EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

6-Hydroxyindole

COLIPA N° A128

Adopted by the SCCP
during the 7th plenary meeting of 28 March 2006

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1. BACKGROUND

Submission I on 6-hydroxyindole was submitted to the Scientific Committee on Cosmetic Products and Non-food Products intended for Consumers (SCCNFP) in November 1997 and Submission II in January 2002. On 9 December 2003, the opinion on that substance was adopted by the SCCNFP (SCCNFP/0667/03). In that opinion, the SCCNFP asked for the additional information in order to be able to assess the safe use of the substance in combination with hydrogen peroxide.

Submission III presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Is 6-hydroxyindole safe for use in hair dye formulations taken into account the data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of 6-hydroxyindole in hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

6-Hydroxyindole (INCI name)

3.1.1.2.	Chemical names

6-hydroxyindole
1H-Indol-6-ol (CAS name)
Indol-6-ol

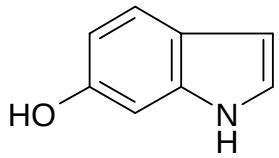
3.1.1.3.	Trade names and abbreviations

Trade name: Imexine® OBA
COLIPA n°: A128

3.1.1.4. CAS / EINECS number

CAS: 2380-86-1
ELINCS: 417-020-4 (Imexine OAB, no link to CAS number)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: C_8H_7NO

3.1.2. Physical form

Light-grey powder (which rapidly darkens on exposure to air)

3.1.3. Molecular weight

Molecular weight: 133.15

3.1.4. Purity, composition and substance codes

Batches used: D89/222 (1992), Op.T2 (1993, 2004), Op.T16 (1995), Op.38 (1977), Pil.10 (1994, 1997), Op.T50 (1997), 05039001 (2001), 0500177 (2001), 0509182 (2004).

The identity of the substance was tested by NMR and MS.

Purity

Titre as determined by HPLC:	97.4 - 99.5% (99.5% in Pil 10)
Water content:	0.05% (w/w) (by the Karl Fischer method)
Ash content:	<0.1%
Heavy metals:	< 10 ppm
Chloride ions:	<0.05% (w/w)

Potential impurities (Not more than 1%)
Reagents and intermediate reaction products (see below 3.1.5)
Impurity A is determined in 2 batches (<0.1 %)
Impurities B, C, D, E are not detected (<0.1 %)

Solvent residues

ethyl acetate:	200 ppm
ethanol:	90 ppm
cyclohexane:	250 ppm
toluene:	50 ppm

isopropanol: < 50 ppm

Comparative table of results

	Pil. 10	0509182	D89/222	Op.T2	Op.T16	Op.38	Op.T50	05039001	0500177
IR spectrum	In accordance with the proposed structure							In accordance with the proposed structure	
UV spectrum	The UV spectra are comparable								
^{1}H an ^{13}C NMR spectra	In accordance with the proposed structure								
Mass spectrum	In accordance with the proposed structure								
Titre (g/100g) HPLC	99.5 (1)	99.7 (2)	98.7 (3)	98.4 (4)	98.0 (4)	97.5 (4)	97.7	98.3 (5)	99.2 (5)
Impurities (g/100g) - A - B, C, D, E	< 0.1 D < 0.1 ND								
Residual solvents (µg/g) GC									
Ethyl acetate	200	1000	< 2000 D	500					
Ethanol	90	< 100 ND							
Cyclohexane	250	1000	< 2000 D	< 2000ND					
Toluene	50	100							
Isopropanol	< 50 ND	50							
DMF		< 500 ND							
Melting point (°C)	126.0	129.8	126.9	125.0	125.4	123.5	124.7		

ND: Not detected – D: Detected

(1) HPLC Titre performed against reference standard JJV XVIII considered as pure (100%)

(2) HPLC Titre performed against reference standard Pil 10 considered as pure (100%)
calculated against ref. JJV XVIII, the titre of batch 0509182 would be 99.2 g/100g

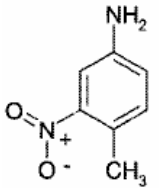
(3) Relative purity – area%

(4) HPLC Titre performed against reference, considered as pure (100%)

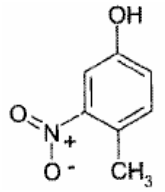
(5) HPLC Titre performed against reference standard E580 considered as pure (100%)

3.1.5.	Impurities / accompanying contaminants

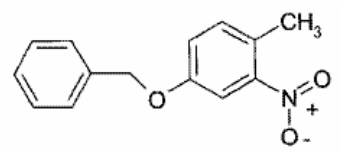
Reagents and intermediate reaction products



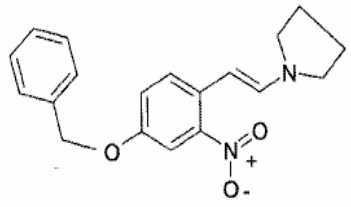
4-methyl-3-nitro-phenylamine: < 500 ppm (Impurity A)



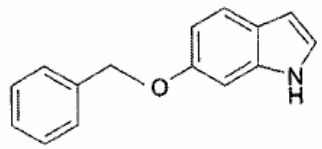
4-methyl-3-nitro-phenol: < 500 ppm (Impurity B)



4-benzyloxy-1-methyl-2-nitro-benzene: < 500 ppm (Impurity C)

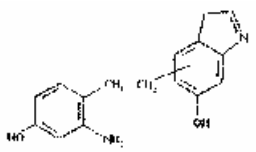


1-[2-(4-benzyloxy-2-nitro-phenyl)-vinyl]-pyrrolidine: < 500 ppm (Impurity D)



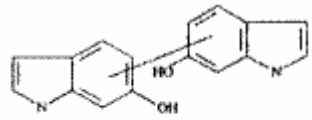
6-benzyloxy-1H-indole: < 500 ppm (Impurity E)

Unidentified impurities observed by HPLC (Pil.10),



$C_{16}H_{16}N_2O_2$
m = 268

Condensation of 2-amino-4-hydroxytoluyl moiety with 6-hydroxyindole (not quantifiable)



$C_{16}H_{12}N_2O_2$
m = 264

Dimeric form of 6-hydroxyindole (not quantifiable)

Another trace impurity, of insufficient quantity to obtain further information

3.1.6.	Solubility

Solubility in water 1.5% (w/v) at 30°C - method A6
Ethanol : ≥ 20
DMSO : ≥ 20

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow} experimental: 1.46 at 23°C (according to OECD method A8)

Note: the experimental data were not submitted

3.1.8.	Additional physical and chemical specifications

Appearance: Beige crystalline powder
Melting point: 123 – 130 °C (by capillary tube method, depending on batch)
Boiling point: /
Density: /
Rel. vap. density: /
Vapour Pressure: /

3.1.9.	Stability

Degradation of the active hair dye is less than 2% in the formulation over 1.5 months

Stability in solution was found > 97 % after storage at room temperature protected from light and under inert gas atmosphere for 6 hours in the dosage forms of 1 and 100mg/mL in 30% PEG 300 in purified water and after storage at +4°C for 9 days in the dosage forms of 3 and 48 mg/ml.

General Comments on Physico-chemical characterisation

* Potential impurities have been identified at the detection limit of 500ppm.

3.2. Function and uses

6-Hydroxyindole is used as a hair colorant requiring the presence of hydrogen peroxide as an oxidant. It is 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.

3.3. Toxicological Evaluation

3.3.1.	Acute toxicity

3.3.1.1.	Acute oral toxicity

Guideline:	/
Species/strain:	Rat, Crl:CD (SD)BR strain (VAF plus)
Group size:	5 males + 5 females
Test substance:	Imexine OBA
Batch:	Pil.10
Purity:	not stated in study report (97.4 % in submission)
Dose:	600 mg/kg bw in a volume of 10 ml/kg
Vehicle:	suspension in 30% aqueous solution PEG
Observation:	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, hypo-activity 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 LD_{50} was higher than 600 mg/kg bw.

