

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

CONCERNING

HC YELLOW N° 11

COLIPA n° B63

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 Yellow n° 11 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 Yellow n° 11 (INCI name)

2.1.2. Chemical names

Chemical name : 1- β -hydroxyethylamino-2-hydroxy-4-nitro-benzene
 CAS name : phenol,2-[(2-hydroxyethyl)amino]-5-nitro-
 Synonyms : 1-(β -hydroxyethyl)-amino-2-hydroxy-4-nitrobenzene
 2-[(2-hydroxyethyl)amino]-5-nitrophenol

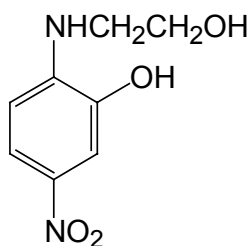
2.1.3. Trade names and abbreviations

Trade name : IMEXINE[®] FW (Chimex)
 COLIPA No. : B63

2.1.4. CAS/EINECS no

CAS No. : 73388-54-2
 EINECS : /

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : C₈H₁₀N₂O₄
 Mol weight : 198

2.1.7. Purity, composition and substance codes

Purity (batch op.16) : 99.4% (titre as determined by potentiometry)
 Water Content (batch op.16) : < 4% (w/w)
 Ash Content (batch op.16) : < 0.1%

Evaluation and opinion on : HC Yellow n° 11

Potential impurities and reaction intermediates

Reagent	:	3,4-methylene-dioxy-nitrobenzene (batch op.2X)	:	0.02%
Solvent residue	:	ethanol (batch op.16)	:	550 ppm
Others	:	Acetic Acid (batch op.16)	:	0.04%
		Free formaldehyde (batch op.16)	:	33 ppm
		Sulphite ions (batch op.16)	:	10 ppm
		Heavy metals (batch op.16)	:	< 10ppm

2.1.8. Physical properties

Appearance	:	Brownish orange/brownish violet crystalline powder; almost odourless
Melting point	:	202-205° C (thermomicroscopic method)
Boiling point	:	/
Density	:	0.6 g/cm ³
Rel. vap. dens.	:	/
Vapour Press.	:	/
Flash point	:	> 218 °C
Log P _{ow}	:	1.2 (calculated)
Storage	:	Protect from light and moisture

2.1.9. Solubility

Water	:	540 mg/l at 30°C
95% ethanol	:	0.5%
Receptor fluid*	:	≥ 133 µg/ml

* receptor fluid used in percutaneous absorption study : Instamed® PBS buffer w/o Ca²⁺, Mg²⁺ 9.55g/l containing 0.25% of Tween 80,

2.1.10 Stability

A loss of 5 % of the dye was noted in the formulation (dye content 1%), used for percutaneous absorption study, over a period of one month at room temperature. The degradation products of the dye in the formulation have not been characterised.

General comments on analytical and physico-chemical characterisation

- * The purity of HC Yellow n° 11 has been determined by potentiometric titration against KOH – phenolic function. Its chromatographic purity has not been reported.
- * Chromatographic impurities in only one batch (op. 2X) of HC Yellow n° 11 has been reported. This batch is different from the one used for the description of purity and physico-chemical properties. Batch op.2X has not been used for any of the studies reported.

Evaluation and opinion on : HC Yellow n° 11

- * Degradation products of the dye in the formulation have not been characterised.
- * HC Yellow n° 11 is a secondary alkanolamine, and thus, it is prone to nitrosation. No information on the nitrosamine content of the dye and the dye formulation has been provided.

2.2. Function and uses

HC Yellow n° 11 will be incorporated in semi-permanent hair dye formulations up to a maximum concentration of 1.1%. It is common practice for 35 ml of undiluted formulation to be applied for a period of 30 minutes before washing.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	OECD 401 (1981)
Species/strain	:	Sprague Dawley rat, strain not specified
Group size	:	5 males + 5 females
Test substance	:	IMEXINE FW in polyethylene glycol 400
Batch no	:	op.6
Purity	:	/
Dose	:	5000 mg/kg bw
Observ. Period	:	14 days
GLP	:	in compliance

The dose group was selected on the basis of a preliminary range-finding study in which rats were given the test compound in polyethylene glycol 400 at dose levels of 500, 1000, 3000 and 5000 mg/kg bw. Groups of 5 male and 5 female rats received a single dose of test substance by gastric gavage. The animals were observed 1 and 4 hours after dosing and thereafter daily for 14 days for mortality and clinical signs. Body weights were recorded on days 0, 7 and 14 of the study. Macroscopic examination of main organs was performed at autopsy. No histological examinations were performed.

