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

Dihydroxyindole

COLIPA N° A111

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

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1. BACKGROUND

Submission I on Dihydroxyindole was submitted by COLIPA (European Cosmetics Toiletry and Perfumery Association) in May 1997 and Submission II in July 2001. On 18 March 2003, the Scientific Committee on Cosmetic Products and Non-food Products intended for Consumers (SCCNFP) adopted the opinion on the above mentioned substance (SCCNFP/0657/03, final).

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 Dihydroxyindole safe for use in hair dye formulations taken into account the data provided?*

2. *Does the Scientific Committee on Consumer Products (SCCP) recommend any restrictions with regard to the use of Dihydroxyindole 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

Dihydroxyindole (INCI name)

3.1.1.2.	Chemical names

5,6-dihydroxyindole
1H-indole-5,6-diol (CAS)
Indole-5,6-diol

Synonyms
Dopamine lutine

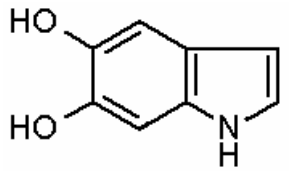
3.1.1.3.	Trade names and abbreviations

IMEXINE® OAY (Chimex)
COLIPA n° A111
Substance code: P39

3.1.1.4. CAS / EINECS number

CAS : 3131-52-0
EINECS : /

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula : $C_8H_7NO_2$

3.1.2. Physical form

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

3.1.3. Molecular weight

Molecular weight: 149.15

3.1.4. Purity, composition and substance codes

Substance codes: P 39, 54422
Purity: 96-100% (titre by potentiometry) *
Water content: ≤ 0.4 %
Ash content: < 0.1% w/w

* Batch numbers: OP 1E (96% pure, 1988), 7X (98.3% pure, 1989-1990), OP 3X (99% pure) 1991-1995). 0509230 (98.7% pure, 2004).
Radiolabelled dihydroxyindole: Batch 92170 of (98% radiochemical pure), Batch "CFQ13624-batch 1" (98.3% radiochemical pure)

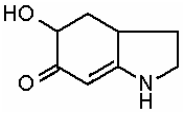
3.1.5. Impurities / accompanying contaminants

Heavy metals: < 20 ppm

Solvent residues
Hexane: < 0.01% (in batch op 3X)
Isopropyl ether: < 0.01% (in batch op 3X)
Ethyl acetate: 0.06% (in batch 0509230), < 0.01% (in batch op 3X)
Dichloroethane: 0.25% (in batch op 3X)
Cyclohexane: 1% (in batch 0509230)
Toluene: 0.05% (in batch 0509230)

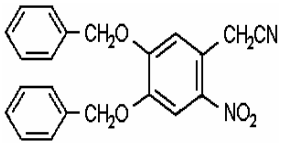
N,N-Dimethylformamide: <0.05% (in batch 0509230)
Chloride ions: < 0.4 %

Intermediates and oxidation products



4-Hydroxy-3-oxo-9-aza-bicyclo-(4,2,0)-nonene-1 (Impurity A): < 0.56 %

(5-Hydroxy-1,2,3,3α,4,5-hexahydro-6H-indol-6-one)



4,5-Dibenzyloxy-2-nitrophenylacetonitrile (Impurity B}: < 0.02 %

	Op.3X	**0509230**	**Op.7X**	**Op.1E**
Appearance	A light grey powder			
IR spectrum	In accordance with the proposed structure			
UV spectrum	Comparable			
^{1}H and ^{13}C NMR spectra	In accordance with the proposed structure			
Mass spectrum	Consistent with the proposed structure			
Titre (g/100 g) Potentiometry	99.0	98.7	98.3	96.0
Impurities (g/100 g) – HPLC)				
- **Impurity A**	0.2	0.56	0.03	< 0.2
- **Impurity B**	< 0.02 ND	< 0.1		
Residual solvents (g/100 g – G.C)				
- **Dichloroethane**	0.25		0.62	0.28
- **Hexane**	< 0.01 ND		0.05	< 0.01 ND
- **Ethyl acetate**	< 0.01 ND	0.06	ND	< 0.01 ND
- **Isopropyl ether**	< 0.01 ND		ND	< 0.01 ND
- **Toluene**		0.05		
- **Cyclohexane**		1		
- **N,N'-Dimethylformamide**		0.05		

ND: Not Detected

3.1.6. Solubility

Solubility in:

- water at 20 °C: 12.3%
- 96% ethanol: 10%
- chloroform: 0.1%

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow}: 0.58 (calculated)

3.1.8.	Additional physical and chemical specifications

Organoleptic properties

Melting point:	134 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	1.28 g/ml
Viscosity:	/
pKa:	/
Refractive index:	/

3.1.9.	Stability

0.5 – 500 mg/ml solutions in acetone/olive oil were stable up to 4 hour study period (maximum deviation from initial concentration ±10%) at room temperature, when stored protected from light and under inert atmosphere.

General comments on analytical and physico-chemical characterisation

The following properties do not or poorly comply with the basic requirements for proper characterisation:

- the stability of the compound in marketed products is not provided.
- No characterisation of the reaction products in the presence of oxygen.
- Log $P_{ow,}$: calculated values can not be accepted as estimates of the true physical constants without justification, indicating that the reported values are realistic.
- No EINECS or ELINCS number reported.

3.2. Function and uses

Dihydroxyindole is used as a hair colorant without the need for an additional oxidant such as hydrogen peroxide. It forms coloured polymers in the presence of atmospheric oxygen.

