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

CONCERNING

6-HYDROXYINDOLE

COLIPA n° A128

adopted by the SCCNFP during the 26th plenary meeting
of 9 December 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 6-Hydroxyindole 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

6-Hydroxyindole (INCI name)

2.1.2. Chemical names

Chemical name : 6-Hydroxyindole
CAS name : 1H-Indol-6-ol
Synonyms : Indol-6-ol

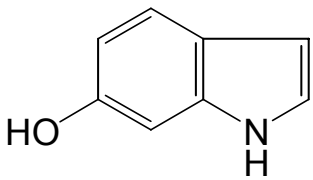
2.1.3. Trade names and abbreviations

Trade name : IMEXINE® OBA
COLIPA n° : A128

2.1.4. CAS n° / EINECS n°

CAS n° : 2380-86-1
EINECS n° : /

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : C_8H_7NO
Mol weight : 133.15

2.1.7. Purity, composition and substance codes

All analytical data relate to batch pil.10.

Purity

Titre as determined by HPLC	:	97.4 %
Water content	:	0.05% (w/w)
Ash content	:	<0.1%
Heavy metals	:	< 10 ppm

Potential impurities
Reagents and intermediate reaction products

4-methyl-3-nitro-phenylamine	:	< 500 ppm
4-methyl-3-nitro-phenol	:	< 500 ppm
4-benzyloxy-1-methyl-2-nitro-benzene	:	< 500 ppm
1-[2-(4-benzyloxy-2-nitro-phenyl)-vinyl]-pyrrolidine	:	< 500 ppm
6-benzyloxy-1H-indole	:	< 500 ppm

Unidentified impurities observed by HPLC,
Condensation of 2-amino-4-hydroxytolyl moiety with 6-hydroxyindole (not quantifiable)
Trace impurity, of insufficient quantity to obtain further information
Dimeric form of 6-hydroxyindole (not quantifiable)

Solvent residues

ethyl acetate	:	200 ppm
ethanol	:	90 ppm
cyclohexane	:	250 ppm
toluene	:	50 ppm
isopropanol	:	< 50 ppm
Chloride ions	:	<0.05% (w/w)

2.1.8. Physical properties

Appearance	:	Beige crystalline powder
Melting point	:	126 °C
Boiling point	:	/
Density	:	/
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	1.47 (calculated)

2.1.9. Solubility

0.1% soluble in water, 10% soluble in 95% ethanol (with stirring for 10 min.), 1% insoluble in chloroform, 10% soluble in DMSO

General comments on analytical and physico-chemical characterisation

* Potential impurities have been identified at the detection limit of 500ppm.
* Degradation of the active hair dye is 2% in the formulation over 1.5 months.
* Purity of all tested batches is not given.
* Total of identified impurities mounts to max. 0.5%. This implies other unknown impurities.

2.2. Function and uses

6-Hydroxyindole is used as a hair colorant requiring the presence of hydrogen peroxide as an oxidant. It will be incorporated in hair dye formulations at a maximum concentration of 1%, for use in a 1:1 mixture with hydrogen peroxide preparation. The concentration on application is therefore 0.5%. It is intended for once monthly use with typical application of 100ml.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	OECD 401 (1987)
Species/strain	:	Rat, Crl:CD (SD)BR strain (VAF plus)
Group size	:	5 males + 5 females
Test substance	:	Imexine OBA in 30% aqueous solution of PEG
Batch no	:	pil.10 (purity not stated)
Dose	:	600 mg/kg bw in a volume of 10 ml/kg
Observ. Period	:	14 days
GLP	:	in compliance

A range finding study resulted in 4/4 deaths at 1200 mg/kg bw, 1/4 deaths at 800 mg/kg bw and clinical signs of toxicity but no mortalities at 600 mg/kg bw. The dose level of 600 mg/kg bw was therefore used in the main study.
Groups of 5 male and 5 female rats received a single dose of test substance by gastric gavage. The animals were observed twice a day for mortalities and daily for clinical signs for 14 days. Bodyweights were recorded weekly and macroscopic abnormalities were recorded at autopsy. No histological examinations were performed.