Results

There were no mortalities and body weight gain was considered normal for the age and strain of rat. No abnormalities were noted at autopsy of animals. The LD₅₀ of the test substance administered to rats by the oral route was greater than 5000 mg/kg bw.

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 rat, Crl: CD (SD) BR
Group size	:	10 males + 10 females
Test substance	:	IMEXINE FW suspended in 0.5% aqueous methylcellulose
Batch no	:	op T 22
Purity	:	99.7 %
Dose	:	0, 50, 200 and 800mg/kg bw/day (5ml/kg)
Exposure period	:	13 weeks (7 days per week)
GLP	:	in compliance

Dose levels were determined following a preliminary 4 week oral study. Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 50, 200 and 800 mg/kg bw/day, 7 days a week for 13 weeks. The dosing solutions were analysed during weeks 1, 4, 8 and 12 for concentration and at the beginning of treatment for stability and verification of homogeneity. During the study, the animals were observed daily for clinical signs and mortality, and weekly for body weight and food consumption. Daily water consumption was recorded in control and high dose males during weeks 9 and 10. During week 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 period 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 females given 800 mg/kg/day were found dead during the treatment period, on day 22 and 63. Prior clinical signs were consistent with other animals of the same dose group. The cause of death could not be established from the macroscopic and microscopic findings, but it was considered that the mortalities were treatment-related.

Hypersalivation was observed in all males and 8/10 females at 800mg/kg/day. Soft faeces were noted from week 9 and persisted until termination of the study in both sexes and at all dose levels. A dose dependent discoloration of the urine was seen from week 1 of treatment,

accompanied by yellow coloration of the tail in animals given 50 and 200 mg/kg bw/day and of the extremities in high dose animals. Other minor clinical signs were reported in low incidence in all groups throughout the study and were not considered to be attributable to treatment.

Bodyweight of all dose groups were reduced in a dose related fashion (-7%, -9% and -17% in males; -8%, -12% and -22% in females, at 50, 200 and 800 mg/kg bw/day, respectively. The terminal bodyweight was significantly reduced for both males and females at 800mg/kg bw/day (approximately 90% of control), but did not differ significantly from control for the lower dose groups. Food consumption was increased in both males (from week 3) and females (from week 6) at 800mg/kg bw/day and was similar to controls for the other test groups. A statistically significant increase in water consumption was recorded in the high dose male group during weeks 9 and 10.

No abnormalities were reported from the ophthalmology examination in week 13. Higher prothrombin time and activated partial prothrombin time were noted in males given 800mg/kgbw/day. The changes were mainly attributable to 2/10 rats with high values and hence this finding was considered to be of minor toxicological significance. Eosinophil levels appeared to show a dose-related increase in both sexes, which was significantly different from control at 800 mg/kg bw/day. Other slight differences were not dose-related and individual values were within or close to the normal range, and therefore, not considered to be treatment-related.

Creatinine concentration showed a dose-related decrease in males, which was significant at 200 and 800 mg/kg bw/day. Glucose content appeared to show a dose-related decrease, in both sexes, but was only significant in the high dose male group. These changes were attributable to a minority of animals and were considered of minor toxicological importance. Other small changes were not dose-related and within or close to the normal range. No changes were noted for the urinalysis; determination of pH and density at high dose levels was not possible due to the discoloration of the urine.

There were statistically significant increases in absolute (110% in males and 118% in females) and relative liver weights (up to 127% in males and 138% in females) at 800 mg/kg bw/day. The relative liver weight was also significantly increased in females at 200 mg/kg bw/day (115%).

Kidney weights were significantly increased in both sexes at 200 (absolute 109% to 112%; relative 116% to 119%) and 800 mg/kg bw/day (absolute 114% to 116%; relative 133%).

Adrenal weights were also increased in high dose groups of both sexes (117% to 126%).

Statistically significant lower absolute thymus weight was noted in females at 200mg/kg bw/day (78%) and in males (70%) and females (79%) at 800mg/kg bw/day.

At autopsy, only treatment-related findings related to the staining properties of the hair dye.

