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

CONCERNING

DIHYDROXYINDOLINE HBR

COLIPA n° : A147

adopted by the SCCNFP during the 23rd plenary meeting
of 18 March 2003

1. Terms of Reference

1.1 Context of the question

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

1.2 Request to the SCCNFP

The SCCNFP is requested to answer the following questions :

* Is Dihydroxyindoline safe for use in cosmetic products?

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

1.3 Statement on the toxicological evaluation

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

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

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

2. Toxicological Evaluation and Characterisation

2.1. General

2.1.1. Primary name

Dihydroxyindoline HBr (INCI name)

2.1.2. Chemical names

Chemical name : 5,6-Dihydroxy indoline x HBr
CAS name : 1H-Indole-5,6-diol,2,3-dihydro-,hydrobromide
Synonyms : KN 17; SAT 940451; Dihydroxyindolin x HBr; SAT 930385

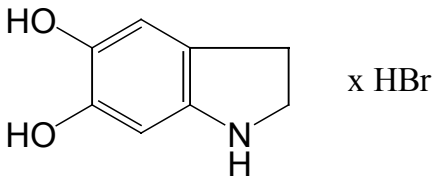
2.1.3. Trade names and abbreviations

Trade name : KN 17
COLIPA No. : A147

2.1.4. CAS n° / EINECS n°

CAS No : 29539-03-5
EINECS n° : /

2.1.5. Structural formula



x HBr

2.1.6. Empirical formula

Emp. Formula : $C_8H_9NO_2$ x HBr
Mol weight : 232.077 (as hydrobromide)

2.1.7. Purity, composition and substance codes

Purity : > 98%, titre as determined by HPLC (batch 4354/22)

2.1.8. Physical properties

Appearance	:	Brown crystals/odourless
Melting point	:	236-238 ^{O}C
Boiling point	:	No information
Density	:	No information
Rel. vap. dens.	:	No information
Vapour Press.	:	No information
Log P_{ow}	:	No information

2.1.9. Solubility

Soluble in water

General comments on analytical and physico-chemical characterisation

* Impurities in the test substance have not been characterised
* Log Pow, density and data on stability have not been provided

2.2. Function and uses

Dihydroxyindoline HBr will be incorporated in oxidative and semi-permanent hair dye formulations at a maximum concentration of 2 % without mixing with developer solutions. A maximum of 50 ml formulation will be used per application, at the most twice a month.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	OECD 401
Species/strain	:	Rat, Wistar Albino (outbred, SPF-Quality)
Group size	:	5 male + 5 female (fasted overnight prior to gavage)
Test substance	:	Dihydroxyindoline in propylene glycol
Batch no	:	4354/6 (Purity 98.7%)
Dose	:	200 and 1000 mg/kg bw in a volume of 10ml/kg bw
Observ. Period	:	14 days
GLP	:	in compliance

Dose levels were selected on the basis of a “Limit study” in which all animals dosed at 2000mg/kg bw died within the first 3 days. Groups of 5 male and 5 female rats were administered a single dose of test substance by gavage at 200 and 1000 mg/kg bw. They were observed at regular intervals immediately after dosing and thereafter twice daily for 14 days.

Body weights were recorded on days 1(pre-administration), 8 and 15 of the study and at death. Macroscopic abnormalities of main organs were recorded at autopsy. No histological examinations were performed.

Results
All females and 2/5 males died within 3 days of dosing at 1000mg/kg bw. Macroscopic abnormalities observed at autopsy included discoloration of the glandular stomach, liver and lungs. Signs of toxicity at both dose levels included lethargy, piloerection and ataxia, with all surviving animals appearing normal by day 3. Body weight and body weight gain of the surviving animals was considered normal. Macroscopic abnormalities observed at necropsy showed green coloration along the limiting ridge of the stomach in 2/3 of the surviving animals dosed at 1000mg/kg bw. No abnormalities were detected in animals dosed at 200mg/kg bw. LD50 values were estimated as 868mg/kg bw for males and 368 mg/kg bw for females.