Results
There were two mortalities: one male rat died immediately after dosing and a second male on the day after dosing. Clinical signs were hunched posture, hypoactivity and prostration on the day of dosing. Surviving animals appeared normal thereafter. Weight gain was normal for the strain used. Autopsy of the animals surviving to day 14 revealed abnormalities in the spleen of one male and in the uterus of one female. All other animals appeared normal.
The maximum tolerated dose was less than 600 mg/kg bw and the oral LD50 was higher than 600 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	:	Crl: CD (SD) BR strain rat, (VAF plus)
Group size	:	10 males + 10 females
Test substance	:	Imexine OBA in 30% aqueous PEG 300
Batch no	:	Pil.10 (purity not stated in study report)
Dose levels	:	0, 30, 100 and 300 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, 30, 100 and 300 mg/kg bw/day, 7 days/week for 90 days. The dosing solutions were analysed at 4-weekly intervals 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 week 13, blood was sampled from the lateral tail vein for haematology and blood biochemistry and urine was collected overnight for urinalysis. 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 in both the control and high dose group animals.

Results
No mortalities or treatment related clinical signs of toxicity were reported. Hair loss and scabbing were noted in some animals of all dose groups. Bodyweight gain was similar for all dose groups. The food consumption was consistently higher in the female rats treated at 300 mg/kg bw/day from week one throughout the study (111% of control overall), but this was not considered to be an adverse effect. Food consumption of other dose groups was comparable to controls. Ophthalmological examinations revealed no differences between control and treated animals. The female rats treated with 300 mg/kg bw/day had minor haematological changes compared to concurrent controls which were within the range of historical control data. Of these, the increase in mean cell haemoglobin was dose-related and statistically higher than control at all dose levels. The maximum increase (high dose) was 7% above control and the change was not considered to be of toxicological significance. Other changes were not considered to be treatment-related.

There was an approximately two-fold increase in both alanine aminotransferase and aspartate aminotransferase levels in plasma of female rats receiving 300 mg/kg bw/day, with no indication of any effect at 100 mg/kg bw/day. The female rats showed dose-related elevation of blood cholesterol and inorganic phosphate levels, which were significantly different from control in the 100 mg/kg bw/day (124% and 126% of control, respectively) and 300 mg/kg bw/day (137% and 130% of control). The authors considered that the relevance of these findings was unclear. The values were within the range of historical control data. Other minor changes were not dose related and also within the historical control range. Urinalysis revealed no treatment-related changes.

Absolute and relative liver weights were increased in both sexes at 300 mg/kg bw/day to 15-18% above controls. Increases in absolute and relative weights of spleen (13-24% above controls) and kidney (11-15% above controls) in both sexes at 300 mg/kg bw/day. The changes in spleen and kidney were within the range of historical controls and in the absence of histopathological changes were not considered to be treatment-related. No treatment-related macroscopic changes were noted at autopsy.

Hepatocyte hypertrophy was reported in 4 of 10 females dosed at 300 mg/kg bw/day. Other occasional minor abnormalities observed in the histopathological examination were within the normal range of background alterations and were not treatment-related.
The authors concluded that the effects seen in liver, and associated elevation of transaminases, in female rats treated at 300 mg/kg bw/day were treatment-related and that the NOAEL was 100 mg/kg bw/day. The SCCNFP concluded that the NOEL was 30 mg/kg bw/day, based upon the observation of dose-related elevation in blood cholesterol and inorganic phosphate.

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	:	OECD 404 (1981)
Species/strain	:	New Zealand albino rabbit
Group size	:	3 males
Test substance	:	Imexine OBA, moistened with water
Batch no	:	pil.10 (purity not stated)
Dose	:	0.5 g
GLP	:	in compliance

0.5 g of moistened neat test substance was applied to 6.25 cm^2 of intact skin of 3 male rabbits. Semi-occlusive patches were applied and left in place for 4 hours. Remaining test substance was rinsed off. The skin was examined for erythema, eschar formation and oedema at 1, 24, 48 and 72 hours after removal of the patches.

Results
No skin reactions were observed in any of the animals. Slight yellow staining was noted in 2 of the 3 rabbits at all observation times. The substance was non-irritating to rabbit skin.

Ref. : 3

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405 (1987)
Species/strain	:	New Zealand albino rabbit
Group size	:	3 females
Test substance	:	Imexine OBA, 5% in 30% aqueous PEG solution
Batch no	:	pil.10 (purity not stated)
Dose	:	0.1 ml
GLP	:	in compliance

0.1 ml of the test substance was applied once to the right eye of 3 female rabbits, without rinsing. The left eye served as control and was untreated. A preliminary study established that the vehicle was not irritating to the rabbit eye. Ocular reactions were recorded at 1, 24, 48 and 72 hours after instillation.