Ref.: 1

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402 (1987)
Species/strain:	Rat, Crl:CD (SD)BR strain (VAF plus)
Group size:	5 males + 5 females
Test substance:	Imexine OBA
Batch:	Pil.10
Purity:	not stated in study report (97.4 % in submission)
Dose:	2000 mg/kg bw
Observation:	14 days
GLP:	in compliance

Dorsal skin of 5 male and 5 female rats had been shaved 24 h prior to dosing. A single dose of the test substance applied and held in place for 24 h by moistened porous gauze dressing. Any residual test substance was removed with water after the exposure period. The animals were examined immediately, 30 minutes, 1, 2 and 4 h post-dosing, and daily for clinical and behavioural signs for 14 days. Bodyweights were recorded Days 1, 8 and 14.

Results
There were no mortalities. The only clinical sign was brown staining of the fur at the application site.
Weight gain was stated to be normal for the strain used, but the results suggest female weight gain was lower than normal. The uterus of 3/5 females showed red discolouration and were distended. These changes were discounted by the study authors.
The study authors considered under the conditions of this study, the maximal non-lethal dose of 6-hydroxyindole following single dermal application to rats was higher than 2000 mg/kg, a dose at which no adverse effects were observed.

Ref.: 2

Guideline:	OECD 402 (1987)
Species/strain:	Rat, Sprague Dawley ICO: OFA-SD (IOPS Caw)
Group size:	5 males + 5 females
Test substance:	Imexine OBA
Batch:	0500177
Purity:	not stated in study report (97.8 % in submission)
Dose:	2000 mg/kg bw
Observation:	14 days
GLP:	in compliance

Dorsal skin (~6 cm x 8cm) of the rats had been clipped 24 h prior to dosing. A single dose of the test substance applied and held in place for 24 h by a semi-occlusive dressing. Any residual test substance was removed with water after the dressing was removed. The animals were examined immediately, 30 minutes, 1, 2 and 4 h post-dosing, and daily for clinical and behavioural signs for 14 days. Bodyweights were recorded Days 1, 8 and 14.

Results
There were no mortalities. Weight gain was normal for the strain used. There were no clinical signs or cutaneous reactions. No skin colouration was recorded. There were no apparent macroscopic abnormalities.
Under the conditions of the study, no adverse effects were observed up to a dose of 2000 mg/kg bw. The LD_{50} was considered to be > 2000 mg/kg bw.

Ref.: 3

3.3.1.3. Acute inhalation toxicity

Guideline:	OECD 403
Species/strain:	Rat, Wistar Crl (WI) BR, SPF
Group size:	5 males + 5 females
Test substance:	Imexine OBA
Batch:	Op.T50
Purity:	≈ 100 % in study report, 97.7% in submission
Dose:	2000 mg/m³
Observation:	14 days
GLP:	in compliance

Animals were exposed in a nose-only exposure chamber system for 4 hours to an aerosol of 6-hydroxyindole powder generated by a cylinder/brush system at 2000 mg/m^3, the maximal achievable concentration (achieved exposure males 2170 ± 240 mg/m^3; females 2440 ± 220 mg/m^3). Measurement of actual concentration of 6-hydroxyindole was carried out on aerosol samples taken at the animal nose, and granulometric distribution of the aerosol was determined with a laser granulometer. The Mass Median Aerodynamic Diameter (MMAD) reached in the aerosol was about 18 µm.
Rats were observed for clinical signs for 14 days. Bodyweight was recorded daily. All animals were killed at the end of the observation period and subjected to macroscopic examination. Gross anomalies and lungs were taken at necropsy; lungs were then weighed and prepared for a subsequent microscopic examination.

Results
There were no mortalities, thus no LC_{50} calculated. There were no significant differences in weight gain compared with the control. Clinical signs were difficult to assess during exposure, but no symptoms were noted during the observation period.
There were no macroscopic abnormalities of the lung tissue or other tissues. The histology of the lungs was normal.
Under the conditions of this study, a single inhalation exposure to an aerosol of 6-hydroxyindole powder to rats, no adverse effects were observed at the maximal non-lethal dose, 2000 mg/m^3.

Ref.: 4

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

From SCCNFP/0667/03

Guideline:	OECD 404 (1981)
Species/strain:	New Zealand albino rabbit
Group size:	3 males
Test substance:	Imexine OBA, moistened with water
Batch:	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 cm² 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.: 5

Guideline:	/
Species /strain:	rabbits
Group size:	6 (3 males and 3 females)
Test substance:	IMEXINE OBA
Batch:	Op.38
Purity:	97.5 %
Dose:	2 ml, 5% (w/w) (day 1 to day 9) and 1.55 (w/w) (day 10 to day 14)
GLP:	in compliance

A volume of 2 ml of preparation of the test substance in paraffin oil was applied to the left flank of the rabbits for 14 consecutive days. The test site was not covered by a dressing.
The concentration of the test substance applied was reduced from 5% (w/w) (day 1 to day 9) to 1.55 (w/w) (day 10 to day 14) in order to preserve the good state of health of the animals.
The right flank received paraffin oil under the same experimental conditions.
All animals wore a protective collar in order to avoid ingestion of the test substance. Cutaneous reactions were evaluated in each animal immediately before each application and approximately 24 hours after the last application. Daily irritation indices were calculated.

Results
On the treated flank, very slight or well- defines erythema was noted on almost all the animals, from day 3 up to the end of the observation period. A moderate erythema was also observed in

2/6 animals, from day 8 up to day 15. A slight oedema was noted in 2/6 animals on day 3; a slight to severe oedema persisted in one of them up to the end of the observation period (day 15). The maximum Weekly Mean Irritation Index was 2.6.
On the flank which received the vehicle, very slight or well- defined erythema was noted in 3/6 animals, from Day 8 (2 animals) or 11 (1 animal up to day 15.A moderate erythema was recorded in one of these animals on day 15.
In a first instance, the cutaneous reactions observed on the treated flank were not attributed to a single effect of the test substance since erythema was also noted on the flank which received the vehicle only, with a lower incidence and severity. However, contact of the test substance with the vehicle- treated flank could not be excluded since rabbits can touch both flanks with their ears and contamination of the vehicle treated sites with the test substance could not be excluded.
Severe erythema and crusts were observed on both ears of all animals from day 9 up to the end of the observation period (day 15).The cutaneous reactions were considered to be due to the contact of the ears with the test substance and/or vehicle when animals were at rest, with the ears touching the back and the flanks.
Body weight gain of the males was normal for animals pf this strain and age. In females, body weight gain was reduced. One female lost weight between day 1 and day 8, possibly related to the cutaneous irritation.

Conclusion
Under experiment conditions, repeated daily cutaneous application of the test substance in paraffin oil was not well tolerated in rabbits.

Ref.: 7

Guideline:	/
Species/strain:	Guinea- pigs
Group size:	10 (5 males and 5 females)
Test substance:	IMEXINE OBA
Batch:	0500177
Purity:	97.8 %
Dose:	0.05 ml of 5 % (w/w) in PEG 300 at 30%
GLP:	in compliance

A dose-volume of 0.05 ml of the test substance at the concentration of 5% (w/w) in PEG 300 at 30% in purified water was applied to the left flank of ten guinea pigs once a day for 14 consecutive days. It was applied over the same area of clipped skin, measuring approximately 2 cm x 2 cm. No rinsing of the test site was performed. The test site was not covered by a dressing. The right flank received the vehicle under the same experimental conditions.

Cutaneous reactions were evaluated on both flanks of each animal before each application and approximately 24 hours after the last application. At the end of the observation period, the animals were killed without examination of internal organ and skin samples were taken from the control and treated sites. No histological examination was performed.

Results
No clinical signs and no deaths were noted during the study. The body weight gain of the animals was within the expected values for animals of this strain and age. No cutaneous reactions were observed on the right controls flank animals.

A very slight erythema was noted o the left treated flank of 5/10 animals on days 9 and 10, a very slight or well-defined erythema was recorded in 7/10 animals on day 11, then all animals exhibited a very slight (6/10), well-defined (3/10) or moderate (1/10) erythema, from day 12.
No oedema was observed. Dryness of the skin was recorded in one animal on day 11 and in 3/10 animals from day 12. A slight orange coloration of the skin was observed in all animals during the observation period. The Maximum Weekly Irritation Index obtained was 1.1 (week 2).

Conclusion
Under experimental conditions, the test substance induces slight to moderate skin irritation in Guinea pigs.