Ref. : 1

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

14 day range finder for 90 day study

Guideline	:	/
Species/strain	:	Sprague-Dawley rat, Ico:OFA,SD (IOPs Caw) strain
Group size	:	5 males + 5 females
Test substance	:	KN 17 in aqueous solution
Batch No	:	4354/70 (purity 98.5%)
Dose levels	:	0, 15, 45 and 135 mg/kg bw/day in 5ml/kg
Treatment period	:	14 days
GLP	:	in compliance

Groups of 5 male and 5 female rats were dosed with the test substance by gavage at 0, 15, 45 and 135 mg/kg bw/day, 7 days a week for 14 days. They were observed daily for clinical signs and mortality, and weekly for body weight and food consumption. Urine was collected overnight for urinalysis and blood was sampled from the retro-orbital sinus for haematology and blood biochemistry. At the end of the treatment period a full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs.

Results
No mortalities occurred. The only clinical sign in the treated animals was coloured urine. Body weight gain and food consumption were comparable in all dose groups. There were no treatment-related changes in haematological, biochemical or urinary parameters. No changes in organ weights were reported. The only histopathological change recorded was discoloration within the kidney tubules of one male rat treated at 135 mg/kg bw/day. Discoloration of the intestinal tract

was noted in controls and treated rats. The authors suggested that dose levels up to 135 mg/kg bw, and possibly above, could be used in a 13 week study.

Ref. : 11

2.3.5. Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline	:	OECD 408
Species/strain	:	Sprague-Dawley rat, Ico:OFA,SD (IOPs Caw) strain
Group size	:	10 males + 10 females
		5 males +5 females (control + high dose recovery groups)
Test substance	:	KN 17 in aqueous solution
Batch no	:	4354/76 (purity 98.5%)
Dose levels	:	0, 20, 60 and 180 mg/kg bw/day in 5ml/kg
Treatment period	:	13 weeks +4 week reversal
GLP	:	in compliance

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 20, 60 and 180 mg/kg bw/day, 7 days a week for 13 weeks. Additionally groups of 10 animals (5 per sex) were used as satellite groups (recovery) for the high dose and control to evaluate the reversibility of symptoms during a subsequent 4 week period without treatment.

During the study, the animals were observed daily for clinical signs and mortality, and weekly for body weight and food consumption. Urine was collected overnight for urinalysis and blood was sampled from the retro-orbital sinus for haematology and blood biochemistry. At the end of the treatment period a full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs. Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on control and high dose animals.

Results

One female (180mg/kg bw/day) died in week 9. The cause of death could not be ascertained. No other mortalities occurred. Salivation was reported at the beginning of dosing but was considered to be a response to the dosing rather than the compound. The only treatment-related clinical sign in animals was coloured urine. A small number of high dose animals were reported either as languid at the beginning of the study or wheezing towards the end, which continued after treatment stopped.

Body weight gain and food consumption were comparable in all dose groups. There were no eye lesions. Animals of both sexes treated at 60 and 180 mg/kg bw/day exhibited a number of significant changes in haematological parameters associated with anaemia. The mean cell volume and mean cell haemoglobin of high dose animals remained above the controls during the recovery period. Treated males of all dose groups had higher serum calcium levels than controls. High dose males and all treated females had higher serum phosphorus concentrations. These changes reversed after the four week recovery period.

The liver and kidney weights tended to be greater in the high dose females and spleen weight was increased in both male and female high dose groups. After 4 weeks without treatment organ weights were comparable to controls.
Histopathological changes were recorded in all treated animals but most notably at 180mg/kg bw/day. In particular discoloration within the kidney tubules and of the duodenum was reported in all treated groups with a dose-related increase in severity. These changes were also seen in treated animals at the end of the recovery period.
The study did not identify a NOAEL.