Results
No reactions were reported in the eyes of any of the test animals. The test substance was reported to be non-irritant to the rabbit eye at a concentration of 5% in 30% PEG.

Ref. : 2

2.5. Sensitisation

Magnusson and Kligman study

Guideline	:	OECD 406 (1981)	
Species/strain	:	Dunkin Hartley guinea pig	
Group size	:	10 male + 10 female in test group, 5 male + 5 female in control group	
Test substance	:	30201 C, in PEG(6OE)/water (85:10)	
Batch no	:	no information	
Concentrations	:	intradermal induction :	0.1 ml Freund's complete adjuvant (FCA)
			0.1 ml 0.5% test substance
			0.1 ml 0.5% test substance/FCA (1:1)
		induction of irritation :	0.5 ml of 10% sodium lauryl sulphate in vaseline
		topical induction :	0.5 ml 5% test substance for 48 hours, occluded
		challenge :	0.5 ml 5% test substance for 24 hours, occluded
GLP	:	in compliance	

Induction commenced with three intradermal injections, of Freund's Complete Adjuvant, test substance (0.5%), and a mixture of these two. Six days later 0.5 ml of 10% lauryl sulphate was applied to the injection site to induce a local irritation, and the next day the induction process

was completed with a single topical application of 0.5ml of the test substance (5%) under occlusive patch for 48 hours. An interval of 2 weeks was allowed after induction and then the animals were challenged by a single 0.5 ml topical application of the test substance (5%) under occlusive patch on the flank for 24 hours. Appropriate controls were treated with vehicle. The skin was examined 24 and 48 hours after removal of the challenge patches.

Results
Twenty four hours after the challenge, 2 male and 2 female test animals showed slight erythema, and 3 females showed well-defined erythema. Slight erythema remained in the same 5 females at 48 hours, whereas the reaction had resolved at that time in the males. No reactions were observed in control animals. The author concluded that the response could be attributed to individual irritation reactions and that the substance was not sensitising.

The author's conclusions are not justifiable. Topical induction and challenge were conducted with the same concentration of test substance. Irritation was not reported after the topical induction. Therefore it should be concluded that the substance caused sensitisation in 40% of the test animals.

Ref. : 4.1

Buehler study

Guideline	:	OECD 406 (1992)	
Species/strain	:	Dunkin-Hartley albino guinea pig	
Group size	:	10 male + 10 female in test group, 5 male + 5 female in control group	
Test substance	:	Imexine OBA in paraffin oil	
Batch no	:	Pil.10 (97.4%)	
Concentrations	:	topical induction :	3 x 0.5 ml 20% test substance for 6 hours
		challenge :	0.5 ml 20% test substance for 6 hours
GLP	:	in compliance	

Topical induction was by three 0.5 ml applications of test substance (20% in paraffin oil), for 6 hours under occluded patch to the left flank on three occasions (days 1, 8 and 15). An interval of 2 weeks was allowed after induction and then the animals were challenged by a single 0.5 ml topical application of the test substance (20% in paraffin oil) under occlusive patch on the right flank for 6 hours. Appropriate controls were treated with vehicle at all stages. The skin was examined 24, 48 and 72 hours after removal of the challenge patches.

Results
During the induction period, very slight erythema was observed in 14/20 animals on day 9 and in 10/20 animals on day 16. Well-defined erythema was observed in 2/20 animals on day 9 and in 3/20 animals on day 16. After the challenge application, very slight erythema was present in 7/20 animals at 24 hours, 2/20 animals at 48 hours and 3/20 animals at 72 hours. Well-defined erythema was observed in 3/20 animals at 24 hours, 5/20 animals at 48 hours and 3/20 animals at 72 hours. Dryness was also reported in one animal at 48 hours and 4 animals at 72 hours. No reactions were reported in control animals.
According to the criteria defined in the report, the observations of very slight erythema do not contribute to quantification of the allergenicity and therefore it was concluded that the substance induced possible sensitisation reactions in 25% of the animals.