Dihydroxyindole may be used in hair colouring products at on-head concentrations up to 0.5%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCNFP/0657/03

Guideline:	OECD 401
Species/strain:	Rat, Sprague-Dawley
Group size:	5 male + 5 female
Test substance:	Imexine OAY in 0.5% aqueous carboxymethyl cellulose
Batch:	Op.7X
Purity:	not stated in study report
Dose:	300, 378, 476 and 600 mg/kg bw in a volume of 10 ml/kg
Observation:	14 days
GLP:	in compliance

Dose groups were selected on the basis of a preliminary range-finding study which indicated an oral LD50 between 400 and 500 mg/kg bw. Groups of 5 male and 5 female rats were fasted overnight before receiving a single dose of test substance by gastric gavage. The animals were observed 1 and 4 hours after dosing and then daily for 14 days. Bodyweights were recorded on days 0, 7 and 14, or at death. Macroscopic abnormalities were recorded at autopsy. No histological examinations were performed.

Results
No deaths occurred at 300 or 378 mg/kg bw. The two highest dose groups showed dose-related mortality (1M + 2F at 476 mg/kg bw; 3M + 4F at 600 mg/kg bw). Probit analysis indicated LD50 values (95% confidence limits) of 593 (451-701) mg/kg bw in male rats and 535 (420 - 682) mg/kg bw in female rats.
Deaths occurred within 5 days of dosing. Surviving animals appeared to show a dose-related decrease in weight gain, although statistical analysis was not presented in the study report. Clinical signs in surviving animals were hunched posture and piloerection in all animals, lethargy at 378 mg/kg bw and higher doses, and ataxia and ptosis at 600 mg/kg bw. No abnormal observations were reported at 5 days or more after dosing. At autopsy, moribund animals exhibited red or haemorrhagic lungs and discoloration of the liver, kidneys or gastric epithelium, and gaseous distension of the stomach and small and large intestines. No abnormalities were observed at autopsy of rats dosed with 300 and 378 mg/kg bw. Rats dosed with 476 and 600 mg/kg bw had dark coloration of the kidneys.

Ref.: 1

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402
Species/strain:	Rat, Sprague-Dawley
Group size:	5 males + 5 females
Test substance:	Imexine OAY
Batch:	Op.7X
Purity:	98.3%

Dose: 2000 mg/kg bw
Observation: 14 days
GLP: in compliance

Rats (5/sex) were given a single 24-hour semi-occlusive dermal application of dihydroxyindole at 2000 mg/kg in its original form. Rats were observed for clinical signs for 14 days and their bodyweight was recorded on days 1 (day of application), 8 and 15. All animals were sacrificed at the end of the observation period and subjected to macroscopic examination.
Results
There were no deaths, no systemic clinical signs or local signs of irritation. Black colouration of the application site was observed in all animals. Under the conditions of this study (limit test), the maximal non-lethal dose of dihydroxyindole following single dermal application to rats was higher than 2000 mg/kg.

Ref.: 2

3.3.1.3. Acute intravenous toxicity

Guideline: OECD 401
Species/strain: Rat, Sprague-Dawley
Group size: 5 males + 5 females
Test substance: Imexine OAY in saline
Batch: Op.3X
Purity: 99 %
Dose: 10 mg/kg bw, i.v.
Observation: 14 days
GLP: in compliance

A single group of five (5) Sprague-Dawley rats/sex was used in this study. Rats (5/sex) were given a single bolus intravenous injection (caudal vein) of the test substance at 10 mg/kg in 0.9% NaCl (5 ml/kg). Rats were observed for clinical signs for 14 days and their bodyweight was recorded on days 1 (day of injection), 5, 8 and 15. All animals were sacrificed at the end of the observation period and subjected to macroscopic examination.

Results
There were no deaths. No systemic clinical signs or gross changes were observed. Under the conditions of this study, the maximal non-lethal dose of dihydroxyindole following single intravenous injection to rats was higher than 10 mg/kg.

Ref.: 3

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Irritancy of 6% solution, *taken from SCCNFP/0657/03*

Guideline: OECD 404
Species/strain: Hybrid New Zealand albino rabbit
Group size: 6 male
Test substance: 5,6-hydroxyindole: 6% in glycerol

Batch: Op.1E
Purity: not stated in study report
Dose: 0.5 ml
GLP: in compliance

Animal treatment was consistent with OECD guideline 404, except that the substance was applied to both intact and abraded skin for 24 hours.
0.5 ml of the test substance was applied to approximately 6.2 cm² intact and abraded (by means 3 incisions in the epidermis) skin of 6 male rabbits. Occlusive patches were applied and left in place for 24 hours. The skin was examined for erythema, eschar formation and oedema at 30 min and 48 hours after removal of the patches, i.e. 24 and 72 hours after commencement of treatment. An index of Cutaneous Primary Irritation was calculated from the mean scores at intact and abraded sites and each examination time.

Results
Slight erythema was observed at the intact application site for one rabbit after 24 hours. No other reactions were reported for intact skin. Mild to moderate reactions were observed in all rabbits at the abraded application sites after 24 hours, most of which had recovered by 72 hours.
The primary irritation score was 0.88 out of a maximum score of 8. According to the defined criteria, the test substance was reported to be slightly irritant to rabbit skin.

