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

Hydroxyanthraquinone-aminopropyl Methyl Morpholinium Methosulfate

COLIPA n° C117

Adopted by the SCCP during the 3rd plenary meeting
of 15 March 2005

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1. BACKGROUND

Submission I on Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate (COLIPA[1] – No. C 117) was submitted in June 2003.
Submission II is an updated dossier in line with the second step of the strategy on the evaluation of hair dyes: http://pharmacos.eudra.org/F3/cosmetic/doc/HairDyeStrategyInternet.pdf.

2. TERMS OF REFERENCE

1. *On the basis of currently available information, the SCCP is asked to assess the risk to consumers of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate, when used in hair dye formulations.*

2. *Does the SCCP recommend any further restrictions with regard to the use of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate 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

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate (INCI name)

3.1.1.2. Chemical names

1-N-Methylmorpholiniumpropylamino-4-hydroxyanthraquinone, methyl sulfate
4-[3-[(9,10-dihydro-4-hydroxy-9,10-dioxoanthryl)amino] propyl]-4-methylmorpholinium methyl sulphate

3.1.1.3. Trade names and abbreviations

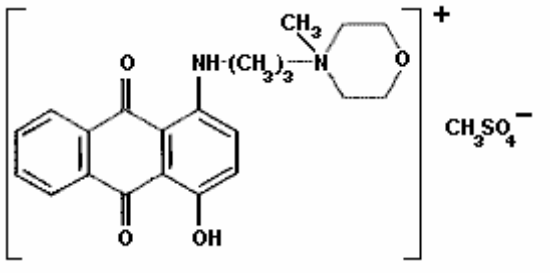
Imexine BD (Chimex)

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3.1.1.4. CAS / EINECS number

CAS : 38866-20-5
EINECS : 254-161-9

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Emp. Formula: $C_{22}H_{25}N_2O_4 . CH_3SO_4$

3.1.2. Physical form

Violet powder

3.1.3. Molecular weight

Mol. Weight : 492.5

3.1.4. Purity, composition and substance codes

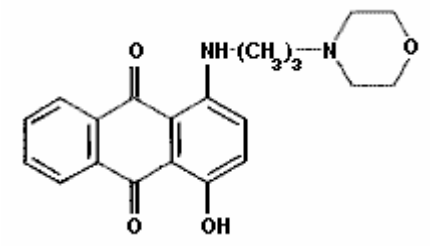
Batch op. T54 was used for all the analytical determinations reported.

Purity : 87.5 % (by HPLC with ref. standard of pure substance - batch RF010)
Water : 2.2 % (Karl Fisher method)
Ash : 0.12%
Methyl Sulphate ions : 22.5 % w/w (theoretical value = 22.5 % w/w)

3.1.5. Impurities / accompanying contaminants

Identified Impurities:

1-Hydroxy-4-(3-morpholin-4-yl-propylamino)-anthracene-9,10-dione : 1.2%



Unidentified Impurities:

At least three hydroxylated derivatives detected by HPLC

Impurity A (ion m/z = 581) : 7.0 % (mole/mole) (semiquantitative)
Impurity B (ion m/z = 507) : 2.6 % (mole/mole) (semiquantitative)
Impurity C (ion m/z = 397) : /

Residual solvents:

Acetone : Detected (Detection Limit < 100 ppm)
Ethanol : Not Detected (Detection Limit < 500 ppm)
Isobutanol : Not Detected (Detection Limit < 500 ppm)

3.1.6.	Solubility

Soluble in water (5 g/100 ml) and in ethanol

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow} : 2 (calculated CLOGPy3 - C. Hansch)

3.1.8.	Additional physical and chemical specifications

Melting point : 215 °C
Boiling point : /
Flash point : /
Vapour pressure : /
Density : 0.35 g/cm³
Viscosity : /
pKa : /
Refractive index : /

General comments on analytical and physico-chemical characterisation

- Hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate is a secondary amine, and thus, prone to nitrosation. The nitrosamine content of the dye is not reported.
- Two of the impurities (total approximately 10 %) are not chemically characterised.