Ref.: 8

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405 (1987)
Species/strain: New Zealand White rabbit (male)
Group size: 1
Test substance: IMEXINE OBA
Batch: Pil.10
Purity: not stated in report
Dose: 0.1 g
GLP: in compliance

A 0.1 g aliquot of the test article was administered to the right eye of one rabbit and resulting reaction to treatment assessed one hour after dosing. Within 15 minutes of dosing, signs of irritation were apparent in the treated eye and became progressively more severe during the next 45 minutes. One hour after dosing, discharge, moderate hyperaemia and chemosis were noted easily discernible areas of opacity covered the majority of the cornea, making observation of the iris difficult. At this point, the eye reaction was considered of greater severity. Due to the severity of the response in this animal no further rabbits were dosed and the study terminated at this stage. As only one animal was dosed, it was not possible to formally classify the irritancy of the test article according to the Kay and Calandra Scale. However, given the severity of the irritant response and rapidity of its onset, it is considered that the test article was moderately to severely irritant to the eye of the albino rabbit under the conditions of the study.

Ref.: 9

From SCCNFP/0667/03

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: Pil.10
Purity: /
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.: 10

3.3.3. Skin sensitisation

Magnusson and Kligman study (*from SCCNFP/0667/03*)

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:	/	
Purity:	/	
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.: 34

Local tolerance after repeated application

Guideline:	/
Species/strain:	Guinea- pigs
Group size:	6 (3 males and 3 females)
Test substance:	IMEXINE OBA
Batch:	Op.38
Purity:	97.5 %
Dose:	0.05 ml at 20% (w/w) on days 1 and 2; 10% (w/w) from day 3 to 14
GLP:	in compliance

A volume of 0.05 ml of a preparation of the test substance in paraffin oil, at the chosen concentration (20% (w/w) on days 1 and 2; 10% (w/w) from day 3 to 14) was applied to the posterior left flank of six Guinea pigs once daily for 14 consecutive days. The concentration was reduced as the first concentration tested (20 %) was badly tolerated.
Each cutaneous application was performed over the same area of clipped skin, measuring approximately 2 cm x 2 cm. The test site was not covering by a dressing. The posterior right flank received paraffin oil under the same experimental conditions.
Cutaneous reactions were observed in each animal immediately before each application and approximately 24 hours after the last application.
Due to skin lesions observed on both flanks, suggestive of sensitization reactions, the animals were challenged with the test substance and vehicle, after a rest period of one week. Three supplementary untreated females were used as controls.
On day 23, 0.5 ml of a preparation of the test substance in paraffin oil was applied (anterior left flank) at the concentration of 10% (w/w).The vehicle was applied under the same experimental conditions to the anterior right flank. The test sites covered an occlusive dressing for 6 hours.
Cutaneous reactions were evaluated 24, 48 and 72 hours after removal of dressing.

Results
No clinical signs or mortality were noted during the study. The body weight gain of the animals was normal.
During the two-week treatment period, moderate skin reactions were observed on the treated flank: well-defined to severe erythema was noted in all animals and a slight oedema was observed on 3/3 males and 1/3 females. First cutaneous reactions were observed between days 3 and 6. Dryness of the skin and/or crusts were also noted in all animals. Weekly mean Irritation Indices were 3.6 for the first week and 2.3 for the second week.
From day 6, cutaneous reactions were also observed on the flank which received the vehicle only: very slight to moderate erythema (2 males and all females), slight oedema (2 males and 1 female), dryness of the skin and crusts. First cutaneous reactions appeared on days 6, 7, 12. After the cutaneous challenge application, no cutaneous reactions were observed in the control animals.
In the treated animals, at the 24-hour reading, a very slight, well-defined or moderate erythema was noted in 1/61/6 and 3/6 animals, respectively. Dryness of the skin was recorded in one animal.
At the 48-hour reading, a very slight, well defined or moderate erythema was noted in 1/6, 1/6 and 2/6 animals, respectively. Dryness of the skin was recorded in three animals.
At the 72-hour reading, a moderate erythema persisted in one animal. Dryness of the skin was observed in three animals; it was severe enough to preclude the evaluation of erythema in two animals. Crusts were also noted in one animal.

Microscopic examinations indicated that the lesions observed were attributable to a sensitization process in two males and one female.

Conclusion
The repeated topical application of IMEXINE OBA at the concentration at 10 and 20 % (w/w) in paraffin oil was badly tolerated in guinea pigs. The cutaneous reactions observed were possibly due to a sensitization process.

Ref.: 6

Guideline:	OECD 406 (1992)
Species/strain:	Guinea- pigs
Group size:	30 (15 males and 15 females)
Test substance:	IMEXINE OBA in paraffin
Batch:	Pil.10
Purity:	97.4 %
Dose:	20 % (w/w) 0.5 ml
GLP:	in compliance

Thirty guinea-pigs were allocated to 2 groups: a control group 1 (10 5 males and 5 females 0 and a treated group 2 (10 males and 10 females). During a 2 week induction period, animals of the treated group 2 received on days 1, 8 and 15 an application of 0.5 ml of the test substance at a concentration of 20% (w/w). The test substance prepared as a formulation in paraffin oil at a concentration of 20% (w/w) was prepared on a dry gauze pad and then applied on an area of the skin approximately 4 cm² on the anterior left flank. After each application, the test substance was held in place for 6 hours by means of am occlusive dressing. The right flank received paraffin oil at the same conditions. After a 14-day rest period (from day 15 to day 28) a cutaneous challenge application of 0.5 ml of the test substance at a concentration of 20% (w/w) (right flank) and the application of 0.5 ml of the vehicle (left flank) were performed on a non-treated area of the posterior region of all animals. Cutaneous reactions were evaluated 24, 48 and 72 hours after the removal of the pads of the challenge application by comparing the reactions on both flanks.
The sensitivity of the guinea-pigs was checked in a study with a positive sensitizer: Dinitro 2.4 Chlorobenzene. During induction period, the test substance was applied at 1% (days 1 and 8) and 0.1% (day 15) concentrations. At cutaneous challenge application, 0.5% was tested on right flank and paraffin oil on the left flank.

Results
No clinical signs and no deaths were noted during the study.
During the induction period very slight a slight erythema was noted in 16/20 and 12/20 animals of the treated group on days 9 and 16, respectively.
After the challenge application, no skin reactions were observed in control animals. In the treated group, slightly erythema was observed in 3/20, 5/20 and 3/20 animals 24, 48 and 72 hours after removal of the dressing, respectively.

Conclusion
Under experimental conditions and according to the method established by Buehler the application of the test substance at a concentration of 20% (w/w), induce sensitization reactions in 25% of the Guinea pigs.

Ref.: 11

Guideline:	OECD 406 (1992)
Species/strain:	Guinea- pigs
Group size:	30 (15 males and 15 females)
Test substance:	IMEXINE OBA in paraffin
Batch:	0500177
Purity:	97.8 %
GLP:	in compliance

Thirty guinea-pigs were allocated to 2 groups: a control group (5 males and 5 females) and a treated group (10 males and 10 females). The test substance induction (treated group) was at the concentration of 50 % (w/w) on days 1, 8 and 15, the challenge (all groups) at the concentration of 50% (w/w) on day 29. The vehicle used was PEG 300 at 30% in purified water.
On days 1, 8 and 15, the animals of the treated group received a topical application of the test substance. The application sites were covered by an occlusive dressing for 6 hours on each occasion. The animals of control group received applications of the vehicle under the same experimental conditions.
On day 29, animals of both groups were challenged by a topical application of the test substance to the right flank. The non-treated posterior region of the left flank served as control and received the vehicle only. Test substance and vehicle were maintained under an occlusive dressing for 6 hours. Skin reactions were evaluated approximately 24, 48 and 72 hours after the removal of the dressing.

Results
No clinical signs and no deaths were noted during the study.
During the induction period, a brown coloration of the skin, which could have masked a possible to intense erythema, was noted in all animals of the treated group.
After the challenge application, a brown coloration of the skin, which could have masked a possible discrete or erythema at the 24- hour reading, was noted in all animals of both groups. In the treated group, a discrete or moderate erythema was noted at the 48 – hour reading in 6/20 and 6/20 animals, respectively. At the 72-hour reading, a discrete, moderate or intense erythema was observed in 4/20, 7/20 and 1/20 animals, respectively. An oedema was recorded in 1/20 animals.
Dryness of the skin was recorded in a few animals of both groups.
The persistent cutaneous reactions observed in 11/20 animals of the treated group were attributed to delayed contact hypersensitivity.