Other observations were common for the strain and age of rat and of comparable incidence in all dose groups. Centrilobular hepatocyte hypertrophy was observed in high dose animals of both sexes (6/10 males and 8/8 females). A dose-related increase in the incidence and severity of accumulation of "acidophilic globules" in the cortical tubular epithelium was noted in male rats, with the incidence greater than control at 200 and 800 mg/kg bw/day. This finding was considered to be treatment related, but of minor toxicological consequence, because the phenomenon occurs with a number of compounds and is specific to the male rat.

The NOAEL was 50 mg/kg bw/day.

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	:	Journal Officiel de la République Française, 21/02/82
Species/strain	:	New Zealand white rabbits
Group size	:	3 males
Test substance	:	IMEXINE FW
Batch no	:	op.6
Purity	:	/
Dose	:	0.5g
GLP	:	in compliance

The substance (0.5 g moistened with water) was applied to 6.25cm² areas of intact and scarified skin of 3 male rabbits. Occlusive patches were applied and left in place for a 24-hour period. Remaining test substance was removed by swabbing with cotton wool swabs soaked in water and diethyl ether. The skin was examined for erythema, eschar formation and oedema at 1, 24 and 72 hours after removal of the patches. An index of Cutaneous Primary Irritation was calculated from the mean scores at the sites and at each time point according to Draize.

Results

Slight erythema and staining of the skin were reported in all animals at 24 hours, but not at 48 hours. There was no oedema at either observation time. The primary irritation index was reported to be 0.5 out of a maximum possible score of 8. The test substance was considered to be non-irritant.

Ref. : 3

2.4.2. Irritation (mucous membranes)

Guideline	:	Journal Officiel de la République Française, 24/10/84
Species/strain	:	New Zealand rabbits
Group size	:	3 males
Test substance	:	IMEXINE FW
Batch no	:	op 6
Purity	:	/
Dose	:	0.1ml (0.057g)
GLP	:	in compliance

0.1 ml (0.057g) of the neat substance was applied once to the right eye of each animal without rinsing. The left eye served as control. Ocular reactions were recorded at 1hour and 1, 2, 3, 4 and 7days after instillation. The mean ocular irritation index was calculated according to Draize.

Results

Instillation of IMEXINE FW affected the cornea and iris of one rabbit, and the conjunctivae of all three rabbits. All reactions had resolved by day 3. The mean ocular irritation index was calculated to be 7 out of a maximum of 110. According to the defined criteria the substance was classified as slightly irritant when applied neat to the rabbit eye.

Ref. : 2

2.5. Sensitisation

Magnusson and Kligman study

Guideline	:	OECD 406 (1981)
Species/strain	:	Dunkin-Hartley guinea pigs
Group size	:	20 test + 10 control, females
Test substance	:	IMEXINE FW in arachis oil
Batch no	:	op.6
Purity	:	/
Concentration	:	intradermal induction : 0.1ml 50% Freund's complete adjuvant (FCA) 0.1ml 5% test substance 0.1ml 5% test substance/50 %FCA topical induction : 0.5ml 75% of test substance challenge : 0.2ml 75% test substance for 24 hours, occluded
GLP	:	in compliance

A preliminary intradermal study indicated that the test substance could be used at a concentration of 5% without provoking an irritant response.

Induction commenced with three pairs of intradermal injections of FCA, test substance (5%) and a mixture of the two. Six days later 0.5 ml of 10% sodium lauryl sulphate was applied to the injection site to induce a local irritation and the following day, the induction process was completed with a single topical application of 0.5 ml of the test substance (75%) under occlusive patch to the shoulder region for 48 hours. An interval of two weeks was allowed after induction and then the animals were challenged by a single topical application of the test substance (75%) under occlusive patch on the right flank for 24 hours. Appropriate controls were treated with vehicle at all stages and the test substance-induced animals received vehicle alone on the left flank.

The skin was examined 24 hours after administration of the intradermal injection and again after removal of the topical patches for signs of irritation. The skin was examined 24 and 48 hours after removal of the challenge patches.

Results

One control animal died during the test period, possibly due to pneumonia. After induction, the yellow staining caused by the test material made the evaluation of erythema difficult in all animals one hour after removal of the patch and in 19/20 animals at the 24 hour observation period. The yellow coloured staining caused by the test material was again noted but was reported not to have precluded evaluation of erythema. A positive skin reaction was noted in 1/20 of the test animals. The study authors concluded that the test substance was a weak sensitiser to guinea pig skin.