3.2. Function and uses

Imexine BD is used in direct hair dye formulations at a maximum concentration of 0.5%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline	:	OECD 401
Species/strain	:	Sprague-Dawley ICO:OFA-SD (IOPS Caw)
Group size	:	5 males + 5 females (2000, 1500 mg/kg bw), 5 females 1000 mg/kg bw
Test substance	:	Imexine BD dissolved in distilled water
Batch no	:	op. T54
Purity	:	87.5 %
Dose	:	1000, 1500, 2000 mg/kg bw by gavage
GLP	:	in compliance

Results
All females given 2000 mg/kg died within 30 minutes of dosing. 80% of the females (four animals) administered 1500 mg/kg died on days 1 and 2; 20% of those (one animal) administered 1000 mg/kg died on day 1. In males, mortality was 40% (two animals) on day 1 at 2000 mg/kg and 80% (four animals) on day 1 at 1500 mg/kg. Males administered 2000 mg/kg were observed to have tremors, hypoactivity, sedation and dyspnoea, one in this group male had a purple-coloured tail from day 2 to day 15 of the study. Sedation and hypoactivity were observed in the males given 1500 mg/kg. Females at both 1500 and 1000 mg/kg showed signs of sedation, hypoactivity, tremors, dyspnoea and piloerection. Clinical signs were observed within 30 minutes of dosing. Recovery in surviving animals was complete by day 2. No effect on body weight was observed. At necropsy, animals were observed to have blue or purple discoloration of the gastrointestinal tract and sometimes of the urinary bladder. With the exception of discolouration no abnormalities were observed at necropsy. The body weight gain of the surviving animals was comparable to that of historical controls.

The acute oral LD50 for Imexine BD (87.5% pure) in both sexes of rats was estimated to be between 200 and 2000 mg/kg (175 and 1750 mg/kg active dye; 95% confidence limit).

Ref.: 1

3.3.1.2. Acute dermal toxicity

/

3.3.1.3. Acute inhalation toxicity

/

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline	:	OECD 404
Species/strain	:	New Zealand White rabbits
Group size	:	3 males
Test substance	:	Imexine BD
Batch no	:	Op T54; 87.5% active
Dose	:	500 mg
GLP	:	in compliance

A group of three male New Zealand White rabbits (mean body weight - 2.5 ± 0.2 kg) was used. 500 mg Imexine BD (437.5 mg active dye) was applied in its original form to a clipped area on the right flank and held in place for 4 hours under a semi-occlusive dressing. The left flank served as a control. When the patches were removed, any residual material was removed with distilled water. The skin was examined at 1, 24, 48 and 72 hours after removal of the dressing.

Results
There was no evidence of oedema at any of the patch test sites during the study. The compound coloured the application sites, making assessment of erythema of grade 1 or grade 2 impossible. No erythema of grade 3 or grade 4 was noted. Because the grade of erythema could not be determined, the compound could not be classified as to its irritant potential.

Ref.: 3

3.3.2.2. Mucous membrane irritation

Guideline	:	OECD 405
Species/strain	:	New Zealand White rabbits
Group size	:	3 males
Test substance	:	Imexine BD
Batch no	:	Op T54; 87.5% active
Dose	:	100 mg
GLP	:	in compliance

A group of three New Zealand White rabbits (mean body weight - 2.6 ± 0.2 kg) was used for this study. 100 mg of Imexine BD in its original form (87.5 mg active dye) was placed into the conjunctival sac of the left eye of the three rabbits. The upper and lower lids were held closed for about 1 second to avoid any loss of the test substance. The eyes were not rinsed after administration of the test substance. The untreated right eye of each animal served as a control. Evaluations of the conjunctiva, cornea and iris were made 1 hour after compound administration, and at 1, 2 and 3 days thereafter.

Results
No signs of ocular irritation were observed during the study. Purple discoloration of the conjunctiva was observed at the 1-hour observation time only. Imexine BD (87.5% active) was non-irritant to the rabbit eye.