Conclusion
Under experimental conditions and according to the method established by Buehler, the test substance induces delayed contact hypersensitivity in 11/20 (55%) of the Guinea pigs.

Ref.: 12

Guideline:	OECD 406 (1992)
Species/strain:	Guinea pigs
Group size:	30 (15 males and 15 females)
Test substance:	IMEXINE OBA in paraffin
Batch:	0500177
Purity:	99.2 %
GLP:	in compliance

Thirty guinea-pigs were allocated to 2 groups: a control group (5 males and 5 females) and a treated group 10 males and 10 females). The test substance induction – (treated group) was at the concentration of 5 % (w/w) in a solution of PEG 300 at 30% in purified water on days 1, 8 and 15. The challenge (all groups) at the concentration of 5% (w/w) in a solution of PEG 300 at 30% in purified water on day 29. The animals of the control group received the vehicle under the same experimental conditions.
On days 1, 8 and 15, the animals of the treated group received a topical application of the test substance. The application sites were covered by an occlusive dressing for 6 hours on each occasion. The animals of control group received applications of the vehicle under the same experimental conditions. The application sites were not rinsed after removal of the dressing.
On day 29, animals of both groups were challenged by a topical application of the test substance to the right flank. The non-treated posterior region of the left flank served as control and received the vehicle only. Test substance and vehicle were maintained under an occlusive dressing for 6 hours. The application sites were not rinsed after removal of the dressing. Skin reactions were evaluated approximately 24, 48 and 72 hours after the removal of the dressing.

Results
No clinical signs and no deaths were noted during the study. During the induction period, discrete skin reactions (erythema, grade 1) were observed in the animals of the treated group.
After the challenge application, a brown coloration of the skin, which could have masked a possible discrete or erythema at the 24- hour reading, was noted in all animals of both groups. In the treated group, a discrete or moderate erythema was noted at the 48 – hour reading in 6/20 and 6/20 animals, respectively. At the 72-hour reading, a discrete, moderate or intense erythema was observed in 4/20, 7/20 and 1/20 animals, respectively. An oedema was recorded in 1/20 animals.
Dryness of the skin was recorded in a few animals of both groups.
The persistent cutaneous reactions observed in 11/20 animals of the treated group were attributed to delayed contact hypersensitivity.

Conclusion
Under experimental conditions and according to the method established by Buehler, the test substance induced cutaneous reactions attributable to delayed contact hypersensitivity and induced delayed contact allergy in 6/20 (30%) of the Guinea pigs.

Ref.: 13

Guideline:	draft OECD 429 (2000)
Species/strain:	CBA/J mice
Group size:	28 (females)
Test substance:	IMEXINE OBA
Batch:	0500177
Purity:	97.8 %
GLP:	in compliance

To induce delayed contact hypersensitivity Murine Local Lymph Node Assay (LLNA) was performed.
Twenty eight female CBA/J mice were allocated to seven groups of four animals each:

- five treated groups receiving the test substance IMEXINE OBA at the concentrations of 0.2,0.5,1.2 and5%,
- one negative control group receiving the vehicle (mixture acetone/olive oil (4/1,(v/v),
- one positive control group receiving the reference substance, ά-hexylcinnamaldehyde (HCA), at the concentration of 25%.
- During the induction phase, the test substance, vehicle or reference were applied over the dorsal side of the ears (25 µL per ear) for three consecutive days (days 1, 2 and 3).After 2 days of resting, the proliferation of lymphocytes in the lymph node draining the application site was measured by incorporation of tritiated methyl thymidine (day 6). The obtained values were used to calculate stimulation indices (SI).
- The irritant potential of the test substance was assessed in parallel by measurement of ear thickness on days 1, 2, 3 and 6.

Results
The test substance was freely soluble in the recommended vehicle, acetone/olive oil (4/1,v/v).
No mortality and no clinical signs were observed during the study.
No cutaneous reactions and no increases in ear thickness were observed in the animals of the treated groups.
A significant dose0related lymphoproliferation was noted at al tested concentrations.
A significant lymphoproliferation was also noted with the reference substance HCA at 25%.

The results are presented in the following table:

Test substance	**Concentration (%)**	**signs of local irritation**	**stimulation index (SI)**
IMEXINE OBA	0.2	no	3.77
IMEXINE OBA	0.5	no	4.63
IMEXINE OBA	1	no	8.82
IMEXINE OBA	2	no	8.07
IMEXINE OBA	5	no	19.17
HCA	25	-	9.62

In the absence of local irritation, the observed lymphoproliferative responses were attributed to delayed contact hypersensitivity.

Conclusion
Under experimental conditions, the test substance IMEXINE OBA (batch 0500177) induces delayed contact hypersensitivity in the Murine Local Lymph Node Assay.

Comment
Also lower concentrations should have been tested.

Ref.:14

Guideline: draft OECD 429 (2000)
Species/strain: CBA/J mice
Group size: 32 (females)
Test substance: IMEXINE OBA
Batch: 0500177
Purity: 99.2 %
GLP: in compliance

To induce delayed contact hypersensitivity murine Local Lymph Node Assay (LLNA) was performed.
Twenty eight female CBA/J mice were allocated to seven groups of four animals each:

- Six treated groups receiving the test substance at the concentrations of 0.02, 0.05,0.1, 0.2, 0.5 and 1%,
- one negative control group receiving the vehicle (mixture acetone/olive oil (4/1,(v/v),
- one positive control group receiving the reference substance, α-hexylcinnamaldehyde (HCA), at the concentration of 25%.
- During the induction phase, the test substance, vehicle or reference were applied over the dorsal side of the ears (25 µL per ear) for three consecutive days (days 1, 2 and 3).After 2 days of resting, the proliferation of lymphocytes in the lymph node draining the application site was measured by incorporation of tritiated methyl thymidine (day 6). The obtained values were used to calculate stimulation indices (SI).
- The irritant potential of the test substance was assessed in parallel by measurement of ear thickness on days 1, 2, 3 and 6.

Results
The test substance was freely soluble in the recommended vehicle, acetone/olive oil (4/1,v/v).
No mortality and no clinical signs were observed during the study.
No cutaneous reactions and no increases in ear thickness were observed in the animals of the treated groups.
A significant dose related lymphoproliferation was noted at concentrations ≥ 0.2 %. No significant effect as observed at concentrations ≤ 0.1%.
A significant lymphoproliferation was also noted with the reference substance HCA at 25%.

The results are presented in the following table:

Test substance	**Concentration (%)**	**sings of local irritation**	**stimulation index (SI)**
IMEXINE OBA	0.02	no	0.77
IMEXINE OBA	0.05	no	0.98
IMEXINE OBA	0.1	no	1.26
IMEXINE OBA	0.2	no	6.78
IMEXINE OBA	0.5	no	10.51
IMEXINE OBA	1	no	8.74

Test substance	Concentration (%)	sings of local irritation	stimulation index (SI)
HCA	25	-	7.75

In the absence of local irritation, the observed lymphoproliferative responses were attributed to delayed contact hypersensitivity.

Conclusion
Under experimental conditions, the test substance induces delayed contact hypersensitivity (stimulation index ≥ 3) in the Murine Local Lymph Node Assay at concentrations ≥ 0.2%.

Ref.:15

3.3.4. Dermal / percutaneous absorption

From SCCNFP/0667/03

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:	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, 14C-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 4.70 ± 2.08 µg/cm² (1.23 – 8.09 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 5.86 ± 2.68 µg/cm² (3.80 – 10.25 µg/cm²) of the applied dose at the end of 24 hours of diffusion after a contact with the skin of 30 minutes.
However, the maximum dermal absorption of 8.09 µg equiv/cm², observed under oxidative conditions in typical hair dye formulations, will be used for the calculation of the Margin of Safety.