Ref. : 4.2

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague-Dawley rat, Crl: CD (SD) BR (VAF plus) strain
Group size	:	24 females (mated)
Test substance	:	Imexine OBA in 30% aqueous PEG300
Batch no	:	Pil.10 (purity 97.4%)
Dose levels	:	0, 50, 150 and 300 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 on days 6 to 15 after mating. Dose levels were initially set at 0, 50, 150 and 450 mg/kg bw/day, but the high dose resulted in an immediate severe reaction in the first 14 dams dosed. They were prematurely sacrificed, and the remaining 10 animals supplemented with spares, which were subsequently dosed with the alternative high dose of 300 mg/kg bw/day. The dams were observed daily for clinical signs and mortality, bodyweight was recorded on days 0, 6-9, 12, 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 (two thirds) and visceral (one third) abnormalities. The concentrations, homogeneity and stability of the dosing formulations were verified analytically.

Results

Other than the mortalities occurring at 450 mg/kg bw, there were no treatment-related mortalities. There were no clinical signs of reaction to the test substance except for greenish discoloration of the urine at 150 and 300 mg/kg bw/day and salivation after dosing at 300 mg/kg bw/day. The high dose group animals exhibited reduced weight gain compared to controls, which was attributed to the higher initial weight resulting from the late entry of 14 of the animals into the study. It was not considered to be treatment-related. Body weight gain at 50 and 150 mg/kg bw/day was not statistically different to control. Food consumption did not differ significantly for any of the dose groups, although the high dose group consumption appeared slightly lower than for controls at the onset of dosing.

No macroscopic abnormalities were observed in any of the dams at autopsy. The mean numbers of corpora lutea, implantations and live foetuses in the dams treated at 300 mg/kg bw/day was higher than for concurrent or historical controls. This observation was attributed to coincidence, since it could not result from an adverse response to the test substance. Results at 150 and 50 mg/kg bw/day were comparable to control. The mean foetal bodyweights were statistically lower in high dose animals than in controls (to 91% of control), and with a small, non-significant difference at 150 mg/kg bw/day and no effect at 50 mg/kg bw/day. There was a low incidence of macroscopic anomalies, consistent with the normal range for the strain, and the distribution between groups (0, 1, 4, and 1 at 0, 50, 150 and 300 mg/kg bw/day, respectively) indicated that it was not treatment-related. The incidences of minor external and visceral abnormalities were also in the normal range. Incomplete ossification was seen in foetuses from the 150 mg/kg bw/day dose group, and to a lesser extent at 300 mg/kg bw/day. This particularly related to the skull, sternum, metacarpals and metatarsals. Some slight changes were also seen at the low dose, but these did not follow a pattern, were not significant and not considered to be treatment-related.

The test substance gave no convincing evidence of maternal toxicity, and no evidence of embryolethality or teratogenicity, but there was a definite retardation of embryonic development at doses of 150 and 300 mg/kg bw. The NOAEL was reported to be 50 mg/kg bw/day.

Ref. : 14

2.7. Toxicokinetics (incl. Percutaneous absorption)

2.7.1 Percutaneous absorption *in vitro*

***In vitro* study without coupler**

Guideline	:	/
Tissue	:	Human abdominal epidermis, heat-separated
Method	:	Franz diffusion cell (static)
Test substance	:	6-hydroxyindole, 1% in formulation/H_2O_2 mix
Batch no	:	DG2 (purity not stated in study report)
Dose levels	:	c. 40mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	7/8
GLP	:	not in compliance

The skin penetration of 6-Hydroxyindole was evaluated in a static Franz diffusion cell using heat-separated human epidermis, with and without addition of finely chopped bleached hair. Integrity of the epidermal membrane was checked by microscopy. The test substance was prepared at a concentration of 2% in a formulation and then mixed 1:1 with hydrogen peroxide to give a final concentration of 1%. Approximately 40 mg of the mixture was applied to 2cm^2 of epidermal membrane 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 containing sodium ascorbate) using HPLC.

Results
The quantity of test substance penetrating through the epidermis to the receptor fluid corresponded to 0.239% of applied dose in the presence of hair and 0.128% of applied dose 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. The study is considered inadequate (see SCCNFP Notes of Guidance).

Ref. : 15.1

***In vitro* study in the presence of p-aminophenol**

Guideline	:	/
Tissue	:	Human abdominal epidermis, heat-separated
Method	:	Franz diffusion cell (static)
Test substance	:	6-hydroxyindole, 1% in formulation with p-aminophenol/H_2O_2 mix
Batch no	:	DG2 (purity not stated in study report)
Dose levels	:	c. 40mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	8
GLP	:	not in compliance

The skin penetration of 6-Hydroxyindole was evaluated in a static Franz diffusion cell using heat-separated human epidermis, with and without addition of finely chopped bleached hair. Integrity of the epidermal membrane was checked by microscopy. The test substance was prepared at a concentration of 2% in a formulation containing 1.64% p-aminophenol and then mixed 1:1 with hydrogen peroxide to give a final concentration of 1%. Approximately 40 mg of the mixture was applied to 2cm^2 of epidermal membrane 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 containing sodium ascorbate) using HPLC.