Ref.: 2

3.3.3. Skin sensitisation

Guinea Pig Maximisation (Magnusson and Kligman)

Guideline	:	OECD 406
Species/strain	:	Dunkin-Hartley guinea pigs
Group size	:	10 females treated; 5 female controls
Test substance	:	Imexine BD
Batch no	:	Op T54; 87.5% active
Dose	:	Induction: intradermal 1% (0.875% active); epicutaneously 30% Challenge: 10% (8.75% active)
GLP	:	in compliance

A preliminary test was performed in two animals to determine the concentration to be used in the principal study.
For the principal study, guinea pigs were allotted to two groups: a control group of five females and a treated group of ten females. On day 1, six 0.1 ml intradermal injections were administered (three on each side) in the scapular region: Freund's complete adjuvant diluted to 50% (v/v) with sterile isotonic saline, a 1% concentration (w/w) of Imexine BD (0.875% active dye) in sterile isotonic saline, and a mixture of 50/50 (w/v) Freund's complete adjuvant in isotonic saline and 1% (w/w) Imexine BD (0.875% active dye) in the vehicle.

In control animals, the vehicle replaced Imexine BD in the mixtures previously described.
On day 7, animals were treated with 10% sodium lauryl sulfate in petrolatum to induce local irritation. On day 8, 0.5 ml of either the vehicle (control group) or a 30% (w/w) concentration of the test substance (26.3% active dye) (treated group) was administered topically in the area of the previous intradermal injections and held in place for 48 hours under an occlusive dressing. One hour after the dressings were removed, cutaneous reactions were recorded.

On day 22, a challenge dose of 0.5 ml of the vehicle was applied to the left flank and 0.5 ml of a 10% (w/w) concentration of Imexine BD (8.75% active dye) in the vehicle was applied to the right flank in both the control and treated groups. These treatments were left in place for 24 hours under an occlusive dressing. Skin reactions were evaluated 24 and 48 hours after removal of the occlusive dressing.

On day 25, animals were killed and skin samples were taken from the application sites on the right and left flanks for each animal. Tissues were preserved for possible microscopic evaluation. Animals were judged to have positive reactions if lesions were clearly visible and more marked than the most severe reaction in control animals or if "doubtful" reactions were confirmed upon histopathological examination.

Results
After the challenge application, purple discoloration that could mask slight to well defined erythema was observed in 3/10 treated animals at the 24-hour observation period. No visible reactions were noted in the control group at any time. Very slight to severe erythema was noted in 7/10 animals at 24 hours and in 9/10 animals at 48 hours. Slight oedema was noted in 6/10 animals at 24 hours; none was observed at 48 hours. Crusts were observed in 2/10 animals with severe erythema, and dryness of the skin was observed in 6/10 guinea pigs at 48 hours.

Cutaneous reactions attributable to the sensitization potential of Imexine BD (87.5% active dye) were observed in 9/10 guinea pigs. The substance is a potent contact allergen.

Ref: 4

Guinea Pig (Buehler)

Guideline	:	OECD 406
Species/strain	:	Dunkin-Hartley guinea pigs
Group size	:	10 females + 10 males treated; 5 female + 5 male controls
Test substance	:	Imexine BD
Batch no	:	Op T54; 87.5% active
Dose	:	Induction: 0.5ml of 30% (26.3% active) on days 1, 8 and 15. 1st Challenge: 0.5ml of 10% (8.75% active) 2nd Challenge: 0.5ml of 2% (1.8% active)
GLP	:	in compliance

Guinea pigs were allocated to two groups: a control group of five males and five females and a treated group of ten males and ten females. During a 3-week induction period, animals of the treated group received a cutaneous application of 0.5 ml of the test substance at a concentration of 30% (w/w) (26.3% active dye) in distilled water on the anterior left flank on days 1, 8 and 15 of the study. Control animals received the vehicle (distilled water). Each application was held in place under an occlusive dressing for 6 hours.