Ref.: 33

3.3.5.	Repeated dose toxicity

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

No data submitted

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

Guideline:	OECD 408 (1981)
Species/strain:	Crl: CD (SD) BR strain rat, (VAF plus)
Group size:	10 males + 10 females
Test substance:	Imexine OBA
Batch:	Pil.10
Purity:	not stated in study report
Dose:	0, 30, 100 and 300 mg/kg bw/day, 7 days/week by gavage
Vehicle:	suspension in 30% aqueous solution PEG, fresh daily
Exposure:	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 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 with 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.

Ref.: 16 (submission II, 5)

3.3.5.3. Chronic (> 12 months) toxicity

See 3.3.7. Carcinogenicity

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. 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:	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.: 17

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: 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.

Ref.: 18

In Vitro Mammalian Chromosome Aberration Test

Guideline: OECD 473
Species/strain: Chinese Hamster Ovary Cells
Replicates: yes
Substance: Imexine OBA
Batch: 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 highest concentration 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 concentrations 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.: 19

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:	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.

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.: 20

3.3.6.2 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:	Op.T16
Purity:	98.0 %
Dose levels:	150, 300 and 600 mg/kg bw
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.: 22

***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:	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.

No statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values was observed.

Groups of mice treated with 6-Hydroxyindole did not exhibit variation of the PCE/NCE ratio and therefore, the test does not indicate whether there was a relevant exposure of 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.: 21

***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:	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 exhibit variation of the PCE/NCE ratio and there is no indication that the test substance 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.: 23

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:	Op.T2
Purity:	98.4%
Dose levels:	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.: 24

General comments

* 6-Hydroxyindole has been tested in bacterial cells for gene mutation in two experiments that gave positive results.
* The *in vitro* 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 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.
The *in vivo* micronucleus assay in bone marrow of rats gave negative results. There is no clear evidence that the bone marrow was effected by the test agent, since a toxic dose was not used. However, a pharmacokinetic and excretion balance study demonstrated a high systemic exposure after oral administration of 6-hydroxyindole to rats.

3.3.7. Carcinogenicity

Guideline:	OECD 451
Species/strain:	Rat, Wistar Han Ico: WI (IOPS AF/Han), 9 weeks old
Group size:	50 + 8 satellite rats/sex
Test substance:	Imexine OBA (11.2.98, +4°C, away from light and under argon atmosphere)
Batch:	0500177
Purity:	not stated in study report; 97.8% in submission
Dose:	0, 6, 25 or 100 mg/kg bw/day, 7 days/week by gavage, made up every 8 days (stored at 4^0C, under argon and protected from light)
Vehicle:	suspension in 0.5% aqueous solution carboxymethylcellulose, 5 mL/kg
Exposure:	104 - 107weeks
GLP:	in compliance

The dose levels were selected on the basis of the results of a preliminary 8-week toxicity study at 30, 100, 250 and 500 mg/kg/day. In this study, deaths were observed at 250 mg/kg/day and higher [40].
The chemical analysis of the dose formulations administered during the study showed that achieved concentrations were close to the intended values (within ± 7% of nominal concentrations), and plasma levels of unchanged 6-hydroxyindole were below the limit of quantification.

In all groups, the 8 satellite animals were used for toxicokinetic investigations: blood samples were taken on day 1 and in weeks 13 and 52, at designated time-points, and plasma levels of the test item were measured. Throughout the study, clinical signs and mortality were checked daily. Detailed physical examination was carried on a weekly basis. Palpation of possible masses was carried out every 2 weeks from 6 months of treatment.
Body weight and food consumption were measured at weekly intervals during the first 13 weeks of the study and then every 4 weeks. Efficiency of food utilization was estimated by calculation of food conversion ratio.
Haematological investigations were carried out in weeks 52 and 78 (Differential White Cell Count in control and high-dose groups) and at the end of the study (quantitative and qualitative measurements of red and white cells in all animals).
After 105 weeks of treatment, all animals were sacrificed and submitted to a macroscopic *post mortem* examination. A full range of organs and any masses or macroscopic lesions were sampled. A microscopic examination was performed on all sampled tissues, in all animals (including decedents).
In comparison with the controls, factors contributing to death or premature sacrifice were similar in the treated groups at 6 and 25 mg/kg/day as regards the distribution and timing. At termination, in the 100 mg/kg/day group, there was a slight increase of mortality rate in the males (56% compared with 38 - 42%). However, these deaths were due to normal (geriatric) causes, rather than to a specific factor, suggesting a slight shortening of the time of onset of death. This was not seen in the females.
The body weight gain was similar in the control and the treated groups at 6 and 25 mg/kg/day and in females at 100 mg/kg/day. Body weight gain was slightly lower for males dosed at 100 mg/kg/day (approximately down 9% from controls; 429 versus 472 g). The food consumption was similar in the control and the treated groups during the study period.

The only clinical sign was increased salivation and/or regurgitation observed in all treated groups. The incidence, time of onset, localization and size of palpable masses was similar in all groups. The only haematological changes were slight decreases (at most -8%) in main red blood cell count, packed cell volume and plasma haemoglobin concentration, in rats given 100 mg/kg/day. These changes indicated anaemia in these animals.
No quantifiable levels of the test item was found in plasma of treated animals.
There were no gross findings, no neoplastic or non-neoplastic lesions attributed to treatment with 6-hydroxyindole.

Conclusion
At 100 mg/kg/day only, a slightly lower survival rate and body weight gain in the males and slight anaemia in the males and the females were noted.
Under the study conditions, the No Observed Adverse Effect Level for general toxicity was 25 mg/kg/day, whereas the No Observed Effect Level for carcinogenicity was 100 mg/kg/day.

Ref.: 25

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	/
Species/strain:	Rat, Crl: CD (SD) BR strain, (VAF plus)
Group size:	24 mated females
Test substance:	Imexine OBA (stored RT in dark)
Batch:	D89/222 (purity 97.2% in report, 98.7% pure in submission), used for dosing GD 6, 7 Pil.10 (purity 97.4%), used for dosing all GD 8 onwards
Dose:	0, 50, 150 or 300/450 mg/kg bw/day, daily by gavage
Vehicle:	suspension in 30% aqueous solution PEG, 5 ml/kg, fresh daily
Exposure:	Gestation Day (GD) 6-15
Observation:	GD 16-20
GLP:	in compliance

The dose levels 0, 50, 150 or 450 mg/kg bw/day were selected on the basis of the results of a preliminary study performed at 100, 250 and 600 mg/kg/day. Excessive maternal toxicity (including deaths) was observed at 600 mg/kg/day whereas no adverse effects were observed at 100 and 250 mg/kg/day [41].
Due to severe clinical reactions at the onset of dosing at 450 mg/kg bw/day, fourteen of the twenty four animals were killed. The remaining 10 and a further 14 were given 300 mg/kg/day from GD 6.
Clinical observations were recorded from GD 0. Bodyweights were recorded GD 0, 6-15, 20. Food consumption measured GD 0 - 6, 6 - 9, 9 - 15, 15 - 20.
On GD 20, all were killed. Post-mortem examination recorded organ abnormalities, pregnancy status, number of corpora lutea, implantation sites, together with foetal weight, sex and abnormalities. Approximately two thirds of the live foetuses were dissected, their viscera were

examined, and their skeletons were observed for anomalies following alizarin red S staining. The remaining foetuses were examined by a combined sectioning/dissection technique (head, thorax and abdomen) following fixation in Bouin's fluid.

Results
The chemical analysis of the dose formulations administered during the study showed that achieved concentrations were close to the intended values (within ± 5% of nominal concentrations).
All females (n=14) given 450 mg/kg on the first day of dosing (GD 6) showed severe clinical signs including hypoactivity, hunched posture and piloerection. They were then treated at 300 mg/kg on GD 7 but, as similar clinical reactions were observed, they were sacrificed on GD 8.
At 300 mg/kg/day, maternal toxic effects were limited to slightly lower body weight gain and food intake from GD 6 to GD 9. Increased salivation at 300 mg/kg/day and greenish urine at 150 mg/kg/day and higher were also observed.
Bodyweights and food consumption were lower at the higher doses.
On GD 20, 22, 23, 24 and 22 females were pregnant in the 0, 50, 150 and 300 mg/kg/day groups, respectively. The dams did not show any treatment related toxic effects. Pre-implantations losses were similar or lower than the controls.
Mean foetal sex ratio was similar in all groups. Mean foetal weight was slightly but statistically significantly reduced at 300 mg/kg/day (approximately 9% lower, $p<0.01$).
Reduced ossification, effecting most of the bones, of the foetal skeleton was noted at the mid and high doses. At 50 mg/kg/day, there was a slight indication, but non-specific incidence of reduced ossification.