Ref. : 12

Guideline	:	OECD 408
Species/strain	:	Sprague-Dawley rat, Ico:OFA,SD (IOPs Caw) strain
Group size	:	5 males + 5 females 3 males+3 females (week 4, interim kill, test groups only) 5 males +5 females (week13-17 recovery groups, control + high dose)
Test substance	:	SAT 930384 in aqueous solution
Batch no	:	4354/76 (purity 98.2%)
Dose levels	:	0, 5, 10 and 20 mg/kg bw/day in 5ml/kg pH2.5
Treatment period	:	13 weeks + 4 week recovery
GLP	:	in compliance

Groups of 5 male and 5 female rats were dosed with the test substance by gavage at 0, 5, 10 and 20 mg/kg bw/day, 7 days a week for 13 weeks. Additional groups of animals (5 per sex) were used as satellite groups (recovery) to evaluate the reversibility of symptoms during a subsequent 4 week period without treatment (controls and high dose). A second satellite group (3 animals per sex-treated only) was killed 4 weeks after commencement of dosing.

The animals were observed daily for clinical signs and mortality, and weekly for body weight and food consumption. Urine was collected overnight for urinalysis in weeks 4, 13, 14 and 17. At the end of the treatment period a full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs. Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on control and high dose animals. Blood was not sampled and therefore there was no haematology or clinical biochemistry.

Results
One low dose male died in week 9, reported due to a perforation of the oesophagus due to dosing. No other mortalities occurred. The only treatment-related clinical sign in animals was coloured urine seen in all the high dose animals and in the female group dosed with 10 mg/kg bw/day. Towards the end of the treatment period there was a dose-related discoloration of urine at 10 and 20 mg/kg bw/day, which was more marked in the males. After the recovery period, the urine colour of all treated animals was comparable to controls.
Body weight gain and food consumption were comparable in all dose groups. There were no eye lesions. Microscopically, dose-related pigmentation was reported in the kidney tubules and duodenal villi of all treated rats, and was also present at the end of the recovery period.
The study did not identify a NOAEL.
No blood analyses were performed and therefore it is not possible to determine whether the observed pigmentation was associated with functional disturbances.

Ref. : 13

Guideline	:	OECD 408
Species/strain	:	Sprague-Dawley rat, Ico:OFA,SD (IOPs Caw) strain
Group size	:	10 males + 10 females 5 males + 5 females (week13-17 recovery groups, control + high dose)
Test substance	:	SAT 940451 (KN 17) in aqueous solution
Batch No	:	KN-Hmu 5145/148 (purity 95%)
Dose Levels	:	0, 10 and 20 mg/kg bw/day in 5ml/kg
Treatment Period	:	13 weeks +4 week reversal
GLP	:	in compliance

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 10 and 20 mg/kg bw/day, 7 days a week for 13 weeks. Additionally groups of 10 animals (5 per sex) were used as satellite groups (recovery) for the high dose and control to evaluate the reversibility of symptoms during a subsequent 4 week period without treatment. During the study, the animals were observed daily for clinical signs and mortality, and weekly for body weight and food consumption. Urine was collected overnight for urinalysis and blood was sampled from the retro-orbital sinus for haematology and blood biochemistry in weeks 3, 7, 13/14 and 18, with special attention to kidney function parameters. At the end of the treatment period a full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs. Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on control and high dose animals.

Results
One control female died on day 49 due to blood sampling error. No other mortalities occurred. The only treatment-related clinical sign in animals was coloration of the urine, particularly in the males. The urine colour was normal at the end of the recovery period. Body weight gain and food consumption were comparable in all dose groups. There were no eye lesions. Pigmentation was reported in the pelvis epithelium and cortex of the kidney and pigmentation of the stroma of the duodenal villi of some animals of all test groups, including the recovery group. There was no indication of functional disorders suggesting nephrotoxicity. In particular, urinary γ-glutamyl transferase, N-acetyl-β-glucosamidase, urea, creatinine, bilirubin, glucose, proteins and ketones showed no consistent changes and were within the range of control levels. Four of 15 high dose males exhibited significantly elevated blood levels of creatinine at the week 7 sampling. In the absence of similar effects at other times, this was considered to be of doubtful toxicological significance. Blood urea was similar to controls at all times and doses.
The authors concluded that the dose of 20mg/kg bw/day was a NOAEL, but this requires clarification.