After a 14-day rest period, 0.5 ml of the test substance at a concentration of 10% (w/w) (8.75% active dye) in distilled water was applied on the posterior left flank and 0.5 ml of the vehicle was applied on the posterior right flank (both previously untreated skin sites) under occlusive dressing for 6 hours. Cutaneous reactions were evaluated 24, 48 and 72 hours after removal of the dressing.
A second challenge was performed and evaluated using this same method, but using the test substance at a concentration of 2% (w/w) (1.8% active dye) on the left flank, with sites being evaluated at 24 and 48 hours only.
Animals were judged to have positive reactions if macroscopic lesions were clearly visible and more marked than the most severe reaction in control animals or if "doubtful" macroscopic reactions were confirmed upon histopathological examination.

Results
After the first challenge (10%), grade 1 erythema was observed in one control animal at the 48-hour observation point. Very slight (grade 1; incidence: 5/20) to well defined (grade 2; incidence: 2/20) erythema was observed in treated animals 24 hours after removal of the test substance. After 48 hours, very slight erythema was observed in 6/20 animals (three of which had no erythema at 24 hours) and well defined erythema was observed in 3/20 animals (one of which has slight erythema at 24 hours). After 72 hours, very slight erythema was noted in 9/20 animals, and dryness of skin was noted in 3/20 animals.

No skin reactions were observed in either the control or the treated group after the second challenge (2%).
Imexine BD (87.5% pure) at a concentration of 10% (8.75% active dye) elicited sensitization

reactions in 9/20 guinea pigs following induction with 30%. No reaction was elicited upon rechallenge with a 2% dilution of the test compound (1.8% active dye).

Ref.: 5

Guinea Pig (Buehler)

Guideline	:	OECD 406
Species/strain	:	guinea pigs – Himalayan Spotted
Group size	:	20 female treated; 10 female controls
Test substance	:	Imexine BD
Batch no	:	Op T54; purity 87.5 %
Dose	:	Induction: 0.5ml of 10% (8.65% active) on days 1, 8 and 15. 1st Challenge: 0.5ml of 3% on day 29 2nd Challenge: 0.5ml of 3% on day 43
GLP	:	in compliance

Each animal's fur was shaved with a fine clipper blade. 0.5 ml of freshly prepared test article was applied to the skin in a 25 mm Hill Top Chamber, which was firmly secured with an occlusive dressing and left in place for 6 hours.
For the induction phase of the study, fur was clipped from the left shoulder and 10% Imexine BD in bi-distilled water was applied once a week for 3 weeks at the same site as described above. Skin responses were graded approximately 24 hours after the compound was removed. A 2-week period elapsed prior to treatment with the challenge dose.

For the first challenge dose (day 29), fur was clipped from the left posterior side and back of each animal of both the control and test groups. The challenge concentration of 3% Imexine BD was
applied for 6 hours on this naive skin site. Skin responses were graded at 24 and 48 hours after removal of the test compound.

For the second challenge dose (day 43), fur was clipped from the right posterior side and back of each animal. The rechallenge concentration of 3% Imexine BD was applied for 6 hours on this naive skin site. Only the test group was rechallenged. Skin responses were graded at 24 and 48 hours after of the test compound.

Results
One animal was found dead on day 23; no abnormal findings were noted at necropsy. The cause of death could not be established.

After induction, no oedema was noted. Discoloration of the application site precluded the evaluation of erythema. At first challenge, discrete/patchy to moderate/confluent erythema was observed in 2/19 treated animals at the 24- and 48-hour readings. Discrete/patchy erythema was observed in an additional treated animal at 48 hours. No reactions were observed in control animals.
At re-challenge, discrete/patchy to moderate/confluent erythema was observed in 7/19 treated animals at the 24-hour reading and in 9/19 treated animals at the 48-hour reading.

A concentration of 3% Imexine, BD elicited allergic reactions following induction with 10%. The substance was considered to be sensitizing in this study.