Conclusion
The No Observed Adverse Effect Level (NOAEL) was considered to be 150 mg/kg/day for maternal toxicity and at 50 mg/kg/day for embryo-foetal toxicity.
Evaluation of this study is hampered by the use of 2 batches of 6-hydroxyindole together with the change in the top dose and new animals recruited into the experiment.

Ref.: 26

Guideline:	/
Species/strain:	Rat, Sprague-Dawley
Group size:	24 mated females
Test substance:	6-Hydroxyindole (received 29/12/03, stored 4°C, protected from light and under nitrogen gas, expiry date: March 2005)
Batch:	0509182
Purity:	99.7%
Dose levels:	0, 15, 60 or 240 mg/kg bw/day, 7 days/week by gavage
Vehicle:	suspension in 30% aqueous solution PEG, 5 mL/kg, fresh daily
Exposure period:	Gestation Day (GD) 6-19
Observation:	GD 20
GLP:	in compliance

Stability of dosage forms had been checked in a separate study [42].
Clinical observations were recorded from GD 0. Bodyweights were recorded GD 3, 6, 9, 12, 15, 18 and 20. Food consumption measured GD 0 - 6, 6 - 9, 9 - 15, 15 - 2 3, 3-6, 6-9, 9-12, 12-15, 15-18 and 18-20.
On GD 20, all were killed. Post-mortem examination recorded organ abnormalities, pregnancy

status, number of corpora lutea, implantation sites, early and late resorptions, dead and live foetuses. The foetuses were weighed, sexed and subjected to external and visceral or skeletal (bone) examinations.

Results
The chemical analysis of the dose formulations showed that achieved concentrations were close to the intended values (within ± 3% of nominal concentrations).
No deaths occurred during the study. The only treatment-related clinical signs observed were ptyalism in four females at 240 mg/kg/day. No treatment-related body weight, body weight gain and food consumption were noted.
A total of 23, 24, 24 and 22 females were pregnant with live foetuses on GD 20 in the 0, 15, 60 and 240 mg/kg/day groups, respectively. No treatment-related necropsy findings were observed.
In the mid and high dose groups, preimplantation loss was significantly different from the controls, but it was not considered toxicologically relevant as this was lower than in the control group. The mean numbers of resorptions and of dead foetuses were not affected by treatment. The foetal sex ratio was normal. The mean foetal body weight was slightly lower but not statistically significant, at the high dose compared with the control group (-4%). No treatment-related visceral anomalies were noted.
At the high dose, retardation of general ossification of foetuses was seen, but there were no bone malformations. These findings were considered consistent with the slightly lower mean foetal weight observed this dose.

Conclusion
Since there was no evidence of maternal toxicity, the No Observed Effect Level (NOEL) for maternal toxicity was considered to be 240 mg/kg/day. Developmental effects (lower foetal weight and reduced ossification of foetal skeleton) were seen at 240mg/kg/day, thus the embryo-foetal NOEL was considered to be 60 mg/kg/day.

Ref.: 27

3.3.9. Toxicokinetics

Pharmacokinetic and Excretion Balance Study after Single Oral Administration to Rats

Guideline:	/
Species/strain:	Rat, Wistar Han Ico: WI (IOPS AF/Han)
Group size:	12 per sex
Test substance:	6-Hydroxyindole
Batch:	0500177 (unlabelled 6-hydroxyindole, 97.8% pure); CFQ10790 (radio-labelled 6-hydroxyindole, 99.5% radiochemical pure, received 10/8/98 stored -20°C, protected from light and under nitrogen gas, expiry)
Dose:	300 mg/kg bw/day, single dose by gavage
Vehicle:	suspension in 0.5% aqueous carboxymethylcellulose, 5 mL/kg
GLP:	in compliance

Nine and 3 rats per sex were dedicated to plasma pharmacokinetics and to excretion balance respectively.
Plasma pharmacokinetics: Blood samples were collected from the orbital sinus of designated animals under light isoflurane anaesthesia at the following time-points: before dosing, and 1, 2,

4, 6, 8, 24, 48, and 72 hours after dosing (3 rats/sex/time-point, each rat bled thrice). All animals were killed after the last blood sample. No further analysis was made of carcasses.
Excretion balance: Urine, faeces and cage washes were collected at the following times: during a 24-hour pre-dose period (urine and faeces), and then during the periods 0-6h, 6-24h (urine) or 0-24h (faeces), 24-48h, 48-72h, 72-96h, 96-120h, 120-144h and 144-168h (urine and faeces) after dosing. After each collection of faeces, the cages were carefully rinsed with approximately 20 ml of water (200 ml at 168h). All samples were stored frozen at -20°C until analysis. All animals were killed and a wide range of tissues/organs were sampled.
For each sample, total radioactivity was evaluated by Liquid Scintillation Counting on plasma, urine, faeces and rinse-off water. The minimal (default) fraction of oral dose absorbed was derived from urine excretion data.

Results
There were no deaths. Clinical signs noted in the first 24 h, included brown greenish urine and piloerection in most animals. Other signs that occurred in some animals about 45 min post-dosing and lasted 3-5 h were tremors, hypersalivation, lacrimation, lateral decubitus, jerking movements, staggering gait, half-closed eyes and coldness to touch. These were seen more frequently in the female. The urine was remained coloured up to 3 days.
All these signs were considered to be treatment related.

Total radioactivity levels in plasma reached a maximum 1 hour after dosing (mean C_{max} value of 132 µg-eq/ml; males 107µg-eq/mL, females 157 µg-eq/ml). Thereafter, plasma radioactivity levels decreased (biphasic manner) at a moderate rate until 24 hours post dosing, followed by a slower decline until the last sampling point mean 0.88 µg-eq/ml (males 0.77 µg-eq/ml, females 0.98 µg-eq/ml; quantification limit >0.084 µg-eq/ml). The mean systemic exposure [AUC(0-∞)] was 799 µg-eq.h/ml (males 638 µg-eq/ml, females 954 µg-eq/ml). The time profiles were similar for both sexes and there was little inter animal variation.
The mean total Cumulative Excretion (CE) of the radioactive dose over the 168-h period monitored was 95.4% (males 94.9 ± 1.4%, females 95.9 ± 1.6%). Most of this dose was excreted rapidly, as an average of 81.9% was excreted within 24 hours of dosing. The radioactivity was mainly excreted in urine (mean 73.8%; males 78.0 ± 2.1%, females 69.6 ± 7.0% over the entire study period), and the CE in faeces was in mean 17.3% (males 13.1 ± 2.5%, females 21.6 ± 8.0%).
No post-mortem anomalies were noted. Since the CE rate was so high during the study period, no further radioactivity analysis was performed.

Conclusion
Slight sex differences in pharmacokinetic parameters and excretion profiles were observed. Most of the radioactive dose was excreted in the urine within 24 hours of dosing. Based on the urinary CE data, the mean fraction absorbed following oral dosing was at least 73.8%. These results confirm high systemic exposure after oral administration of 6-hydroxyindole to rats.