Ref. : 14

Dihydroxyindoline is an indol derivative with chemical similarities to melanin precursors. Brownish pigments occur frequently in rodent's (rats) kidneys representing e.g. lipofuscine, hemosiderine or even $\alpha_{2\mu}$-globulin-containing inclusions – all appearing as "brownish pigment". At a request of the SCCNFP, industry further investigated the brownish pigment observed in the organ slides of the duodenum and in the kidneys as well as their potential biological importance.

The SCCNFP asked to look into and evaluate again the respective organ slides of the 13-week studies carried out earlier (1993). Organ samples of kidneys and duodenum were recut and stained by H&E; also four additional histological stainings for a discrimination of the observed pigments (A 147) against others, above mentioned compounds were carried out. Notable is that

in a comparison test-study it could be stated that staining intensity remained over the years in the organs embedded in paraffin blocks.

From the reported results it seems to be worth to mention that using the more subtle technique in the repetition carried out in 2002 that also in the respective organs of control rats "brownish" pigments were observed. But nevertheless in the treated groups, beginning with the (10)/20 mg/kg/day group and higher in addition, a dose related pigmentation was confirmed; the incidence decreased not fully during a recovery period of 4 weeks.

The present investigation is based on three 90-days (13-week) studies; A 147 was administered by gavage to Sprague Dawley rats, dose levels 0, 20, 60, 180 mg/kg/day (study I), 0, 5, 10, and 20 mg/kg /day (study II), 0, 10, 20 mg/kg/day (study III). As already described earlier (submission I, 1995) there were no other pathological findings which could be attributed to the test substance with the exception of coloured urine; this coloration disappeared during the recovery period. No other histological or functional signs, compared with the controls were observed.

Conclusion
After sub-chronic oral dosing of the test substance A 147, brownish pigments were found in kidneys of all treated and also in control groups of rats using H&E histochemical staining. Pigments were discovered in animals treated with 0, 5, 10, 20, 60 and 180 mg/kg/day within three different 90-day studies (gavage). In the duodenum, the brownish pigment was recorded only in treated animals.

In general, brownish pigments in kidneys and duodenum are inconspicuous, corresponding to the existence of physiological brownish pigments like lipofuscine and hemosiderin, and even $\alpha_{2\mu}$-globulin-containing hyaline inclusions preferable in male rats' kidneys.
New histochemical investigations in rats were performed (2002) using the original organs of three 13-weeks studies performed earlier in order to clarify the endogenous or exogenous origin of this pigmentation.
After clarifying the physiological background of brownish pigmentation, treatment-related exogenous pigments were found in kidneys of animals receiving doses of at least 20 mg/kg/day, with only occasional pigments found at the lowest dose (10 mg/kg/day).

There were neither functional nor histological findings indicative for intestinal or renal injury, whether in treated or untreated animals.

Due to the fact that if even minor signs of exogenous pigments were found in kidneys of rats dosed with 20 mg/kg/day, the no-observed effect-level (NOEL) was established at 10 mg/kg/day.
Ref. : 19

2.3.8. Sub-chronic dermal toxicity

No data

2.3.9. Sub-chronic inhalation toxicity

No data

2.3.10. Chronic toxicity

No data

2.4. Irritation & corrosivity

2.4.1. Irritation (skin)

Guideline	:	OECD 404
Species/strain	:	New Zealand White rabbits (SPF-Quality)
Group size	:	3 female
Test substance	:	Dihydroxyindoline moistened with propylene glycol
Batch no	:	4354/6 (Purity: 98.7%)
Dose	:	0.5g
GLP	:	in compliance

0.5g of the test compound moistened with propylene glycol was applied to 6 cm^2 clipped skin on one flank of 3 female rabbits. Semi-occlusive patches were applied and left in place for a 4-hour period. The contralateral flank of each rabbit served as control. Remaining test substance was removed by swabbing with tissues moistened with tap water and subsequently with a dry tissue. In order to facilitate the scoring the skin area was reshaved before observation on days 2 and 3. The skin was examined for erythema, eschar formation and oedema at one, 24, 48 and 72 hours after removal of the occlusive patch. An index of Primary Irritation was calculated according to the scheme of Draize.