Ref. : 4

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague Dawley CrL CD (SD) BR
Group size	:	25 females (mated)
Test substance	:	IMEXINE FW suspended in 0.5% aqueous methylcellulose
Batch no	:	Pil.1 CBX
Purity	:	98.3 %
Dose levels	:	0, 20, 200 and 2000 mg/kg bw/day
Treatment period	:	Days 6-15 of pregnancy, inclusive
GLP	:	in compliance

Groups of 25 female rats were dosed with the test substance by gavage on days 6 to 15 after mating. The control group received the vehicle alone.

The dams were observed daily for clinical signs and mortality, and for food consumption and body weight on days 0, 6, 9, 12, 15 and 20. The dams 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 end point).

Results

No deaths or abortions occurred at any dose level. No clinical signs were observed in the low dose group. Dark yellow discoloration of the urine was noted at 200 and 2000 mg/kg bw/day (from day 4 and day 2, respectively). Food consumption and body weight gain were reduced in the group treated with 2000mg/kg bw/day, particularly during the first 3 days of treatment. The mean bodyweight of the high dose group was significantly lower than control on day 15 (95% of control), but comparable by day 20. At autopsy, no abnormalities were observed in the control and low dose group animals. One animal treated at 200 mg/kg bw/day exhibited an adherent and hypertrophic spleen, and one at 2000 mg/kg bw/day exhibited dilated kidneys. These effects were not considered to be treatment related because of the single incidence. No other macroscopic findings were reported.

No dead foetuses were reported for any groups. The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses and foetal body weights were similar for control and treated groups. There was a very low incidence of malformations and abnormalities which were not dose related were not considered to be treatment-related.

The test substance elicited maternal toxicity at the highest dose level tested but was not embryo-toxic or teratogenic. The NOAEL for maternal toxicity is considered to be 200 mg/kg bw/day.

Ref. : 11

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.1 Percutaneous Absorption *in vitro*

Guideline	:	/
Tissue	:	Human dermatomed skin
Method	:	Flow through diffusion cell, thermostated at 32°C
Test substance	:	IMEXINE FW, 0.954% in formulation
Batch no	:	0500776
Purity	:	99.2 % (potentiometric)

Evaluation and opinion on : HC Yellow n° 11

Dose levels	:	circa 20 mg formulation/cm ²
Replicate cells	:	8
GLP	:	in compliance

The study was performed according to COLIPA guidelines on percutaneous absorption. The skin penetration of HC Yellow n° 11 was evaluated on human dermatomed skin samples mounted on flow through diffusion cells. The cell was maintained at 32°C and the receptor fluid was constantly stirred. A hair dye formulation containing 0.954% HC Yellow n° 11 was applied at a dose of approximately 20 mg/cm² (hair dye content 184.57±4.83 µg/cm²) for 30 minutes and then excess washed off with water and 2% sodium lauryl sulphate solution; and dried. Twenty four hours later the levels of the substance were measured in the receptor fluid (physiological saline containing 0.25% Tween 20), epidermis and dermis using HPLC-UV. The HPLC method for the analysis was validated and the required solubility of B63 in the receptor fluid has been documented.

Results

The total recovery of the applied dose was 99.93%. The distribution of the applied dye was:

Removed excess	:	184.1±5.07 µg/cm ²
Stratum corneum	:	0.213±0.182 µg/cm ²
Epidermis + dermis	:	0.091±0.049 µg/cm ²
Receptor fluid	:	0.037±0.018 µg/cm ²

The percutaneously absorbed (PA) IMEXINE FW, which is the amount of the dye found in epidermis, dermis and receptor fluid = 0.13±0.07 µg/cm²

Ref. : 12

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline	:	OECD 471 (1983)
Species/strain	:	<i>S. typhimurium</i> , TA98, TA100, TA1535, TA1537; <i>E. coli</i> , WP2uvrA
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	IMEXINE FW in DMSO solution
Batch no	:	op 16
Purity	:	99.4 %
Concentrations	:	156.25-5000 µg/plate with and without metabolic activation
GLP	:	in compliance

HC Yellow n° 11 has been investigated for gene mutation in *S. typhimurium* and *E. coli*, using the direct plate incorporation method and the preincubation method.