Ref.: 28

Pharmacokinetic, Excretion Balance and Tissue Distribution Study after Single Dermal Administration to Rats

Guideline: /
Species/strain: Rat, Wistar Han, Ico: WI(IOPS AF/HAN
Group size: 12 per sex

Test substance:	6-Hydroxyindole
Batch:	CFQ10790 (radio-labelled 6-hydroxyindole, 99.5% radiochemical pure, received 10/8/98, stored -20°C, protected from light, humidity and under nitrogen gas) 0500177 (unlabelled 6-hydroxyindole, 97.8% pure received 11/3/98, stored 4°C, protected from light and under argon, expiry date Dec 1998));
Dose:	25 mg/kg bw/day, single application
Vehicle:	suspension in 0.5% aqueous carboxymethylcellulose, 5 ml/kg
GLP:	in compliance

The application site (dorsum area) was clipped free of hair 24 hours before application, and test formulations were applied under a non-occlusive dressing left in place for 30 minutes. The dressing was then removed and the application site was rinsed-off with water. Blood samples were collected from the orbital sinus of designated animals under light ether anaesthesia at the following time-points: 1, 2, 4, 6, 8, 24, 48, and 72 hours after dressing removal (3 rats/sex/time-point, each rat bled thrice). Urine, faeces and cage washes were collected from designated animals at the following times: during a 24-hour pre-dose period (urine and faeces), and then during the periods 0-6h, 6-24h (urine) or 0-24h (faeces), 24-48h, 48-72h, 72-96h, 96-120h, 120-144h and 144-168h (urine and faeces) after dosing. After each collection of faeces, the cages were carefully rinsed with approximately 20 ml of water (200 ml at 168h). All samples were stored frozen at -20°C until analysis. Thereafter, animals were killed and a wide range of tissues/organs were sampled for tissue distribution analysis.
For each sample, total radioactivity was evaluated by liquid scintillation counting. The minimal (default) fraction of topical dose absorbed was derived from excretion data.

Results
Total radioactivity levels in plasma reached a maximum 1 or 2 hours after application (C_{max} males 233 ± 167, females 396± 50, mean both sexes 282 ± 137 ng-eq/ml). Then, plasma radioactivity levels decreased, biphasically. By 4 h post-application, C_{max} was 33%, with a slower decline until the last quantifiable time point, 24 hours post-application (males 30.1 ± 8.6, females 39.4 ± 20.2, mean both sexes 35.7 ± 15.8 ng-eq/ml, quantification limit <21.0 ng-eq/ml). At 6h, the males showed a second minor peak. There was moderate inter-animal variation.
There were minor gender-related differences in pharmacokinetic/excretion profiles and parameters, and mean systemic exposure [$AUC_{(0-\infty)}$] was 2809 ng-eq.h/ml (both sexes).
The mean total Cumulative Excretion (CE) of urine, faeces and cage wash over the 168-h period was 4.6 % ± 0.78 (males), 6.77 % ± 0.67 (females) and 5.6 % ± 0.16 (both sexes combined) of the radioactive dose. Radioactivity was excreted equally in urine and faeces with slow elimination over 96 h (males 82%, females 74%, mean both sexes 77%). Most of the administered radioactive dose was recovered in the dressing and in the skin at the application site (mean 68% and 11% of the dose administered, respectively). The maximal fraction of the dose absorbed was 16 % males, 19 % females and 18% both sexes; the minimal fraction was 5% males, 7% females and 6% both sexes. This was estimated by the sum of urine, faeces and carcass together with skin stripping, organs and tissue. Tissue levels of radioactivity were virtually non-quantifiable.

Conclusion
After a single dermal application of [^{14}C]-6-hydroxyindole at 25 mg/kg to rats, plasma total radioactivity levels were maximal 1 or 2 hours after dosing (C_{max} of 282 ng-eq/ml in mean), and mean systemic exposure [$AUC_{(0-\infty)}$] was 2809 ng-eq.h/ml. Minor gender differences in pharmacokinetic parameters and excretion profiles were observed. Most of the radioactive dose

was recovered in dressings and skin at the application site, and virtually no radioactivity was found in the tissues/organs analysed.

Ref.: 29

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

Dermal Phototoxicity and Photoallergenicity, study 1

Guideline:	/	
Species/strain:	Guinea pig, Dunkin Hartley	
Groups:	Group 1, Control irradiated	5
	Group 2, test substance	5
	Group 3, test substance, irradiated	10
	Group 4, vehicle control, irradiated	5
Test substance:	6-Hydroxyindole	
Batch:	Op.38	
Purity:	97.5%	
Dose:	Days 1, 2, 3, 4: 5% (w/w)	
	Days 7, 8: 0.1% (w/w)	
Vehicle:	paraffin oil, 0.2 ml	
GLP:	in compliance	

Test System
The 5% concentration was determined in a preliminary test to be the maximal non-irritant concentration after single application.
The study consisted of three different phases:

a) for phototoxicity, single dermal application of 6-hydroxyindole with ultra-violet (UV) irradiation to assess phototoxic potential, Day 1. Reactions recorded at 1, 6 and 24;
b) induction phase consisting of 6 dermal applications of 6-hydroxyindole with UV irradiation over 8 days,
c) challenge dermal application of 6-hydroxyindole with UV irradiation to assess photoallergenic potential.

Irridation was with 'Toxicotronic' UV lamp, 312/365 nm. Irridation was 2 stage, UVB (312 nm) followed by UVA (365 nm).

Phase a: The phototoxic potential was determined after single cutaneous application 0.2 mL 6-hydroxyindole solution in paraffin oil at 0% (group 4) or 5% (groups 2 and 3) on Day 1 to of the anterior scapula (9 cm^2). Irradiation (UVB, 0.1joules/cm^2 followed by UVA 9 joules/cm^2 at a sub-erythemal dose) of the application site (anterior scapular region) was performed 30 minutes after application of the test substance (groups 1, 3 and 4). Cutaneous reactions were evaluated at 1, 6 and 24 h.

Phase b: Continued application as in Phase a for Days 2, 3, 4. Due to the reaction observed, dose applied reduced to 0.1% Days 7, 8.

Phase c: After a rest period of 20 days (day 29), a 0 or 5% challenge dermal applicationwas made on the distal part of the back. The designated groups were then irradiated after 30 minutes of application at sub-erythemal doses of UVA (right flank) or UVB (left flank), and cutaneous reactions were evaluated 1, 6, 24 and 48 hours after challenge. Animals were then killed; skin samples were taken from posterior treatment sites, but no microscopic examination was performed.

Results
After the dermal application on Day 1, very slight cutaneous reactions were observed in most animals. 6-hydroxyindole was therefore considered to have no phototoxic potential.
A weak to severe erythema with oedema was noted in all Group 3 animals on days 5, 8 and 9, and in Group 2 animals on day 5. These reactions were due to a poor local tolerance to 6-hydroxyindole following repeated dermal application After the challenge application, a well-defined to severe erythema was observed in Group 3 (5/10 animals treated and then irradiated) at the 48 h, whereas no such reactions were observed in any other group.

Conclusion
Under the conditions of the study and based on the severity of the cutaneous reactions observed, the repeated topical application of 6-hydroxyindole in 5% in paraffin oil was considered to induce photo-sensitisation reactions in 50% of the test animals.

Ref.: 30

Dermal Phototoxicity and Photoallergenicity, study 2

Guideline:	/	
Species/strain:	Guinea pig, Dunkin Hartley	
Groups:	Group 1, Control irradiated	5
	Group 2, test substance	5
	Group 3, test substance, irradiated	10
	Group 4, vehicle control, irradiated	5
Test substance:	6-Hydroxyindole	
Batch:	Op.38	
Purity:	97.5%	
Dose levels:	Days 1, 2, 3, 4, 6, 7, 8: 2% (w/w)	
Vehicle:	paraffin oil, 0.2 ml, 0.1ml in controls	
GLP:	in compliance	

Irradiation was with ‘Toxicotronic’ UV lamp, 312/365 nm. Iridation was 2 stage, UVB (312 nm) followed by UVA (365 nm).

The study consisted of three different phases:

a) for phototoxicity, single dermal application of 6-hydroxyindole with ultra-violet (UV) irradiation to assess phototoxic potential, Day 1. Reactions recorded at 1, 6 and 24;
b) induction phase consisting of 6 dermal applications of 6-hydroxyindole with UV irradiation over 8 days,
c) challenge dermal application of 6-hydroxyindole with ultra-violet (UV) irradiation to assess photoallergenic potential.

Phase a: The phototoxic potential was determined after single cutaneous application 0.2 mL 6-hydroxyindole solution in paraffin oil at 0% (group 4) or 5% (groups 2 and 3) on a Day 1 to 9 cm^2 of the anterior scapula,. Irradiation (UVB followed by UVA at a sub-erythemal dose) of the application site (anterior scapular region) was performed 30 minutes after application of the test substance (groups 1, 3 and 4). Cutaneous reactions were evaluated at 1, 6 and 24 h.
Phase b: Continued application as in Phase a for Days 2, 3, 4. Due to the reaction observed, dose applied reduced to 0.1% Days 7, 8.
Phase c: After a rest period of 20 days (day 29), a 0 or 2% challenge dermal application was made on the distal part of the back. The designated groups were then irradiated after 30 minutes of application at sub-erythemal doses of UVA (right flank) or UVB (left flank), and cutaneous reactions were evaluated 1, 6, 24 and 48 hours after challenge. Animals were then killed; skin samples were taken from posterior treatment sites, but no microscopic examination was performed.