Results
Very slight erythema was observed in all 3 rabbits one hour after exposure, accompanied by slight oedema in one of them. Both erythema and oedema were reversed within 24 hour after exposure. The skin was stained brown by the test substance at the site of treatment. This was considered not to be related to toxicity. There was no evidence of corrosion or systemic toxicity during the test period. The primary irritation index for dihydroxyindoline was calculated to be 0.0 (non-irritating).
The study was initially carried out by application of the test substance moistened with distilled water instead of with propylene glycol. The results of this study were included in appendix to the study report and also gave an irritation index of 0.

Ref. : 2

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405
Species/strain	:	New Zealand White rabbits (SPF-Quality)
Group size	:	3 female
Test substance	:	Dihydroxyindoline
Batch no	:	4354/6 (Purity >98.7%)
Dose	:	73mg
Observ. Period	:	1 hour and 1, 2, 3, 7, 14, and 21 days after instillation
GLP	:	in compliance

73 mg of the test substance was instilled once to one eye of each animal without rinsing. The other eye served as control. Ocular reactions were recorded at one hour and 24,48 and 72 hours after application. Immediately after the 24 hour observation, a solution of 2% fluorescein in water (pH 7) was instilled into both eyes of each animal to quantitatively determine corneal epithelial damage, and then the eyes were rinsed to remove the residual test substance. Not all the test substance was removed during this procedure as it was mixed with the discharge, which adhered to the conjunctivae.
The degree of ocular irritation was calculated according to the method of Draize, from which a Kay and Calandra interpretation was obtained to account for time required for healing.

Results
Reactions were reported in the cornea, iris and conjunctiva of all three rabbits one-hour after instillation. Opacity of the cornea and injection of the iris were reversed within 21 days. Grey/white discoloration of the conjunctivae as a sign of necrosis was observed in 2 animals from day3 onwards which was not reversed within the study period. The irritation index of the conjunctivae noted in the third animal was reversed within 21 days. No ocular corrosion was observed.
The substance was considered moderately irritating to the rabbit eye. The Kay and Calandra interpretation of the calculated maximum Draize score was reported to be 32.3.

Ref. : 3

2.5. Sensitisation

Magnusson and Kligman Test

Guideline	:	OECD 406	
Species/strain	:	Guinea pig, Himalayan strain	
Group size	:	20 test + 10 control, female	
Test Substance	:	Dihydroxyindoline in propylene glycol (unstable)	
Batch No	:	4354/6 (Purity >98.7%)	
Concentration	:	intradermal induction :	0.1ml 50% Freund's complete Adjuvant (FCA)
			0.1ml 5% (w/v) test substance in PG
			0.1ml 10% (w/v) test substance/FCA
		topical induction :	0.5ml 10%(w/w) test substance in PG
		challenge :	0.5%-5% (w/v) test substance for 24 hours
GLP	:	in compliance	

A study was initially undertaken to identify the concentration of test substance suitable for the induction and challenge phases of the main study. In the main study induction commenced with three pairs of intradermal injections of FCA, test substance and a mixture of the two. One week later the induction process was completed with single topical application of the test substance under occlusive patch to the shoulder region for 48 hours. An interval of two weeks was allowed after induction and then the animals were challenged by a single topical application of the test substance at 0.5, 1 and 5% in propylene glycol under occlusive patch on the left flank for 24 hours. The test substance was removed with tissues moistened with water. Appropriate controls were treated with vehicle and the test substance-induced animals received vehicle alone on the right flank. The skin was examined 24 and 48 hours after removal of the challenge patches.
Results
During the induction phase of the study all experimental animals showed slight or well defined erythema. Two animals also showed slight oedema after the 48 hours occluded epidermal

induction exposure. The challenge resulted in 11/20 positive sensitisation reactions (eschar formation) in response to 5% and 10/20 positive reactions in response to 1% test substance. Animals treated with 0.5% solution showed no signs of delayed hypersensitivity.
Under the conditions of the study COLIPA A147 resulted in a sensitisation rate of 50%, indicating that it has moderate sensitising properties applying the rating of allergenicity described by Kligman (1966).