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. The concentration range 156 - 5000 µg/plate was selected on the basis of a preliminary toxicity indicating moderate cytotoxicity at 2500 - 5000 µg/plate.

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*).
- with S9 mix : a significant and reproducible increase in revertant numbers was observed only in TA98 tester strain. However, this increase exceeded 2 fold in test # 1 (2.2 – 3.5), and in test # 3 (1.9 – 3.0) but not in the preincubation test # 2 (1.2 – 1.6) or in the direct incorporation test # 4 (0.7 – 1.5).

Conclusions

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 substance is positive in the *Salmonella typhimurium* TA 98 frameshift tester strain 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 FW in DMSO solution
Batch no	:	op 16
Purity	:	99.4 %
Concentrations	:	125 to 1000 µg/ml with and without metabolic activation
GLP	:	in compliance

IMEXINE FW has been investigated for gene mutation at the TK locus in L5178Y (TK^{+/-}) mouse lymphoma cells. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

Exponentially growing suspension cultures of L5178Y were treated with the test agent for 2 hours in the culture medium containing horse serum in the presence and absence of S9 mix.

The concentration range 125-1000µg/ml was selected on the basis of a preliminary toxicity study indicating that 1000 µg/ml was the lowest precipitating dose. Negative and positive controls were in accordance with the OECD guideline.

Results

At the top dose of 1000 µg/ml, a strong precipitate occurred. pH measurement of post-treatment medium was between 8 - 8.5.

Cytotoxicity

In the range finding experiment

- without S9 mix : no toxicity as evidenced by a reduction in the cloning efficiency (CE) was noted.
- with S9 mix : the toxicity as evidenced by the CE was of 50 % of control at the top precipitating dose of 1000 µg/ml.

At lower non precipitating doses, no CE reduction was noted.

In the mutagenicity tests

- without S9 mix : no appreciable toxicity as evidenced by a reduction in the CE was noted. The top dose tested yielded 64 % relative survival in one test
- with S9 mix : the toxicity as evidenced by the CE was of 58 % of controls at the top precipitating dose of 1000 µg/ml.

At lower non precipitating doses, no CE reduction was noted.

Viability at day 2 was 68 % and 82 % to that of the controls.

Mutant frequency

- without S9 mix : no statistical or biological significant increase in mutant frequency was observed over the concurrent solvent controls in test #1. In test # 2, mutant frequency was increased by a factor of 3.5.
- with S9 mix : a statistical and reproducible significant increase in mutant frequency was observed over the concurrent solvent controls in the 2 assays (1.7 x ; 2.7 x).

Conclusions

From the results generated in 2 experiments, it is concluded that HC YELLOW n° 11 shows reproducible positive results in this test. However, it should be noted that the increased mutant frequencies were observed at precipitating concentrations, under basic pH conditions but at acceptable levels of toxicity. Therefore, HC YELLOW n° 11 is considered mutagenic in this test.

Ref. : 7

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

Guideline	:	OECD 473 (1983)
Species/strain	:	Human peripheral blood lymphocytes from 2 donors (exp #1) + 2 donors (Exp #2)
Replicates	:	2 independent tests
Test substance	:	IMEXINE FW
Batch no	:	op T 22
Purity	:	100 %
Concentr. scored	:	250 - 800 µg/ml with and without metabolic activation
GLP	:	in compliance

Doses selected. Samples were cultured during 48 h and then exposed as follows :

Test # 1

Fixation time	Exposure period	Concentrations in µg/ml with and without S9 mix
20 hrs	3 hrs	125 250 500 750 1000

Test # 2

Fixation time	Exposure period	Concentrations in µg/ml without S9 mix
20 hrs	20 hrs	12.5 25 50 100 200
44 hrs	44 hrs	12.5 25 50 100 200
with S9 mix		
20 hrs	3 hrs	400 600 800

44 hrs 3 hrs 400 600 800

IMEXINE FW has been investigated for induction of chromosomal aberrations in human peripheral blood lymphocytes - of 2 different donors. - with exposure times of 3 hours with S9 and 20 hours without S9, and harvest times of 20 and 44 hours. Liver S9 fraction from Aroclor1254-induced S9 was used as the exogenous metabolic activation system. Test concentrations were based on the maximum concentration associated with a reduction in mitotic index of greater than 50%. Negative and positive controls were in accordance with the OECD guideline.