Ref.: 4

Irritancy of undiluted substance

Guideline: OECD 404 (1981)
Species/strain: New Zealand albino rabbit
Group size: 1 male + 2 female
Test substance: IMEXINE OAY
Batch: Op.7X
Purity: not stated in study report
Dose: 0.5 g of pure test substance moistened with 0.5 ml distilled water
GLP: in compliance

0.5 g of moistened test substance was applied to 6.25 cm² of intact skin of 1 male and 2 female rabbits. Semi-occlusive patches were applied and left in place for 4 hours. Remaining test substance was removed by swabbing with cotton wool swabs soaked in diethyl ether. The skin was examined for erythema, eschar formation and oedema at 1, 24, 48 and 72 hours after removal of the patches. A Primary Irritation Index was calculated from the mean scores.

Results
Very slight erythema was observed on one of the two female rabbits after 1 hour and on the male rabbit after 24 and 48 hours.
No other reactions were reported. Black staining due to the test substance was reported to be present throughout the study, but no comment was made on whether this interfered with the observations.
The primary irritation index was 0.2 out of a maximum score of 8. According to the defined criteria, the test substance was reported to be a mild irritant to rabbit skin.

Ref.: 5

3.3.2.2. Mucous membrane irritation

Taken from SCCNFP/0657/03

Guideline: OECD 405
Species/strain: Hybrid New Zealand albino rabbit
Group size: 6 male
Test substance: 5,6-hydroxyindole: 6% in glycerol
Batch: Op.1E
Purity: not stated in study report
Dose: 0.1 ml
GLP: in compliance

0.1 ml of the test substance was applied once to the right eye of 6 male rabbits, without rinsing. The left eye served as control. Ocular reactions were recorded at one hour and 1, 2, 3, 4, 7, days after instillation, and the Mean Ocular Irritation Score and Maximum Score of Ocular Irritation were calculated.

Results
Reactions were reported in the conjunctiva of all rabbits and the iris of 2 rabbits 1 hour after instillation. Observations on the iris returned to normal after 2 days and on the conjunctiva by 7 days. No corneal reactions were reported. According to the defined criteria, the test substance was reported to be slightly irritant to the rabbit eye.

Ref.: 6

Guideline: OECD 405 (1987)
Species/strain: White New Zealand rabbit
Group size: 1
Test substance: Dihydroxyindole
Batch: Op.7X
Purity: 98.3%
Dose: 60 mg (corresponding to approximate volume of 0.1 ml)
GLP: in compliance

A 60 mg sample of dihydroxyindole in its original form (corresponding to an approximate volume of 0.1 ml) was instilled in the conjunctival sac of the right eye of the first test animal. The ocular reactions were assessed 1, 24 and 48 hours after instillation. Because of the severe reactions observed at 48 hours and on the basis of obvious ethical reasons of animal welfare, the animal was humanely killed and no additional animals were treated.

Results
Translucent corneal opacity at the 24-hour observation turned totally opaque at 48 hours and covered most of the corneal surface. Sloughing of the cornea, iridial inflammation as well as signs indicative of moderate to severe conjunctival irritation (redness, chemosis) were also noted at these observation times. For human reasons, this animal was sacrificed following the 48 – hour observation and the study was then terminated.

Conclusion

Dihydroxyindole is severely irritant to rabbit eye when tested undiluted.

Ref.: 7

3.3.3.	Skin sensitisation

Magnusson and Kligman study (*Taken from SCCNFP/0657/03*)

Guideline:	OECD 406
Species/strain:	Dunkin-Hartley albino guinea pig
Group size:	20 treated + 10 control females
Test substance:	IMEXINE OAY
Batch:	Op.7X
Purity:	not stated in study report
Concentrations:	intradermal induction: 0.1 ml 50% FCA 0.1 ml 0.1% test substance in distilled water 0.1 ml 0.1% test substance/FCA topical induction: 0.2-0.3 ml 50% test substance in distilled water challenge: 0.1-0.2 ml 10% test substance for 24 hours
GLP:	in compliance

Induction commenced with three intradermal injections, of FCA, test substance, and a mixture of these two. One week later the induction process was completed with a single topical application of the test substance (50%) under occlusive patch to the shoulder region for 48 hours. An interval of 2 weeks was allowed after induction and then the animals were challenged by a single topical application of the test substance (10%) under occlusive patch on the right flank for 24 hours. Appropriate controls were treated with vehicle at all stages and the test substance-induced animals received vehicle alone on the left flank. The skin was examined 24 and 48 hours after removal of the challenge patches.

Results
A slight brown staining was observed due to the test substance. This was reported to preclude accurate assessment of erythema after the induction, but not after the challenge application. No adverse reaction was observed in any of the 20 treated Guinea pigs. The test substance was not shown to be a sensitiser to Guinea pig skin under the test conditions.

Ref.: 8

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species:	CBA/J mice
Group size:	Fifty-six (56) females, separated in two independent experiments conducted on 7 groups of 4 animals each
Substance:	dihydroxyindole
Batch:	0509230
Purity:	98%
GLP:	in compliance

The 7 groups used in a first experiment consisted of:

- 5 treated groups receiving dihydroxyindole at the concentrations of 0.5, 1, 2.5, 5 and 10% (w/v) dihydroxyindole in a mixture of acetone:olive oil (4:1, v/v). This vehicle was selected in a previous solubility study [32], and 10% (w/v) was determined in a preliminary test to be the highest practicable concentration whilst avoiding excessive local skin irritation;
- A negative control group receiving the vehicle acetone:olive oil (4:1, v/v) alone;
- A positive control group receiving alpha-hexylcinnamaldehyde at 25% (v/v) in acetone:olive oil (4:1, v/v).