Results
The quantity of test substance penetrating through the epidermis to the receptor fluid corresponded to 0.08% of applied dose in the presence of hair and 0.335% of applied dose 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. The study is considered inadequate (see SCCNFP Notes of Guidance).

Ref. : 15.2

Study 3

Guideline	:	OECD 2000
Test substance	:	Imexine OBA
Tissue	:	Human abdominal (kept frozen at - 20°C) dermatomed skin
Skin integrity	:	TEWL measurement
Method	:	flow through diffusion cell 2 cm²
Receptor fluid	:	PBS buffer w/o Ca^{++}, Mg^{++} Instamed 9.55 g/l
Concentration	:	1 % after dilution with hydrogen peroxide developer (“complete formulation” study) 1 % after dilution in water (“coupler alone” study)
Batch no	:	05039001 (unlabelled compound) CFQ 12351 (radiolabelled compound)
Dose applied	:	20 mg/cm²
Replicate cells	:	“complete formulation” : 4 skin donors, 2 cells/donor, 8 cells interpreted “coupler alone” : 5 skin donors, 2 cells/donor, 11 cells mounted, 8 cells interpreted , 3 cells were discarded because of leakage or abnormal data in one analyzed compartment (elimination documented by Dixon’s test)
Analytical method	:	liquid scintillation, ^{14}C-6 Hydroxyindole (radiochemical purity checked by HPLC : 99.1 %)
Limit of detection	:	not applicable for radioactivity measurements
Solubility	:	270 µg/ml in the receptor fluid
Stability	:	HPLC control of the stability of unlabelled 6-hydroxyindole in the formulations after 1.5 month at room temperature, no significant degradation was observed (decrease of 2 % and 1.1 % respectively for the “complete formulation” and for the “coupler alone” formulation.). The degradation products are not documented.

GLP : in compliance

The skin penetration of the test substance was evaluated in a flow through diffusion cell system. Human abdominal skin previously frozen was dermatomed to a constant thickness (604 ± 122 µm). The integrity of the skin was evaluated by the measurement of the TEWL, the skin surface temperature was monitored (32.5 ± 0.2 °C). The solubility in the receptor fluid (PBS buffer) was checked in the range of the concentration used.

The test substance was prepared at a concentration of 2 % in :

* A "commercial type" formulation containing the coupler (6-Hydroxyindole unlabelled and labelled) associated with a primary intermediate (toluene-2,5-diamine sulfate 3.3 %). After a 50 % dilution with the developer (hydrogen peroxide) the formulation was applied on the skin. The final concentration, of 6-Hydroxyindole was 1.08 %. Approximately 19.70 ± 1.31 mg/cm² of the formulation i.e. 211.9 ± 14 µg/cm² (exactly measured by weight) were applied to 2 cm² of skin for 30 minutes.

* The same "commercial type" formulation containing only the coupler (6-Hydroxyindole unlabelled and labelled) without any intermediate compound. The formulation was applied on the skin after a 50 % dilution with water. The final concentration, of 6-Hydroxyindole was 1.06 %. Approximately 18.52 ± 1.42 mg/cm² of the formulation i.e. 195.5 ± 15.5 µg/cm² (exactly measured by weight) were applied to 2 cm² of skin for 30 minutes.

After 30 minutes of contact, 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 cotton swabs. 24 hours after the application the substance was measured using liquid scintillation in the receptor fluid, in the horny layer collected by tape stripping, in the epidermis/dermis and in the remaining skin outside the application area (washings). After assay of 6-Hydroxyindole in the washing material (skin excess) the mass balance of the study was calculated: 98.2 ± 4.9 % of the applied dose for the complete formulation, 101.4 ± 1.7 % of the applied dose for the coupler alone formulation.

Results
When 6-Hydroxyindole is formulated with H_2O_2 (the complete formula), the absorbed amount (epidermis + dermis + receptor fluid) represents 2.23 ± 0.99 % (5.40 ± 2.36 µg/cm²) of the applied dose at the end of 24 hours of diffusion after a contact with the skin of 30 minutes.