Ref. : 4

Buehler Test

Guideline	:	OECD 406
Species/strain	:	Guinea pig, Dunkin-Hartley strain
Group size	:	10 male + 10 female, tests and controls
Test substance	:	Dihydroxyindoline in aqueous solution
Batch no	:	4354/50 (purity 98.7%)
topical induction	:	0.5ml 40%(w/w) test substance in water for 6 hours on days 1, 8 and 15 challenge: 0.5ml 10% (w/v) test substance for 6 hours
GLP	:	in compliance

A preliminary study established the minimum irritant and the maximum non-irritant concentration. Topical induction was performed on one flank on days 1, 8 and 15 by applying 0.5ml of 40% aqueous test substance under occlusive patch for 6 hours. Control animals received equal amounts of vehicle. The challenge was carried out 14 days later by applying 0.5ml of 10% aqueous test substance under occlusive patch for 6 hours to the untreated flanks. Animals were examined 24 and 48 hours after removal of the patches for signs of erythema and oedema.

Results
Signs of irritation were noted during the induction phase. The macroscopic and histopathological examinations after the challenge did not show any evidence for delayed hypersensitivity in the 20 guinea pigs of the treated group. No cutaneous abnormalities were noted in the control group.
The test substance did not provoke any cutaneous sensitisation reaction under the test conditions.

Ref. : 5

2.6. Teratogenicity

Guideline	:	OECD 414
Species/strain	:	Sprague-Dawley rat, Ico:OFA, SD(IOPs Caw) strain
Group size	:	25 females (mated)
Test Substance	:	KN 17 in aqueous solution, pH 2.5
Batch No	:	4354/76 (purity 98.5%)
Dose Levels	:	0, 15, 60 and 240mg/kg bw/day
Treatment Period	:	Days 6-15 of pregnancy, inclusive
GLP	:	in compliance

The dose levels were determined from a preliminary study with 6 mated rats per group, indicating no adverse effects over the same dose range (Ref. 15). In the main study (Ref. 16), groups of 25 mated female rats were dosed with the test substance by gavage on days 6 to 15 after mating. The dams were observed daily for clinical signs and mortality; bodyweights and

food consumption were recorded on days 0, 6, 16 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 end point).

Results
Treatment-related clinical signs were limited to discoloured urine in all treated animals. One animal treated with 60 mg/kg bw/day was found dead on day 17. At autopsy, multiple dark areas were observed on the lungs. As this was not seen in any other treated rat the authors concluded that it was not treatment-related. No abortions were observed. Food consumption and body weight gain were not affected by treatment. No macroscopic abnormalities were observed in any treatment group.
The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses and foetal body weights were similar for control and treated groups. No treatment-related foetal malformations were observed. The only effect noted was generalised oedema in one foetus from a dam treated with 60mg/kg bw/day.
The NOAEL for maternal toxicity and for embryo-foetal development was 240 mg/kg bw/day.

Ref. : 15, 16

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.1 Percutaneous absorption *in vivo*

Guideline	:	OECD 417
Species/strain	:	Sprague-Dawley rat, Him:OFA, SPF strain
Group size	:	6 females
Route	:	Topical
Test substance	:	^{14}C-labelled dihydroxyindoline (radiochemical purity >98%) Unlabelled material charge no: 1; SAT No. 920933 (purity not stated)
Dose	:	205 mg on 9 cm^2
Exposure period	:	30 minutes
GLP	:	in compliance

Labelled and unlabelled test substance were mixed 1:1 in a hair dye formulation such that the final concentration of dye was 1%. 205mg of the formulation were applied to a 9cm^2 area of clipped skin of anaesthetised rats. The area of 9 cm^2 was calculated to correspond to a proportion of the rat's total skin equivalent to the scalp area as a proportion of total human skin area. After 30 minutes under open conditions the material was scraped off with a spatula and then rinsed off with water and dabbed dry until no further colour was seen in the rinsings and the tissues. The application site was then covered and the animals placed into metabolism cages. Faeces and urine samples were analysed as daily fractions over 72 hours and then the animals were sacrificed. Radioactivity was measured in the rinsing water, treated (hair, stratum corneum and hair stubs) and untreated skin, urine, faeces, 13 organs and the carcass.