As positive results were obtained in the first experiment, a second experiment was performed similar to the above but using concentrations of 0.05, 0.1, 0.25, 0.5, and 1%. These concentrations were selected on the basis of the results of the first experiment to determine the EC_3 value of dihydroxyindole.

In each experiment, dihydroxyindole in acetone:olive oil (4:1, v/v) or alpha-hexylcinnamaldehyde was applied over the ears (25 µL per ear) for three consecutive days (designated as days 1, 2 and 3). After 2 days of resting, the proliferation of lymphocytes in the lymph nodes draining the application sites was measured by incorporation of tritiated methyl thymidine (^{3}H-TdR, day 6). The values obtained were used to calculate stimulation indices (SI), and the EC_3 was estimated (concentration resulting in a SI of 3). The irritant potential of the test item was assessed in parallel by measurement of ear thickness on days 1, 2, 3 and 6.

Results
In the first experiment performed at 0.5, 1, 2.5, 5 and 10%, SI values were above the threshold positive value of 3 at all tested concentrations (SI ranging from 5.9 at 0.5% to 45.6 at 10%).

In the second experiment performed at 0.05, 0.1, 0.25, 0.5, and 1%, SI values were above the threshold positive value of 3 at the three higher concentrations, in the absence of local irritation. The EC_3 value for dihydroxyindole was calculated to be 0.17%.

Conclusion
Dihydroxyindole induced delayed contact hypersensitivity in the murine Local Lymph Node Assay. The EC_3 value estimated in this study was 0.17% indicating that dihydroxyindole is a strong sensitiser.

Ref.: 9

3.3.4. Dermal / percutaneous absorption

Guideline:	/
Species/strain:	human abdominal skin from female donors
Group size:	4 samples from female donors
Test substance:	^{14}C-dihydroxyindole
Batch:	CFQ13624 Batch 1
Purity:	98.3% radiochemical pure
GLP:	in compliance

Skin samples were kept frozen at about –18 ºC until their use. Skin samples were dermatomed (approximately 400µm in thickness) and mounted in diffusion cells, using Phosphate Buffered

Saline containing 0.01% (w/v) sodium azide as a receptor of fluid. Their integrity was checked before application of the formulation by measuring the permeation coefficient for tritiated water (Kp < 2.5 x 10^{-3} cm/h for all selected samples).eight skin samples were used (two replicates per donor) ,and skin was maintained at 32ºC.
About twenty (20) mg/cm² of a typical hair dye formulation at a target concentration of 0.5% ^{14}C-dihydroxyindole (actual concentration of 0.41%, corresponding to 80.7µg/cm³ of dihydroxyindole) was applied to the skin surface and left for 30 minutes. After this time period, the remaining formulation on the skin surface was removed using a standardized washing procedure. Twenty four hours after application, the percutaneous absorption of dihydroxyindole was estimated by measuring its concentration by liquid scintillation counting in the following compartments: skin excess, stratum corneum (isolated by tape strippings), epidermis + dermis and receptor fluid.

Results
Seven out of eight (7/8) skin samples yielded data that could be analysed. Most of the dihydroxyindole applied on the skin surface was removed with the washing procedure (96.4% of the applied dose, in mean), and the mean recovery rate was 98.6%. The mean absorbed amounts of dihydroxyindole were estimated as follows (sum amounts measured in epidermis, dermis and receptor fluid): 0.66 ± 0.24 µg-eq/cm² (0.83 ± 0.30% of the applied dose)

Conclusion
The dermal absorption of dihydroxyindole contained at 0.41% in a hair dye formulation was estimated to be 0.66 ± 0.24 µg-eq/cm² (0.5 – 1.0 µg-eq/cm²).

Comment
The study is not performed according to the SCCNFP Notes of Guidance. Only 4 skin samples of 4 donors were used. The maximum observed value of 1.0 µg/cm² is used for the calculation of the Margin of Safety.

Ref.: 22

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

13-week oral toxicity study in rats, *taken from SCCNFP/0657/03*

Guideline:	OECD 408
Species/strain:	Sprague-Dawley rat, Crl:CD (SD) BR strain
Group size:	16 male + 16 female
Test substance:	P39 in aqueous solution
Batch:	Op.3X
Purity:	99 %
Dose:	0, 3, 10, 30 and 100 mg/kg bw/day, 7 days/week
Exposure:	92 - 96 days
Recovery:	4 weeks (6 males + 6 females from each dose group)

GLP: in compliance

Groups of 16 male and 16 female rats were dosed with the test substance by gavage at 0, 3, 10, 30 and 100 mg/kg bw/day, 7 days/week for 92 to 96 days. After 13 weeks treatment 6 rats of each sex from each dose group were kept for 4 weeks without treatment in order to observe for potential recovery. The animals were observed twice daily for clinical signs and mortality, and weekly for bodyweight and food and water consumption. At weeks 4 and 8, the animals were subjected to orbital bleeding for haematology and blood biochemistry analyses, and analyses were conducted on urine collected for 18 hour (overnight). These analyses were repeated at the end of the treatment and recovery periods, and 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 full treatment period.