Ref.: 6

Guinea Pig (Buehler)

Guideline	:	OECD 406
Species/strain	:	guinea pigs – Himalayan Spotted
Group size	:	20 female treated; 10 female controls
Test substance	:	Imexine BD
Batch no	:	Op T54; 87.5% active
Dose	:	Induction: 0.5ml of 5% (4.4% active) on days 1, 8 and 15. 1st Challenge: 0.5ml of 1% on day 29
GLP	:	in compliance

The same patching method was used for the induction and challenge phases of the study.
Each animal's fur was shaved with a fine clipper blade. 0.5 ml of freshly prepared test article was applied to the skin in a 25 mm Hill Top Chamber, which was firmly secured with an occlusive dressing and left in place for 6 hours.
For the induction phase of the study, fur was clipped from the left shoulder and 5% Imexine BD (4.4% active dye) in bi-distilled water was applied once a week for three weeks at the same site as described above. Skin responses were graded approximately 24 hours after the compound was removed. Challenge was 2-week later.
For the challenge dose (day 29), fur was clipped from the left posterior side and back of each animal of both the control and test groups. The challenge concentration of 1% Imexine BD (0.875% active dye) was applied for 6 hours on this naive skin site. Skin responses were graded at 24 and 48 hours after removal of the test compound.

Results
After induction, discoloration of the application site precluded the evaluation of erythema. No oedema was noted.
No skin reactions were observed in either control or treated animals after challenge with the concentration of 1% Imexine BD.
The concentration of 1% Imexine BD (87.5% active dye) did not elicit allergic reactions following induction with 5% of the substance

Ref.: 7

3.3.4. Dermal / percutaneous absorption

***In Vitro* Percutaneous Absorption Study using Human Dermatomed Skin**

Guideline	:	/
Species/strain	:	human dermatomed abdominal skin
Group size	:	4 donors; 2 samples from each donor
Test substance	:	Imexine BD 0.5% in hair dye formulation 175325 (= 0.5% Imexine BD; 2.5% Benzyl alcohol; 10% Deceth 5; 4.0% Propylene glycol; 83.0% aqua)
Batch no	:	Op T54; purity 87.5 %

GLP : in compliance

Human skin samples from four donors were obtained from abdominal plastic surgery. They were transported at 4°C and kept frozen at –20°C until they were used.
Two dermatomed skin samples per donor were used. Samples were mounted in flow-through diffusion chambers with physiological saline as the receptor fluid. Their integrity was verified by measuring Trans-Epidermal Water Loss.

Twenty (20) mg/cm² of a hair dye formulation 175325 containing 0.50% (w/w) Imexine BD (equivalent to 98.5 ± 1.0 µg/cm² Imexine BD), were applied to the skin surface for 30 minutes. The dye was 87.5% pure; thus the actual amount of Imexine BD applied was ~86.7 µg/cm² active dye.

After 30 minutes, any of the hair dye formulation 175325 remaining on the skin was removed using a standardized washing procedure. Twenty-four (24) hours after application, the percutaneous penetration of Imexine BD was determined by measuring the concentration of the compound by HPLC and UV-Visible detection in the following compartments: skin excess, stratum corneum, epidermis + dermis, and receptor fluid.

Results
Seven of the eight samples tested yielded data that could be used. Most of the hair dye remaining on the skin after the application period was removed in the washing procedure.

The cutaneous distribution of Imexine BD (mean ± SD) was as follows:

Skin excess	
µg/cm²	102.09 ± 2.33
% of the applied dose	103.62 ± 2.39
Stratum corneum	
µg/cm²	1.50 ± 0.36
% of the applied dose	1.52 ± 0.36
Epidermis + dermis	
µg/cm²	0.86 ± 0.34
% of the applied dose	0.87 ± 0.34
Receptor fluid	
µg/cm²	0.083 ± 0.025
% of the applied dose	0.085 ± 0.027
Total recovery	
% of the applied dose	106.0 ± 2.1

The absorbed amount (epidermis + dermis + receptor fluid) or dermal absorption was 0.90 ± 0.31% of the applied dose (or 0.89 ± 0.31 µg/cm²).