Results

Structural chromosome aberrations

Test # 1

- without S9 mix : a statistically and biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control for the top dose of 750 µg/ml. (0.0 % - 9.5 % aberrant cells gaps excluded).
- with S9 mix : a statistically and biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control for the top dose of 750 µg/ml. (0.0 % - 4.5 % aberrant cells gaps excluded).

Test # 2 (the donors are not identical as in test # 1)

- without S9 mix : 20 h continuous exposure. No statistically or biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control for any dose (0.0 % , 0.5 % , 1 % , 1 % aberrant cells gaps excluded).
- without S9 mix : 44 h continuous exposure. No statistically or biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control for any dose (0.0 % , 0.5 % , 0.0 % , 0.5 % aberrant cells gaps excluded).
- with S9 mix : 20 h continuous exposure. A statistically and biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control for the top dose of 800 µg/ml. (0.0 % - 3.0 % aberrant cells gaps excluded).
- with S9 mix : 44 h continuous exposure. No statistically or biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control for any dose (0.0 % , 0.0 % , 0.0 % , 0.0 % aberrant cells gaps excluded).

Conclusions

IMEXINE FW is considered positive for clastogenic activity in non-pooled human lymphocytes after a short exposure time in the presence or in the absence of activation under the conditions of the test.

Ref. : 8

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline : OECD 474 (1983)

Evaluation and opinion on : HC Yellow n° 11

Species/strain	:	Mouse, OF1/ICO:OF1 (IOPS Caw)
Group size	:	5 males + 5 females
Test substance	:	IMEXINE FW suspended in 0.5% aqueous carboxymethylcellulose
Batch no	:	op T 22
Purity	:	100 %
Dose levels	:	0, 500, 1000 and 2000 mg/kg bw/day, on two days.
Sacrifice times	:	24 hours after last dosing
GLP	:	in compliance

HC Yellow n° 11 has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined by a preliminary range finding study in which no toxic effects were seen at 2000 mg/kg bw/day in either sex. The test doses were administered by gavage twice at a 24 hour interval and groups of animals sacrificed 24 hours after the last administration for harvest of bone marrow cells. Negative and positive controls were in accordance with the OECD guideline.

Results

Maximum Tolerated Dose (MTD)

The top dose of IMEXINE FW was chosen on the basis of the lack of clinical signs.
2 x 2000 mg/kg

Test doses

IMEXINE FW was administered by 1 single oral dose daily during 2 consecutive days. Dose: 2000, 1000 and 500 mg/kg.

1 sacrifice times was chosen : 24 h after the last dosing. Bone marrow smears were obtained from the positive control group 24 hours after dosing only.

Number of cells scored:

A total of at least 2000 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:

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 (control PCE 1.8 per 1000 / 1.05 per 1000 / 1.3 per 1000) (positive control :34.7)

PCE/NCE ratio:

A highly statistically significant reduction in the PCE/NCE ratio ($p < 0.001$) was observed in the top dosage group of mice treated with IMEXINE FW. Slightly statistically significant reduction ratio was also observed in the 2 other dosage groups ($p < 0.05$)

Under the conditions of the test it can be concluded that with IMEXINE FW at doses at which no signs of clinical toxicity were recorded, but with significantly changed PCE/NCE ratio were observed, does not induce statistically significant increase in the frequency of PCE. The negative and positive controls gave the expected results. Therefore, IMEXINE FW is not clastogenic and/or aneugenic in this mouse bone marrow micronucleus test.

Ref. : 9

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

Guideline	:	Draft OECD guideline of 1991
Species/strain	:	Wistar rat, HanIbm:WIST
Group size	:	4 males
Test substance	:	IMEXINE FW suspended in 0.5% aqueous carboxymethylcellulose
Batch no	:	op T 22
Purity	:	> 98 %
Dose levels	:	0, 200 and 2000 mg/kg bw, by gavage
Sacrifice times	:	16 hours: all dose groups; 2 hours: high dose group
GLP	:	in compliance

IMEXINE FW 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 2000mg/kg bw and therefore this was used as the highest dose, in accordance with the OECD draft guideline. Negative and positive controls were in accordance with the OECD guideline. Animals were sacrificed after 16 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 ³H-thymidine *in vitro*. Incorporation of radiolabel was assessed using autoradiography.