Results
One animal (female, 3 mg/kg dose group) was found dead in week 13. Death was ascribed to an eye infection resulting from orbital bleeding. No treatment related mortalities occurred.
Hypersalivation was noted in all males of the highest dose group during weeks 2 to 3, and in 15 of the 16 females of the highest dose group during weeks 3 to 5. Also in the 100 mg/kg bw/day group, black discoloration of the hair and tail and cage bedding was observed from week 10 until the end of the study. These observations were considered to be related to the presence of test substance or metabolites in the urine and/or faeces. Bodyweight gain and food and water consumption were similar for all dose groups.
Male rats treated with 30 and 100 mg/kg bw/day exhibited higher serum triglyceride at the weeks 4, 8 and 12 bleeds, which persisted after the recovery period. Other minor changes in haematological parameters and blood biochemistry were within the historical control range and not considered to be of toxicological significance. Overnight urine samples were dark coloured for males at 10 and 30 mg/kg bw/day and for all animals at 100 mg/kg bw/day, consistent with excretion of the dye and the reported observations on hair, tail and bedding. Colour was normal at the end of the recovery period. A dose-related increase in urinary ketones was evident in males at 10, 30 and 100 mg/kg bw/day, and to a lesser extent in females at the highest dose only. Reversibility was not complete. The authors concluded that, in the absence of other abnormalities, the ketonuria could be related to the mobilisation of free fatty acids, possibly related to the increase in plasma triglyceride.
No changes in organ weights were reported. Black discoloration of the kidneys was noted at autopsy, consistent with the microscopic observation of intracellular pigment accumulation in the cortical tubular epithelium. These changes occurred in some animals of both sex in the 30 and 100 mg/kg bw/day dose groups, and persisted at the end of the recovery period. No other histological abnormalities were reported.
On the basis of the increased triglyceride and accumulation of pigment in the kidney at 30 and 100 mg/kg bw/day, the study authors concluded that the dose level of 10 mg/kg bw/day was the NOAEL.
A significant change in urinalysis (ketones) was observed at 10 mg/kg bw/day, which could be related to the effects seen at higher doses, and therefore the NOEL is 3 mg/kg bw/day. Male rats appear to be more sensitive than females to the effects of this substance.

Ref.: 10

In the complementary dossier from 2004 the analytical interference between dihydroxyindole and/or its degradation products and the determination of urinary ketones using N-Multistix SG test strips was investigated. From the results it can be concluded that false positive reactions for

ketone bodies are obtained in the presence of hydroxyindole (see 3.3.12). In consequence the SCCP sets the NOAEL of the oral subchronic toxicity study at the dose of 10 mg/kg bw/day.

13-week subcutaneous toxicity study in rats

Guideline:	OECD 408
Species/strain:	Sprague-Dawley rat, Crl:CD (SD) BR strain
Group size:	16 males + 16 females
Test substance:	dihydroxyindole (P39) in saline
Batch:	OP3X
Purity:	99 %
Dose:	0, 1.5, 5, 15 mg/kg bw/day 3 days/week and 15 mg/kg bw/day, 7 days/week
Exposure:	13 weeks
Recovery:	4 weeks (6 males + 6 females of the control and the 15 mg/kg (7 days/week) dose group)
GLP:	in compliance

Test solutions were made daily immediately before dosing by dissolving the test substance in saline. Groups of 16 male and 16 female rats were dosed with the test substance subcutaneously at 0, 1.5, 5 and 15 mg/kg bw/day 3 days /week or 7 days/week for 13 weeks. After 13 weeks treatment 6 males + 6 females of the control and the 15 mg/kg (7 days/week) dose group were kept for 4 weeks without treatment in order to observe for potential recovery. The animals were observed twice daily for clinical signs and mortality, and weekly for bodyweight and food and water consumption. Ophthalmoscopy was conducted before the start of the study and at week 12 of the treatment period. At weeks 4, 8, 12 and at the end of the recovery period the animals were investigated for haematology and blood biochemistry analyses, and urinalyses. Full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs.

Results
No treatment related mortalities occurred.
At the injection sites erythema and scabs (except at 1.5 mg/kg bw/day), black staining, thickening and nodules were observed. Bodyweight gain was reduced at the dose 15 mg/kg bw/d. accompanied by a decrease in food and water consumption.
Haematology showed increase in fibrinogen and neutrophils and a decrease in haemoglobin associated with decrease in erythrocytes and packed cell volume (5 and 15 mg/kg bw/d). Further slight changes in thrombocytes and reticulocytes count were observed in the high dose group. Biochemistry revealed changes in cholesterol, total protein content and bilirubin (high dose group). Increases in the relative weights of spleen, kidney and liver accompanied by pigmentation were seen at the high dose and for the spleen also at 5 mg/kg bw/d (males). Substance-treated animals showed inflammation in the subcutaneous tissues and pigmentation in lymph nodes (pigmentary lymphadenopathy), spleen, liver thyroids. Kidney, adrenal cortex and ovaries, pigmentation was present even in the lowest dose group. In the recovery group the effects were maintained in some cases. No NOEL can be derived. The NOAEL was set by SCCP at 1.5 mg/kg bw/d.

Ref.: 11

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline: OECD 471
Species/strain: *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537; *E. Coli* WP2 uvrA
Test substance: Imexine OAY
Batch: Op.7X
Purity: not stated
GLP: in compliance

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

Results

In the first experiment:	- S9:	negative in all tester strains
	+ S9:	negative in all tester strains
Second experiment:	- S9:	negative in all tester strains
	+ S9:	negative in all tester strains

Conclusions
Based on the reversion rate, it is concluded that the test substance does not show evidence of mutagenic activity in two bacterial test systems in the presence or in the absence of activation.
Ref.: 12

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

Guideline: OECD 473
Species/strain: Chinese Hamster Ovary (CHO) cells
Replicates: yes
Test substance: P39
Batch: Op.3X
Purity: 99 %
Exposure time: - S9: 21 h
+ S9: 3 h
GLP: in compliance

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

Results

The test substance has been investigated for induction of chromosomal aberrations in Chinese Hamster Ovary cells. Liver S9 fraction from rats (origin not described) was used as the exogenous metabolic activation system.