Results
The total recovery was 97.3% of applied dose. The mean percutaneous absorption of the test substance was 0.78% of dose, of which 0.23% permeated through the skin and 0.55% remained in the dermis. The stratum corneum contained 1.1% which was not considered to be

systemically available, 4.8% was in the hair stubs and 90.6% in the rinsings. Excretion was mainly via the urine (90% of eliminated radioactivity) and to a lesser extent via the faeces. The majority (77% of eliminated amount) was excreted within the first 24 hours. The mean concentrations of radiolabel in the 13 analysed organs was below or near the detection limit, the highest content (0.00019%) being in the kidneys in line with the major route of excretion.

The study was performed with a 1.0 % formulation, where in semi-permanent hair dyes, the foreseen use concentration is 2.0%.

Ref. : 10

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guidelines	:	OECD 471
Species/strain	:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA 1538
Batch no	:	Ko 3658/127 first test 1990; 4354/6 second test 1991
Purity		First test : not given; second test 99.8 % (HPLC)
GLP	:	in compliance

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

Results
In the first experiment
- S9 : Negative for all tester strains
+ S9 : Positive in 2 doses in TA100, TA1537

Second experiment
- S9 : Positive in TA1535n negative for other tester strains
+ S9 : Positive in TA1535, TA100,
Conclusions
Based on the reversion rate, it is concluded that the test agent A 147 shows evidence of reproducible mutagenic activity in this bacterial test system in the presence or in the absence of activation system.

Ref. : 6

Bacterial Reverse Mutation Test, second study

Guidelines	:	OECD 471
Species/strain	:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA 1538
Batch no	:	4354/76
Purity	:	100 %
GLP	:	in compliance

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

Results

In the first experiment
- S9 : Negative for all tester strains except for one dose for TA100.
+ S9 : Positive in 2 doses in TA100, TA1537, and one dose for TA 1535

Second experiment
- S9 : Negative for all tester strains
+ S9 : Positive in one dose of TA1537.

Third experiment
- S9 : Negative for all tester strains
+ S9 : Negative for all tester strains.

Conclusions
Based on the reversion rate, it is concluded that the test agent A 147 shows evidence of reproducible mutagenic activity in this bacterial test system in the presence or in the absence of activation system). It should be noted that the experiments were performed under different technical conditions regarding adjustment of pH.

Ref. : 7

In vitro Mammalian Cell Gene Mutation Test

Guideline	:	OECD 476
Species/strain	:	V79 cell line / HGPRT Locus
Replicates	:	2 independent tests with and without metabolic activation
Test substance	:	KN 17
Batch no	:	4354/22, purity > 98 %
Treatment time	:	4 hours
GLP	:	in compliance

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

Results
The compound shows negative effects both in the absence and in the presence of S9 in both experiments. According to the authors, no biologically significant relevance was associated with the positive results obtained.

Conclusions
Based on the mutation frequency rate, no biologically significant relevance was associated with the positive results obtained. The compound is considered as non mutagenic *in vitro*.

Ref. : 9

In vitro Mammalian Chromosome Aberration test

Guideline	:	OECD 473
Species/strain	:	human peripheral lymphocytes

Replicates	:	no
Exposure time		3 h
Test substance	:	KN 17
Batch no	:	36645, purity > 99 %
GLP	:	in compliance

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

Results
COLIPA A 147 has been investigated for induction of chromosomal aberrations in human peripheral lymphocytes. The preliminary toxicity study was performed. The exposure time of cells to the test agent was 3 h in the presence or absence of metabolic activation. No increase in the frequency of cells displaying chromosomal aberrations was observed.