Ref.: 17

3.3.5. Repeated dose toxicity

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

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3.3.5.2. Sub-chronic (90 days) oral toxicity

Dose range finding study (2 weeks)

Guideline	:	/
Species/strain	:	Sprague-Dawley rats Crl CD (SD) BR
Group size	:	6 males + 6 females
Test substance	:	Imexine BD suspended in water for injection
Batch no	:	op. T54
Purity	:	87.5 %
Dose	:	0, 50, 200, 800 mg/kg bw/day by gavage
GLP	:	in compliance

The study protocol was based on OECD 407.
At 800 mg/kg/day ptyalism, pink coloured urine, blue coloured faeces and purple coloured body extremities were noted. No mortalities occurred. Food consumption and body weight gain were similar to the controls. Slightly lower neutrophil and monocyte counts in females and slightly higher glucose levels in males were noted at 800 mg/kg bw per day. With the exception of discolouration of some organs no relevant macroscopic as well as microscopic findings were reported. The same doses were chosen for the main study.

Ref.: 8

Main study (13 weeks)

Guideline	:	OECD 408 (1981)
Species/strain	:	Sprague-Dawley rats Crl CD (SD) BR
Group size	:	10 males + 10 females
Test substance	:	Imexine BD suspended in water for injection
Batch no	:	op. T54
Purity	:	87.5 %
Dose	:	0, 50, 200, 800 mg/kg bw by gavage
GLP	:	in compliance

Three groups of 10 male and 10 female rats received Imexine BD daily by gavage at 0, 50, 200, 800 mg/kg bw/day for 13 weeks, a further group treated with aqua by injection served as control. A recovery group was not included. The animals were checked daily for clinical signs and mortality. Body weight and food consumption were recorded once per week. Ophthalmological examinations were performed before treatment and on week 13 in the control and the high dose group. Haematology, blood biochemistry and urinalysis were determined in week 13. At the end of the treatment period the animals were sacrificed, macroscopically examined and organs were weighed. Microscopic examination was performed on the control and the high dose group animals and all animals with macroscopic lesions.

Results

No substance-related mortality was observed. Discolouration of tail, fur, extremities, urine and faeces was observed in animals of the high dose and (partially) in the 200 mg/kg dose. All further clinical signs were judged as not being substance-related. The findings on food consumption and ophthalmoscopy were not considered treatment-related. The body weight of the males in the 200 and 800 mg/kg bw/d groups was slightly decreased (weight change compared with controls -15 %) as well as the thymus weight of females (absolute and relative) and males (absolute) at 800 mg/kg bw/d. A statistically significant dose-related decrease in the number of monocytes of females was found at 800 mg/kg bw/d while biochemistry and urinalysis values were not changed. The microscopic pathology findings revealed no substance-related effects. The NOAEL is 200 mg/kg bw/d.

Ref.: 9

3.3.5.3. Chronic (> 12 months) toxicity

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3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline	:	OECD 472
Species/strain	:	*S. typhimurium*, TA98, TA100, TA1535, TA1537 ; *E. coli*, WP2uvrA
Replicates	:	Two independent tests
Test substance	:	IMEXINE BD in distilled water
Batch No.	:	op. T54 (purity: 87.5%)
Concentrations	:	312.5 - 5000 µg/plate without metabolic activation 62.5 - 2000 µg/plate with metabolic activation
GLP	:	in compliance

IMEXINE BD (C 117) has been investigated for the induction of gene mutation in *Salmonella typhimurium* and *Escherichia coli*. Liver S9 fraction from rats induced with Aroclor was used as the exogenous metabolic activation system. A preliminary study revealed no toxicity in the absence of S9 mix and therefore the concentration range was based on the recommended maximum of 5000 µg/plate. In the presence of S9 mix the maximum concentration was 2000 µg/plate in the first test (direct plate incorporation) and 1000 µg/plate in the second test (preincubation method). Negative and positive controls were in accordance with the OECD guideline.