When 6-Hydroxyindole is formulated in water, the absorbed amount (epidermis + dermis + receptor fluid) represents 3.03 ± 1.43 % (6.88 ± 3.11 µg/cm²) of the applied dose at the end of 24 hours of diffusion after a contact with the skin of 30 minutes.

Ref. : 16

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, TA 1538 and *E. coli*. WP2 uvrA.
Substance	:	Imexine OBA
Batch no	:	Pil.10
Purity	:	97.4%
GLP	:	in compliance

Liver S9 fraction from Fischer 344 rats induced with β-naphthoflavone/phenobarbitone was used as the exogenous metabolic activation system. Dose related positive results were found in the presence or the absence of activation system in TA1535 (base-substitution tester strain) in both experiments.

Conclusions
Based on the reversion rate, it is concluded that 6-Hydroxyindole shows evidence of reproducible mutagenic activity in the presence or in the absence of activation system in *S. typhimurium*, TA 1535.

Ref. : 6

In Vitro Mammalian Cell Gene Mutation Test

Guideline	:	/
Species/strain	:	L5178Y cell line / $TK^{+/-}$ Locus
Replicates	:	2 independent tests with and without metabolic activation
Substance	:	Imexine OBA
Batch no	:	Pil.10
Treatment time	:	3 hours
Purity	:	97.4%
GLP	:	in compliance

Liver S9 fraction from Fischer 344 rats induced with β-naphthoflavone/phenobarbitone was used as the exogenous metabolic activation system.

First experiment : at 10 and 40 µg/ml, in the presence of activation system, the compound shows statistically significant positive effects but without dose-effect relationship.
Second experiment : Imexine OBA did not show any statistically significant positive effects in mutant frequency with or without S9 mix.

Conclusions
According to the fact that no trend for positivity was found in the first experiment and that the increased frequencies observed in experiment # 1 were not observed in experiment # 2, the positive results obtained may be considered as devoid of biological significance. Therefore, it may be concluded that Imexine OBA give negative results in this test. However, sizing of colonies was not evaluated.

Ref. : 7

In Vitro Mammalian Chromosome Aberration Test

Guideline	:	OECD 473
Species/strain	:	Chinese Hamster Ovary Cells

Replicates	:	yes
Substance	:	Imexine OBA
Batch no	:	Pil.10 (purity 97.4%)
Harvest times		24 and 48 hours
GLP	:	in compliance

Liver S9 fraction from Fischer 344 rats induced with β-naphthoflavone/phenobarbitone was used as the exogenous metabolic activation system.

First experiment : at the top dose of 100 µg/ml, in the presence of activation system, the compound induced statistically significant increase in the number of cells with structural chromosomal aberrations. While this frequency fell in the historical control range, the number of cells displaying aberrations is elevated and, qualitatively speaking, the type of rearrangements observed is accepted as indicator of clastogenicity.
In the absence of S9 mix, at 10 µg/ml increased a frequency of aberrations was observed at the second sampling time (48h). It should be noted that only 21 cells have been scored but that the percentage of aberrations was very high (14 %).

Second experiment : increased number of cells with structural chromosomal aberrations were observed at the highest concentration with S9 mix at the 48h harvest time and without activation at the 24h harvest time.

Conclusions
The study provided gives positive results at different doses and harvest times. However, while consistent statistically reproducible results were not achieved, the number, type and the presence of cells with multiple aberrations are indicators of clastogenic properties of Imexine OBA.
The results provided in this study are therefore considered equivocal.

Ref. : 8

***In Vitro* Mammalian Chromosome Aberration Test**

Guideline	:	OECD 473
Species/strain	:	Peripheral lymphocytes cells of 2 different donors (1 woman & 1 man)
Replicates	:	yes
Substance	:	Imexine OBA
Batch no	:	Pil.10
Purity	:	97.4 %
Exposure time	:	20 h without activation system, 3 h with activation system.
GLP	:	in compliance

Liver S9 fraction from Sprague Dawley liver rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.

First experiment
Without S9 mix : Statistically and biologically significant increased number of cells with structural chromosomal aberrations were observed; the frequency was outside the control values. The types of aberrations included mainly chromatid and chromosome deletions; only 1 exchange was scored.