Conclusions
While the study provided gives negative results, there is no independent repeat of the experiment. Moreover, the 2 dose range finding studies were performed with blood withdrawn from 2 different individual donors, and the final cytogenetic assay with another volunteer. This study is therefore considered inadequate.

Ref. : 17

2.8.2. Mutagenicity/Genotoxicity *in vivo*

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

Guideline	:	OECD 486
Species/strain	:	hepatocytes of male Wistar rats
Replicates	:	no
Test substance	:	KN 17
Exposure time	:	2-4 h and 12-16 h
Batch	:	36645, purity > 99 %
GLP	:	in compliance

COLIPA A 147 has been investigated for induction of unscheduled DNA synthesis in rat hepatocytes at 3 doses 150, 375 and 750 mg/kg the latter being little toxic. Positive controls are in accordance with OECD guideline and UDS analyzed by autoradiography. 3 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.

Ref. : 18

Mammalian Erythrocytes Micronucleus Test

Guideline	:	OECD 474
Species/strain	:	Mouse, Crl: OF-1 mice

Group size	:	5 male + 5 female
Test substance	:	KN 17,
Batch no	:	4354/17purity: 98.1 %
Dose levels	:	750 mg/kg bw, single intragastric gavage
Sacrifice times	:	24, 48 and 72 hours after dosing
GLP	:	in compliance

COLIPA A 147 has been investigated for induction of micronuclei in the bone marrow cells of OF-1 mice. The substance was administered once by single gavage at 750 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
Clinical signs of toxicity
Beside coloured urine, no clinical signs were recorded.

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 COLIPA A 147 did not exhibited variation of the PCE/NCE ratio.; however, from the toxicokinetic study, it was shown the presence of the radiolabelled compound in the femur.

Conclusions
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.

Ref. : 9

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

CALCULATION OF THE MARGIN OF SAFETY

(Dihydroxyindoline HBr)
(Air oxidative/semi-permanent)

Based on a usage volume of 50 ml, containing at maximum 2 %

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**1766 µg/cm^2**
Typical body weight of human		**=**	**60 kg**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1236 µg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.021 mg/kg**
No observed effect level (mg/kg) (rat, 90 day, oral)	**NOEL**	**=**	**10 mg/kg**

Margin of Safety	**NOEL / SED**	**=**	**476**

2.12. Conclusions

Insufficient information is provided on potential impurities. The database on toxicological properties is poor. Acute toxicity, irritation, sensitisation and embryotoxicity have been adequately investigated, following appropriate guidelines and GLP where relevant. It was irritant to the rabbit eye, but not skin when tested neat, and it is unlikely that similar results would occur under realistic conditions of use. It has shown sensitising potential and formulations should be labelled with a warning in this respect. Three subchronic oral dosing studies in rats indicate a NOEL of 10 mg/kg bw/day. Skin penetration has been investigated in the rat *in vivo*. Sufficient information on tissue distribution and recovery is provided to assume that the results are acceptable.

The percutaneous absorption study was performed with a 1.0 % formulation, where, in case of a semi-permanent hair dye, the foreseen use concentration is 2.0%.

Comparing the NOEL with the maximum systemic exposure dose in humans indicates a safety margin of 476.
A 147 was tested in bacterial cells for gene mutation in different experiments and gave positive results. The *in vitro* test for mammalian gene mutation assay is negative. The *in vitro* test for clastogenicity in human lymphocytes was negative but the study is considered inadequate.
The in vivo UDS on rats hepatocytes is negative. The in vivo micronucleus test in mice gave negative results.
Dihydroxyindoline HBr may be considered not to show clastogenic/genotoxic potentials in the submitted *in vivo* studies.

2.13. References

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3. Opinion of the SCCNFP

The SCCNFP is of the opinion that, based on the present available information, Dihydroxyindoline HBr does not pose a health risk when used as a semi-permanent hair dye at concentrations not exceeding 2.0 %.

4. Other considerations

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5. Minority opinions

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