Results
IMEXINE BD did not induce gene mutations in the E.coli strain WP2uvrA and the S. typhimurium strain TA 1535. It induced mutations in the absence and presence of S9 mix in TA 1537 and in TA 98. Positive results were obtained in TA 100 and TA 102 in experiments with S9 mix.
IMEXINE BD (C 117) is mutagenic in the bacterial gene mutation assay.

Ref.: 10

***In vitro* chromosome aberration test**

Guideline	:	OECD 473
Cells	:	Chinese Hamster Ovary Cells (CHO)
Replicates	:	2 independent tests with and without S9 mix
Test substance	:	IMEXINE BD
Batch No.	:	op. T54 (purity 87.5%)
Concentr. Scored	:	50 -500 µg/ml without metabolic activation; treatment for 20 h 125 - 375 µg/ml without metabolic activation; treatment for 44 h 500 - 5000 µg/ml with metabolic activation
GLP	:	in compliance

IMEXINE BD (C 117) has been investigated for induction of chromosomal aberrations in CHO cells with a harvest time of 20 and 44 hours (continuous exposure without metabolic activation, 2h exposure with metabolic activation). Liver S9 fraction from Aroclor1254-induced rats was used as the exogenous metabolic activation system. Test concentrations for scoring of experiments without metabolic activation were based upon a 38-65% reduction in the mitotic index. Negative and positive controls were in accordance with the OECD guideline.

Results
The test substance induced a significant increase in the frequency of chromosome aberrations in experiments with and without S9 mix at two harvest times. Under the experimental conditions used, IMEXINE BD (C 117) was clastogenic in mammalian cells (CHO cells) *in vitro*.

Ref.: 11

***In vitro* mammalian cell gene mutation test**

Guideline	:	OECD 476
Cells	:	L5178Y mouse lymphoma cells (TK+/-)
Replicates	:	2 independent tests with and without S9 mix
Test substance	:	IMEXINE BD
Batch No.	:	op. T 54 (purity: 87.5%)
Concentr. Tested	:	500 -4000 µg/ml without metabolic activation 187.5 - 4000 µg/ml with metabolic activation
GLP	:	in compliance

IMEXINE BD (C117) has been investigated for induction of gene mutations at the TK-locus in L5178Y mouse lymphoma cells after exposure for 3 hours without and with metabolic activation. Liver S9 fraction from Aroclor1254-induced rats was used as the exogenous metabolic activation system. Test concentrations were based on the level of toxicity (10-20% relative cloning efficiency on day 0 post-treatment). Negative and positive controls were in accordance with the OECD guideline.

Results
In the first experiment without S9 mix no clear increase in relative mutant frequency was determined despite an increase in the absolute mutant frequency – obviously due to a reduced

cloning efficiency in the vehicle control. A significant and concentration-related increase in the mutant frequency was measured in the second experiment without S9 mix.
In the first test with S9 mix a clear positive result was obtained while in the second test the increase in mutant frequencies was less than a doubling of the control value. An increased number of small colonies was observed in all experiments.
Under the experimental conditions used, IMEXINE BD (C 117) was mutagenic in mammalian cells (L5178Y mouse lymphoma cells) *in vitro*.

Ref.: 12

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline	:	OECD 474
Species/strain	:	Mouse, Swiss OF1
Group size	:	5 males + 5 females
Test substance	:	IMEXINE BD
Batch No.	:	op. T 54 (purity: 87.5%)
Dose levels	:	0, 500, 1000 and 2000 mg/kg bw (twice, 24 hour interval by gavage)
Sacrifice time	:	24 hours after the second treatment
GLP	:	in compliance

IMEXINE BD (C 117) has been investigated for induction of micronuclei in the bone marrow cells of mice. Since preliminary toxicity tests did not indicate toxic effects, 2000 mg/kg bw was selected as the top dose-level. Negative and positive controls were in accordance with the OECD guideline.