Results

Clinical Reactions:

No adverse reactions to treatment was observed

UDS assay:

Negative control animals gave a group mean NNG value of less than zero. Positive control animals gave a group mean positive NNG value (35.02)

Treatment with IMEXINE FW at doses of 200 & 2000 mg/kg yielded group mean NNG values less than and caused no significant increases, as compared to control, in the mean nuclear grain counts.

Conclusions

The study is adequate. Data indicate that single oral gavage treatment of male rats dosed once with 1000 & 2000 mg/kg of IMEXINE FW did not induced increased unscheduled DNA synthesis in hepatocytes isolated approximately 2 or 16 hours after dosing. It is concluded that IMEXINE FW did not display DNA repair activities detectable by this assay under the experimental conditions

Ref. : 10

2.9. Carcinogenicity

No data

2.10 Special investigations

No data

2.11. Safety evaluation**NOT APPLICABLE****2.12. Conclusions**

HC Yellow n° 11 is a secondary alkanolamine, and thus, it is prone to nitrosation. No information on the nitrosamine content of the dye and the dye formulation has been provided.

The purity of HC Yellow n° 11 has been determined by potentiometric titration, its chromatographic purity has not been reported. Chromatographic impurity in only one batch of HC Yellow n° 11 (op.2X) has been reported. This batch is other than the batch used for the description of purity and physico-chemical properties. The batch op.2X has also not been used for any of the other studies reported. A 5% loss of HC Yellow n° 11 in a hair dye formulation was noted over a period of 1 months. The degradation products of the dye in the formulation should be characterised and quantified.

HC Yellow n° 11 was not toxic in an acute rat oral toxicity test with dosing up to 5000 mg/kg bw. It was slightly irritating when applied neat to the rabbit eye but not to rabbit skin. It did not show sensitising potential in a Magnusson and Kligman study.

When administered during organogenesis, the substance affected maternal food consumption and bodyweight gain at a dose of 2000mg/kg bw/day; there was no evidence of foetotoxicity or teratogenicity. In a 13 week oral rat study, there was evidence of effects on the liver, kidney and thymus and the NOAEL was 50mg/kg bw/day.

Percutaneous penetration has been investigated using human dermatomed skin *in vitro*. The study revealed very little absorption of HC Yellow n° 11 through the skin, $0.13 \pm 0.07 \mu\text{g}/\text{cm}^2$.

HC Yellow n° 11 was tested in procaryotic cells for gene mutation in several tester strains of *S. typhimurium* and *E. coli* WP2 uvrA. It is positive in the *S. typhimurium* TA 98 frame shift tester strain in the presence of S9 mix and positive in the *in vitro* Mammalian Cell Gene Mutation Test. The substance is considered clastogenic in the *in vitro* mammalian chromosomal aberration test. Two *in vivo* genotoxicity studies using complementary species and endpoints indicated that the *in vitro* mutagenic properties are not expressed *in vivo*. Therefore, the substance can be considered non-mutagenic *in vivo*.

2.13. References

1. Safepharm Laboratories Ltd, UK. Study No 109/282 (June 1989)
2. Safepharm Laboratories Ltd, UK, Study No 109/276 (June 1989)
3. Safepharm Laboratories Ltd, UK. Study No. 109/279 (June 1989)
4. Safepharm Laboratories Ltd, UK, Study No 109/276 (Oct 1989)
5. C.I.T., France. Report 11395 TCR (May 1995)
6. C.I.T., France Report 11281 MMJ (Sept 1994)
7. C.I.T., France Report 11822 MLY. (Jan 1995)
8. C.I.T., France Report 13347 MLH. (Nov 1995)
9. C.I.T., France. Report 12605 MAS. (Oct 1995)

10. Cytotest Cell Research GmbH, Germany, Report No. 499200 (May 1995)
11. C.I.T., France. Study No. 7203 RSR (July 1992)
12. L'Oreal, France. Study No. 16084; 14/02/01

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 :

- * chromatographic purity of HC Yellow No. 11;
- * nitrosamine content in various batches of HC Yellow n° 11 and in the hair dye formulations containing this chemical;
- * characterisation and quantification of degradation products of HC Yellow n° 11 in hair dye formulations,
- * 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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