Results
After the dermal application on Day 1, very slight cutaneous reactions were observed in most animals. 6-hydroxyindole was considered to have no phototoxic potential.
A weak to severe erythema with oedema was noted in all Group 2and 3 animals on Days 8 and 9. These reactions were due to a poor local tolerance to 6-hydroxyindole following repeated dermal application.
After the challenge application, no distinct cutaneous reactions were observed in irradiated controls (Group 1) and in vehicle-treated and irradiated controls (Group 4). In Group 2 (treated, non-irradiated animals), most of the animals showed weak to well-defined erythema at some observation times. In Group 3 (treated and irradiated animals), weak to severe erythema was observed in over half the animals.

Conclusion
Under the conditions of the present study, based on the cutaneous reactions observed, it was concluded that the repeated dermal application of 6-hydroxyindole at 2% in paraffin oil was not phototoxic or cause photo-sensitisation.
However, if there was no phototoxicity of 6-hydroxyindole at this dose level, cutaneous reactions due either to sensitisation or poor local tolerance to repeated dermal applications may have masked photo-sensitisation reactions.

Ref.: 31

Dermal Phototoxicity and Photoallergenicity, study 3

Guideline:	/	
Species/strain:	Guinea pig, Dunkin Hartley	
Groups:	Group 1, Control irradiated	5
	Group 2, test substance	5
	Group 3, test substance, irradiated	10
	Group 4, vehicle control, irradiated	5
Test substance:	Imexine OBA	
Batch:	0500177	
Purity:	not reported (in Submission III, 97.8%)	
Dose levels:	Days 1, 2, 3, 4, 6, 7, 8: 2% (w/w)	
Vehicle:	30% aqueous PEG 300 0.2 ml, 0.1ml in controls	
GLP:	in compliance	

Irradiation was with 'Toxicotronic' UV lamp, 312/365 nm. Irradiation was 2 stage, UVB (312 nm) followed by UVA (365 nm).

The study consisted of three different phases:

a) for phototoxicity, single dermal application of 6-hydroxyindole with ultra-violet (UV) irradiation to assess phototoxic potential, Day 1. Reactions recorded at 1, 4 and 24 h;
b) induction phase consisting of 6 dermal applications of 6-hydroxyindole with UV irradiation over 8 days;
c) challenge dermal application of 6-hydroxyindole with ultra-violet (UV) irradiation to assess photoallergenic potential. Reactions recorded at 1, 4, 24 and 48h

Phase a: The phototoxic potential was determined after single cutaneous application 0.2 mL 6-hydroxyindole solution in paraffin oil at 0% (group 4) or 5% (groups 2 and 3) on a Day 1 to 9 cm^2 of the anterior scapula,. Irradiation (UVB followed by UVA at a sub-erythemal dose) of the application site (anterior scapular region) was performed 30 minutes after application of the test substance (groups 1, 3 and 4). Cutaneous reactions were evaluated at 1, 6 and 24 h.
Phase b: Continued application as in Phase a for Days 2, 3, 4. Due to the reaction observed, dose applied reduced to 0.1% Days 7, 8.
Phase c: After a rest period of 20 days (day 29), a 0 or 2% challenge dermal application was made on the distal part of the back. The designated groups were then irradiated after 30 minutes of application at sub-erythemal doses of UVA (right flank) or UVB (left flank), and cutaneous reactions were evaluated 1, 6, 24 and 48 hours after challenge. Animals were then killed. Skin samples were taken from posterior treatment sites, but no microscopic examination was performed.

Results
After the dermal application on Day 1, very slight cutaneous reactions (erythema grade 0.5-1) were observed in most animals. 6-hydroxyindole was considered to have no phototoxic potential. No cutaneous reactions due the photoirritant effect of the test substance were observed.

A weak to defined erythema (grade 1-2) with dryness was noted in all Group 2and 3 animals on Days 8 and 9.

After the challenge application, no distinct cutaneous reactions were observed in Group 1 (irradiated controls) and Group 4 (vehicle-treated and irradiated controls). In Groups 2 (treated, non-irradiated animals) and 3 (treated and irradiated animals), a persistent weak to well-defined erythema was noted in only 1/5 and 1/10 animals, respectively.
After the challenge application, no distinct cutaneous reactions (questionable or weak erythema, grade 0.5 or 1) were observed in irradiated controls (Group 1) and in vehicle-treated and irradiated controls (Group 4). In Group 2 (treated, non-irradiated animals), a weak erythema (grade 1) was noted in one animal at 1 h and 4 h, with another animal showing erythema (grade 1 or 2) was recorded at the 24 and 48 h readings.
In Group 3 In the treated group 3 (treated and irradiated), a questionable or weak erythema (grade 0.5 or 1) was noted in 7/10 animals and a weak or well-defined erythema (grade 1 or 2) was observed in 1/10 animals at the 1 and 4 h readings. A weak erythema (grade 1) persisted in 2/10 animals at the 24 and 48 h readings.

Conclusion

Under the conditions of the present study, and based on the severity of the cutaneous reactions observed, the study authors concluded that the repeated dermal application of 6-hydroxyindole at 5% in PEG 300 was not phototoxic or photo-sensitising.

Ref.: 32

3.3.10.2.	Phototoxicity / photomutagenicity / photoclastogenicity

No data

3.3.11.	Human data

No data

3.3.12.	Special investigations

No data

3.3.13.	Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY
6-Hydroxyindole)
(Oxidative/permanent)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**8.09 µg/cm^2**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**5.663 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.094 mg/kg**
No observed adverse effect level (mg/kg) (rat, oral, prenatal developmental)	**NOAEL**	**=**	**60 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**638**

3.3.14. Discussion

Physico-chemical specifications
6-Hydroxyindole is used as a hair colorant requiring the presence of hydrogen peroxide as an oxidant. It is 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.

General Toxicity
The oral LD50 was found to be higher than 600 mg/kg bw and the dermal LD50 to be higher than 2000 mg/kg bw. The NOAEL was set at 100 mg/kg bw/day (rat, 13 week, oral). For maternal toxicity, the NOEL was considered to be 240 mg/kg/day and 60 mg/kg bw/day for embryo-foetal toxicity.

Phototoxicity
6-Hydroxyindole in 5% in paraffin oil was considered to induce photo-sensitisation reactions in 50% of the test animals. In 5% aqueous solution and 2% in paraffin oil was not phototoxic or photo-sensitising.

Irritation, sensitisation
The substance was slightly irritating to rabbit skin and slightly to severe irritating to the rabbit eye under the test conditions.
The substance showed to be a skin sensitiser in Guinea pig and mouse. The LLNA shows that it is a strong sensitiser when tested at concentrations higher than 0.1%.

Percutaneous absorption
The dermal absorption was estimated to be 3.03 ± 1.43 % (6.88 ± 3.11 µg/cm² of the applied dose) under use conditions.

Mutagenicity
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*.
The *in vivo* micronucleus assay in bone marrow of rats gave negative results. There is no clear evidence that the bone marrow was effected by the test agent, since the toxic dose was not used.
However, a pharmacokinetic and excretion balance study demonstrated a high systemic exposure after oral administration of 6-hydroxyindole to rats.

Carcinogenicity
No induction of treatment related tumours was found in rats in a two-year gavage study with doses up to 100 mg/kg/d.

4. CONCLUSION

The SCCP is of the opinion that the use of 6-hydroxyindole itself as an oxidative hair dye at a maximum concentration of 0.5 % in the finished cosmetic product (after mixing with hydrogen peroxide) does not pose a risk to the health of the consumer, apart from its sensitising potential.

Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

6. REFERENCES

References in italics are not submitted as full reports in the present dossier. They consist of reports for studies considered to be inadequate (34), reports for range finding toxicity studies (40, 41) or publications (35-39). They can be provided upon request.

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7. ACKNOWLEDGEMENTS

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