With S9 mix : Statistically and biologically significant increased number of cells with structural chromosomal aberrations were observed, the frequency was outside the control values. Qualitatively, the types of aberrations observed in the presence of activation are different from the one observed without S9 mix: more exchanges have been observed.

Second experiment
Without S9 mix : Statistically and biologically significant increased number of cells with structural chromosomal aberrations were observed, the frequency was outside the control values. The types of aberrations included mainly chromatid and chromosome deletions and some exchanges figures.
With S9 mix : Statistically and biologically significant increased number of cells with structural chromosomal aberrations were observed, the frequency was outside the control values. Qualitatively, the types of aberrations observed in the presence of activation are different from the one without S9 mix: more chromatid exchanges have been observed.

Conclusions
6-Hydroxyindole has been investigated for induction of chromosomal aberrations in human peripheral lymphocytes from 2 donors (man & woman). The study is considered adequate and gives clear positive results in 2 donors. Imexine OBA is considered clastogenic under the conditions of this study.

Ref. : 9

2.8.2. Mutagenicity/Genotoxicity *in vivo*

***In Vivo* Mammalian Bone Marrow Chromosome Aberration Test.**

Guideline	:	OECD 475
Species/strain	:	Sprague-Dawley rats
Group size	:	5 males + 5 females
Test substance	:	Imexine OBA in 0.5% aqueous methylcellulose solution
Batch no	:	op T16 (purity 98.0%)
Dose levels	:	0, 150, 300 and 600 mg/kg bw
Sacrifice times	:	18 and 42 hours
Administration	:	single, intragastric gavage
Sacrifice times	:	18 and 2 hours after dosing
GLP	:	in compliance

Clinical signs of toxicity at 600 mg/kg bw were recorded.

Structural chromosome aberrations : no statistically significant or biologically relevant increase in the incidence of cells displaying chromosome aberrations over the concurrent vehicle control values was observed. This is valid for any of the doses or time-points.
Mitotic Index : 25 % reduction of the mitotic index was observed for the high dose group. This indicates satisfactory the bioavailability of the substance to the bone marrow.

Conclusions
Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal damage in the bone marrow of rats treated by single intragastric administration of Imexine OBA. 6-Hydroxyindole is considered not clastogenic under the conditions of this study.

Ref. : 10

***In Vivo* Mammalian Erythrocyte Micronucleus test**

Guideline	:	OECD 474
Species/strain	:	Mouse, CD-1 mice
Group size	:	5 males + 5 females
Test substance		Imexine OBA in 30% aqueous polyethyleneglycol
Batch no	:	Pil.10 (purity 97.4%)
Dose levels	:	0, 50, 250 and 500 mg/kg bw
Sacrifice times	:	24, 48 and 72 hours
GLP	:	in compliance

6-Hydroxyindole has been investigated for induction of micronuclei in the bone marrow cells of CD-1 mice. The substance was administered once by single intragastric gavage at 50, 250 and 500 mg/kg bw and the bone marrow harvested after 24, 48 and 72 hours. Negative and positive controls were in accordance with the OECD guideline.

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 was observed.

PCE/NCE ratio
Groups of mice treated with 6-Hydroxyindole did not exhibited variation of the PCE/NCE ratio and it cannot be estimated if the test substance has reached the bone marrow.

Conclusion
Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal or other damage leading to the micronucleus formation in polychromatic erythrocytes treated mice.
However, there is no evidence that the test agent has reached the target organ, the maximum tolerated dose chosen is also questionable, the first deaths having occurred at 800 mg/kg and the top dose chosen in this assay is 500 mg. This study is considered inadequate

Ref. : 11

***In Vivo* Mammalian Erythrocyte Micronucleus test**

Guideline	:	OECD 474
Species/strain	:	Sprague-Dawley rat, ICO: OFA-SD (IOPS Caw) strain
Group size	:	5 male + 5 female
Test substance	:	Imexine OBA in 0.5% aqueous methylcellulose solution
Batch no	:	op T16 (purity 98.0%)
Dose levels	:	0, 75, 150 and 300 mg/kg bw, on two consecutive days, by gavage
Sacrifice times	:	24 hours
GLP	:	in compliance

6-Hydroxyindole has been investigated for induction of micronuclei in the bone marrow cells of rats. The substance was administered twice by repeated intragastic gavage at 75, 150 and 300 mg/kg bw and the bone marrow harvested 24 hours, after last dosing. Negative and positive controls were in accordance with the OECD guideline.