Without S9: clastogenic activity was observed for the highest concentration in both experiments.

With S9: clastogenic activity was observed for 2 concentrations in the second experiment.

Conclusions

This study is adequate. The test agent displays clastogenic properties both in the presence or absence of activation systems.

Ref.: 13

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

In Vivo Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	Mouse, Albino BKW mice
Group size:	5 male + 5 female
Test substance:	Imexine OAY
Batch:	Op.7X
Purity:	not given
Dose levels:	400 mg/kg bw, single intragastric gavage
Sacrifice times:	24, 48 and 72 hours after dosing
GLP:	in compliance

The test substance has been investigated for induction of micronuclei in the bone marrow cells of Albino BKW mice. The substance was administered once by single intragastric gavage at 400 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.

Results

Mean values of micronucleated PCE.

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

PCE/NCE ratio

Groups of mice treated with Imexine OAY did not exhibit a significant variation of the PCE/NCE ratio.

Conclusions

Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice. The PCE/NCE ratio did not indicate cytotoxic effects in the bone marrow and does not provide information on systemic availability of the test substance. However, appropriate systemic exposure is suggested by the observed clinical signs in animals given Imexine OAY OP 7X. Supportive evidence is also provided by an oral toxicokinetic study in rats which showed high plasma levels of radioactivity following a single oral administration of Imexine OAY OP 7X at 50 mg/kg.

Ref.: 14

***In Vivo* Mammalian Erythrocyte Micronucleus Test**

Guideline:	not indicated but in line with OECD 474
Species/strain:	Mice, Swiss OF1
Group size:	5 males + 5 females
Test substance:	P 39
Batch:	Op.3X
Purity:	not stated in report
Dose:	20, 50 and 100 mg/kg bw; single subcutaneous administration
Sacrifice time:	24, 48 and 72 hours after the treatment
GLP:	in compliance

The test substance has been investigated for induction of micronuclei in the bone marrow cells of mice after a single subcutaneous administration. Since in a preliminary toxicity test 4/6 animals died after exposure to 150 mg/kg, 100 mg/kg was defined as the maximum tolerated dose and the top dose-level in the main study. Negative and positive controls (Cyclophosphamide, 50 mg/kg, administered subcutaneously) were in accordance with the OECD guideline.

Results
A statistically significant decrease in the PCE/NCE ratio was observed at the 24 and 72 hours sampling times in the animals treated with 100 mg/kg. At the sampling time 48 hours the PCE/NCE ratio was also slightly reduced after treatment with 100 mg/kg. This finding might indicate a slight cytotoxic effect of the test compound on the bone marrow.
The mean MNPCE frequencies were significantly increased only in the low dose group (25 mg/kg) at one preparation time point (48 hours). The micronucleus frequencies in all other groups treated with the test substance were in the range of negative controls. The isolated positive finding has no biological relevance. The positive control substance gave the expected result and indicated the sensitivity of this experimental protocol.

Conclusions
The study indicates that the test substance did not induce chromosome aberrations or damage to the mitotic apparatus in bone marrow cells of mice after subcutaneous treatment under the test conditions used. The effect on the PCE/NCE ratio might suggest that there was relevant exposure of the bone marrow. This view is indirectly supported by the oral toxicokinetic study in rats which showed high plasma levels of radioactivity following single oral administration of the test substance at 50 mg/kg.

Ref.: 15

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

Guideline: OECD 486
Species/strain: hepatocytes of male Wistar rats
Test substance: KN 17
Batch: Op.3X
Purity: 99 % (potentiometer)
Exposure time: 2-4 h and 12-14 h
GLP: in compliance

The test substance has been investigated for induction of unscheduled DNA synthesis in Wistar rats hepatocytes at 2 doses of 316.2, and 1000 mg/kg. Positive controls are in accordance with OECD guideline and UDS analysed by autoradiography. 5 animals were used per dose/time sampling.
Results
No evidence of UDS induced by the test agent was observed.

Conclusions
This study is adequate and the results negative. The test agent does not induce UDS under the *in vivo* conditions used.

Ref.: 16

3.3.7.	Carcinogenicity

No data submitted

3.3.8.	Reproductive toxicity

3.3.8.1.	Two generation reproduction toxicity

No data submitted

3.3.8.2.	Teratogenicity

Embryo-foetal developmental toxicity study by oral route in rats, t*aken from SCCNFP/0657/03*

Guideline: OECD 414
Species/strain: Sprague-Dawley rat, Crl CD (SD) BR strain
Group size: 25 females (mated)
Test substance: P39 in aqueous solution
Batch: Op.3X
Purity: 99 %
Dose: 0, 20, 100 and 500/250 mg/kg bw/day
Treatment: Days 6 to 15 of pregnancy, inclusive
GLP: in compliance

Groups of 25 female rats were dosed with the test substance by gavage on days 6 to 15 after mating. Dose levels were initially set at 0, 20, 100 and 500 mg/kg bw/day. Four of nine animals

died 1 day after administration of the first dose at 500 mg/kg bw and the top dose was then reduced to 250 mg/kg bw for the remaining period. Sixteen animals allocated to the top dose received 250 mg/kg bw throughout the dosing period.
The dams were observed daily for clinical signs and mortality, and for bodyweight and food consumption on days 2, 6, 9, 12, 15 and 20. They were sacrificed on day 20 of pregnancy, and examined for number of corpora lutea, number and distribution of live and dead foetuses, of early or late resorptions and of implantation sites, and for macroscopic observations. The foetuses were examined for bodyweight, sex and macroscopic external observations, and for skeletal and visceral abnormalities (half for each endpoint).

