg/kg bw/day.

The test substance was non-irritant to intact rabbit skin when applied neat and slightly irritating to the rabbit eye at a concentration of 4%.

A percutaneous penetration study has been performed in compliance to GLP, using human dermatomed skin *in vitro* and a receptor fluid composed of INSTAMED® PBS buffer w/o Ca^{2+} Mg^{2+}, 9.55 g/l with 0.25 % Tween 80, in which the solubility of HC Red n° 7 has been found adequate (0.12 mg/ml). A low penetration rate of HC Red n° 7 through the skin was found (0.178 µg/cm²).

HC Red n° 7 was tested in procaryotic cells for gene mutation in several tester strains of *S. typhimurium* and *E. coli* WP2 uvrA. The test agent is positive in *S. typhimurium* TA 1537 and TA 98 frameshift tester strain in the presence of S9 mix .
HC Red n° 7 is positive in the *in vitro* Mammalian Cell Gene Mutation Test.
HC Red n° 7 (IMEXINE FZ, batch no op 2 brut or recrystallized) is considered equivocal for clastogenicity in the *in vitro* mammalian chromosomal aberration test as conducted. The study is considered unsuitable for evaluation.
HC Red n° 7 (IMEXINE FZ, batch no op 2 brut) gave negative results in the mammalian erythrocyte micronucleus test. However, the study did not demonstrate that bone marrow was reached by the test agent. For this reason, the study is considered unsuitable for evaluation.
HC Red n° 7 (IMEXINE FZ, batch no op T 81) gave negative results in the unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo*.
In view of the above, the data are insufficient/inadequate for evaluation.

2.13. References

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14. L'Oréal Recherche, France. In vitro percutaneous absorption of HC Red n° 7 (IMEXINE FZ, Colipa n° B36). Study n° 16082; 05/02/2001

3. Opinion of the SCCNFP

The SCCNFP is of the opinion that the information submitted is inadequate to assess the safe use of the substance.

Before any further consideration, the following information is required :

* quantitative data on solubility in e.g. in 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

4. Other considerations

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5. Minority opinions

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