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 was observed.

PCE/NCE ratio
Groups of rats treated with 6-Hydroxyindole did not exhibited variation of the PCE/NCE ratio and there is no indication the test substance has reached the bone marrow.

Conclusion
Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal or other damage leading to the micronucleus formation in polychromatic erythrocytes treated rats. However, there is no evidence that the test agent has reached the target organ. The protocol being different, it is difficult to compare these *in vivo* studies because of difference in strains, species, endpoints, mode of administration and batches. This study is considered inadequate.

Ref. : 12

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

Guideline	:	draft OECD 486 (1991)
Species/strain	:	Wistar rat, HanIbm: WST (SPF) strain
Group size	:	3 male per dose per harvest time
Test substance	:	Imexine OBA in polyethyleneglycol 300
Batch no	:	T2 (purity 98.4%)
Dose levels	:	0, 150 and 1500 mg/kg bw, by gavage
Exposure time	:	16 hours: all dose groups; 2h: high dose group
GLP	:	in compliance

6-Hydroxyindole has been investigated for induction of unscheduled DNA synthesis in rats hepatocytes at 2 doses 150 and 1500 mg/kg. Positive controls are in accordance with OECD guideline and UDS analyzed by autoradiography. 3 males were used per dose/time sampling. No evidence of UDS induced by the test agent was observed.

Conclusion
This study is adequate and the results negative.

Ref. : 13

General comments
* 6-Hydroxyindole has been tested in bacterial cells for gene mutation in two experiments that gave positive results.
* The *in vitro* test for mammalian gene mutation assay is accepted as being negative.
* The results of the *in vitro* test for clastogenicity in Chinese Hamster Ovary cells are equivocal.
* The *in vitro* test for clastogenicity in human lymphocytes from 2 volunteers is clearly positive.
* The *in vivo* chromosome aberration assay in bone marrow of rats gave negative results. The 25 % reduction of the mitotic index may be considered as an evidence that the bone marrow was reached by the test agent.

* The *in vivo* micronucleus test in mice gave negative results; no firm evidence that the bone marrow was reached by the test agent was noted.
* The *in vivo* micronucleus assay in bone marrow of rats gave negative results. There is no clear evidence that the bone marrow was reached by the test agent.
* The *in vivo/in vitro* UDS on rat hepatocytes is negative.

6-Hydroxyindole may be considered to show mutagenic and clastogenic potentials *in vitro* but these properties have not been observed in *in vivo* assays with different endpoints and species/or strains.

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

CALCULATION OF THE MARGIN OF SAFETY

Not Applicable

2.12. Conclusion

The overall package of tests is adequate and most have been conducted to GLP and appropriate guidelines.

The substance was moderately toxic by ingestion. A 13-week oral repeat dose study in rats showed liver changes in female rats and the NOEL was 30 mg/kg bw/day.

It was non-irritating to rabbit skin when applied neat and non-irritating to the rabbit eye at a concentration of 5%, which provides an adequate margin of safety compared with the 0.5% intended for use. It is a sensitiser.
The substance was embryotoxic, resulting in delayed ossification, with a NOEL of 50 mg/kg bw/day. It gave no evidence of teratogenicity.
Percutaneous penetration has been investigated using human skin *in vitro*. When formulated with H_2O_2, the absorbed amount after 24 hours, and after contact of 30 minutes, represents 2.23 % (5.40 µg/cm²) of the applied dose.

The substance induced gene mutations in bacteria and chromosomal aberrations in human cells *in vitro*. *In vivo* genotoxicity studies using complementary species and endpoints indicated that the *in vitro* mutagenic potential was not expressed *in vivo*.

2.13. References

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3. Opinion of the SCCNFP

6-Hydroxyindole has not been found mutagenic. However, no tests have been performed in the presence of hydrogen peroxide.

Consequently, the SCCNFP is of the opinion that the information submitted is insufficient to assess the safe use of the substance in combination with hydrogen peroxide.

Before any further consideration, the following information is required :

* data on the genotoxicity/mutagenicity following the SCCNFP opinion "Proposal for a Strategy for Testing Hair Dye Cosmetic Ingredients for their Potential of Genotoxicity / Mutagenicity", doc. n° SCCNFP/0566/02 of 4 June 2002, and in accordance with the Notes of Guidance, regularly updated by the SCCNFP (doc. n° SCCNFP/0321/00).

4. Other considerations

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5. Minority opinions

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