Results
No clinical signs were observed in control or 20 mg/kg bw/day dose groups. At 100 mg/kg bw/day, all females exhibited hypersalivation for the final 1 to 3 days of dosing. This was considered to be treatment related but of no toxicological significance. Clinical signs (piloerection, tremors, hypotonia, blackish coloured faeces, etc.) were reported in 4 of the 9 top dose group animals treated with 500 mg/kg bw for one day. No deaths occurred in the 0, 20 or 100 mg/kg bw/day dose groups. All of the 9 animals treated with 500 mg/kg bw for one day died, including the 4 animals exhibiting clinical signs: 5 on day 7, 2 on day 9, 1 on day 10, a on day 12 and 1 on day 15. One top dose animal receiving 250 mg/kg bw on all dosing days died on day 9. All deaths were attributed to treatment. No abortions were observed.
Food consumption and bodyweight gain were reduced in the surviving top dose group animals, but not at 20 or 100 mg/kg bw/day. No macroscopic abnormalities were observed in the 20 or 100 mg/kg bw/day dams. In the highest dose group, 19 animals were reported to have blackish kidneys, and 8 with blackish stomach or intestines. Of the animals that died during the course of the study, 5 suffered autolysis and the others exhibited the macroscopic changes noted above. The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses and foetal bodyweights were similar for control and treated groups. No treatment-related foetal anomalies or malformations were observed. The authors concluded that the test substance was well tolerated by the pregnant female rat at dose levels of 20 and 100 mg/kg bw/day. The dose of 250 mg/kg bw/day was considered to be toxic for the pregnant female but not embryo-toxic or teratogenic. The NOAEL was defined as 100 mg/kg bw/day for maternal toxicity and 250 mg/kg bw/day for embryo-foetal development.

Ref.: 17

3.3.8.3	Combined fertility, embryo-foetal and postnatal developmental toxicity

Combined fertility, embryo-foetal and postnatal developmental toxicity by subcutaneous route in rats

Guideline:	/
Species/strain:	Sprague-Dawley rat, Crl CD (SD) BR strain
Group size:	10 males + 10 females
Test substance:	dihydroxyindole (P39) in saline
Batch:	Op.3X
Purity:	99 %
Dose:	0, 5, and 15 mg/kg bw/day
Treatment:	15 days prior to mating, during mating period, pregnancy, lactation until day 7 post partum
GLP:	in compliance

Test solutions were made daily immediately before dosing by dissolving the test substance in saline. Animals were examined once per day for clinical signs and mortality. Body weights were measured regularly. Pregnant females were allowed to deliver their litters (F1) which were monitored for survival and growth. F0 males and females and F1 pups were sacrificed between day 8 and 10 post partum.

Local signs were observed at the injection sites (erythema, scabs, discolouration, nodules, oedema, and thickening). Body weight gain and food consumption was decreased in the 15 mg/kg group. Reproductive performance of the parent as well as survival and growth of the offspring was not affected.
The NOAEL was set at 15 mg/kg bw/d.

Ref.: 18

3.3.9. Toxicokinetics

Toxicokinetic study after single cutaneous application to rats

Guideline:	/
Species/strain:	Sprague-Dawley rat
Group size:	12 males + 12 females
Test substance:	[^{14}C] dihydroxyindole (P39) in PEG 300
Batch:	92170 ([^{14}C]-labelled) and Op.3X
Dose:	25 mg/kg bw (18.5 MBq/kg), single dermal application
Sampling time:	1.8, 2.24, 4.48, 96, 144 and 216 h
GLP:	in compliance

Test batches Op.3X (unlabelled dihydroxyindole, 99% pure) and 92170 (radiolabelled dihydroxyindole, 98% radiochemically pure) were used in the present study. The plasma profile of radioactivity and plasma levels of unchanged dihydroxyindole were determined in Sprague-Dawley rats (12/sex) given a single dermal dose (10 % of body area, clipped dorsal area) of [^{14}C]-dihydroxyindole at 25 mg/kg in PEG 300 (0.5 ml/kg bw). A dressing was kept over an 8-h period. Blood samples were collected 1.8, 2.24, 4.48, 96, 144 and 216 h following application.

In many plasma, blood and erythrocyte samples, the total radioactivity was below or near to the limit of quantification. Therefore, no AUC (Area Under Curve) could be calculated. The maximum value in plasma was 7.15 ng-eq/g at 4 h. 52.2 % (males) and 55.0 % (females) of the applied dose was found in the dressing and 0.053 % (males) and 0.10% were found in rinsing water.

Ref.: 19

Toxicokinetic study after single oral administration to rats

Guideline:	/
Species/strain:	Sprague-Dawley rat
Group size:	12 males + 12 females
Test substance:	[^{14}C] dihydroxyindole (P39) in saline
Batch:	92170 ([^{14}C]-labelled) and Op.3X

Dose: 50 mg/kg bw (11.1 MBq/kg), oral gavage
Sampling time: 20 min, 4, 24 and 144 h
GLP: in compliance

Test batches Op.3X (unlabelled dihydroxyindole, 99% pure) and 92170 (radiolabelled dihydroxyindole, 98% radiochemically pure) were used in the present study. The plasma profile of radioactivity and plasma levels of unchanged dihydroxyindole were determined in Sprague-Dawley rats (12/sex) given a single oral dose by gavage of [^{14}C]-dihydroxyindole at 50 mg/kg in saline. Blood samples were collected from the orbital sinus of animals under light ether anaesthesia.

Total radioactivity was maximum 20 min after dosing (exception erythrocytes in males). Between 20 min and 144 h radioactivity decreased from 19 to 0.022 µg-eq/g with a mean AUC of 67.5 µg-eq.h/g and mean $t_{1/2}\alpha$: 0.56 h, $t_{1/2}\beta$: 20.7 h and Vd/f: 20.6 l.kg^{-1}.

Ref.: 20

Toxicokinetic study after single intravenous administration to rats

Guideline: /
Species/strain: Sprague-Dawley rat
Group size: 12 males + 12 females
Test substance: [^{14}C] dihydroxyindole (P39) in saline
Batch: 92170 ([^{14}C]-labelled) and Op.3X
Dose: 5 mg/kg bw (11.1 MBq/kg), i.v.
Sampling time: 5 min, 1, 4, 24 and 96 h
GLP: in compliance

Test batches Op.3X (unlabelled dihydroxyindole, 99% pure) and 92170 (radiolabelled dihydroxyindole, 98% radiochemically pure) were used in the present study. The plasma profile of radioactivity and plasma levels of unchanged dihydroxyindole were determined in Sprague-Dawley rats (12/sex) given a single intravenous dose (bolus injection, caudal vein) of [^{14}C]-dihydroxyindole at 5 mg/kg in 1.5 mL/kg isotonic saline. Blood samples were collected from the orbital sinus of animals under light ether anaesthesia (or from abdominal aorta for terminal sample).

Results
Plasma total radioactivity levels were maximal 5 minutes after injection (C_{max} of 12.5 µg-eq/mL in mean), and mean systemic exposure (total AUC)] was 37.2 µg-eq.h/mL. s.
Radioactivity was found almost exclusively in plasma samples within 30 minutes of dosing, and the plasma/erythrocyte distribution ratio decreased steadily thereafter. In the 96-hour samples, approximately 90% or total radioactivity was detected in erythrocytes.

Ref.: 21

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

In vitro Analytical Interference Study

The possible analytical interference between dihydroxyindole and/or its degradation products and the determination of urinary ketones using N-Multistix SG test strips was investigated. Urine of 3 male and 3 female Sprague-Dawley rats was collected over at least a 38-h period. The test substance (dihydroxyindole, batch number 0509230, 98 % purity, HPLC) was diluted in urine of males, urine of females and water to target concentrations 0, 3, 10, 30, 100 and 300 µg/ml and tested for the presence of ketones using N-Multistix SG test strips. Positive reactions were observed in rat urine at and above 10 µg/ml and in water at and above 100 µg/l. It can be concluded that false positive reactions for ketone bodies were induced in the presence of hydroxyindole.

Ref.: 23

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**1.0 µg/cm^2**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.7 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.012 mg/kg**
No observed adverse effect level (mg/kg) (rat, 13-week, subcutaneous)	**NOAEL**	**=**	**1.5 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**125**

3.3.14. Discussion

Physico-chemical specifications
0.5 – 500 mg/ml solutions in acetone/olive oil were stable up to 4 hour study period (maximum deviation from initial concentration ±10%) at room temperature, when stored protected from light and under inert atmosphere.
No characterisation of the reaction products in the presence of oxygen.

General toxicity
A range-finding study indicated an oral LD50 between 400 and 500 mg/kg bw. The NOAEL was set at 10 mg/kg bw/day (oral subchronic study), at 1.5 mg/kg bw/day (rat, 13 week subcutaneous study), at 100 mg/kg bw/day for maternal toxicity and at 250 mg/kg bw/day for embryo-foetal developmental toxicity

Irritation / sensitisation
The test substance was slightly irritant to rabbit skin and rabbit eye. It was severely irritant to rabbit eye when tested undiluted.
It was not a sensitiser to guinea pig skin in a Magnusson Kligman study. It was, however, a strong sensitiser in an LLNA.

Dermal absorption
The dermal absorption of dihydroxyindole contained at 0.41% in a hair dye formulation was estimated to be 0.66 ± 0.24 µg-eq/cm² (0.83±0.30% of the applied dose). However, the study is not performed according to the SCCNFP Notes of Guidance. 4 skin samples of only 4 donors have been used. The maximum observed value of 1.0 µg/cm² was used for the calculation of the Margin of Safety.

Mutagenicity
The test substance did not induce mutations in a bacterial gene mutation test but revealed a clastogenic potential in an *in vitro* chromosome aberration test. Two *in vivo* micronucleus tests (one after oral administration, the other after subcutaneous administration) indicate the clastogenic potential is not expressed under appropriate *in vivo* test conditions. Furthermore, an in vivo UDS test with rat liver cells also did not provide any evidence for genotoxic effects induced by the test substance *in vivo*. It can be concluded that dihydroxyindole is not an *in vivo* somatic cell mutagen.

4. CONCLUSION

Based on the information provided, the SCCP is of the opinion that the use of dihydroxyindole itself as a hair dye at a maximum concentration of 0.50% in the finished cosmetic product does not pose a risk to the health of the consumer, apart from its sensitising potential.

5. MINORITY OPINION

Not applicable

6. REFERENCES

References in italics were not submitted as full reports in the dossier. They consist of reports for studies considered inadequate (24-26), reports for range finding toxicity studies (33, 34) or publications (27-31). They can be provided upon request.

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