Results
In all treated groups, the PCE/NCE ratio was lower than in the negative control group indicating toxicity to the bone marrow and relevant exposure of the target cells. The mean MNPCE frequencies were not significantly increased in any of the groups treated with the test substance. The positive control substance gave the expected result.
The study was conducted appropriately. IMEXINE BD (C 117) did not induce chromosome aberrations or damage to the mitotic apparatus in bone marrow cells of mice after oral treatment under the test conditions used.

Ref.: 13

Rat liver in vivo/in vitro UDS assay

Guideline	:	draft OECD guideline 486 (1991)
Species/strain	:	Wistar rat, HanIbm: WST (SPF)
Group size	:	3 males
Test substance	:	IMEXINE BD in deionised water
Batch No.	:	Op T 54 (purity: 87.5%)
Dose levels	:	0, 200 and 2000 mg/kg bw, by gavage
Sacrifice times	:	16 hours: both dose groups; 2h: high dose group
GLP	:	in compliance

IMEXINE BD (C 117) has been investigated for induction of unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro* following *in vivo* dosing. A preliminary toxicity study indicated slight toxic reactions. Therefore 2000 mg/kg bw was selected as the upper dose for the UDS study. Negative and positive controls were in accordance with the OECD guideline. Animals were sacrificed after 16 hours, and for an additional high dose group after 2 hours. With the exception of the test group 200 mg/kg b. w. in which only preparations from 2 animals were scorable, hepatocytes from three animals were evaluated per group.

Results
In none of the groups treated with the test substance there was a significant induction of UDS compared to the control group. There were no differences in the viability of hepatocytes isolated from rats of different dose groups. The results met all the pre-defined criteria for a negative response and therefore the test substance was not found to induce UDS. The positive control substance agent gave the expected results. The negative test result indicates that IMEXINE BD (C 117) does not induce DNA damage that is detectable with the UDS test.

Ref.: 14

3.3.7. Carcinogenicity

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3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

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3.3.8.2. Teratogenicity

Preliminary study

Guideline	:	/
Species/strain	:	Sprague-Dawley rats Crl CD (SD) BR
Group size	:	7 mated females
Test substance	:	Imexine BD suspended in water for injection
Batch no	:	op. T54
Purity	:	87.5 %
Dose	:	0, 50, 200, 800 mg/kg bw by gavage
GLP	:	not in compliance

The pregnant animals were treated daily by gavage from day 6 to 15 of gestation. Clinical signs including mortality were daily checked. Food consumption was recorded from days 2-6, 6-9, 12-15, and 15-20 of gestation. Body weights were recorded on days 2, 6, 9, 12, 15, and 20 of gestation. On day 20 the dams were sacrificed, the foetuses were removed by Caesarean section and the number of implantations was determined. The foetuses were weighed, checked for external abnormalities and sexed.

Results

No mortality and no clinical signs with the exception of ptyalism and some discolouration were observed. No changes in food consumption and body weight gain were noted. The resorption rate, mean number of foetuses, mean foetal body weight and the sex ratio was similar to controls. No external foetal anomalies were observed.

Ref.: 15

Main study

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague-Dawley rats Crl CD (SD) BR
Group size	:	25 mated females
Test substance	:	Imexine BD suspended in water for injection
Batch no	:	op. T54
Purity	:	87.5 %
Dose	:	0, 50, 200, 800 mg/kg bw by gavage
GLP	:	in compliance

The pregnant animals were treated daily by gavage from day 6 to 15 of gestation. Clinical signs including mortality were twice a day checked. Food consumption was recorded from days 2-6, 6-9, 12-15, and 15-20 of gestation. Body weights were recorded on days 2, 6, 9, 12, 15, and 20 of gestation. On day 20 the dams were sacrificed, the foetuses were removed by Caesarean section and the number of implantations was determined. The foetuses were weighed, checked for external abnormalities and sexed. Half of the foetuses were submitted to soft tissue examination, one half to skeletal examination.

Results
No mortality and no clinical signs with the exception of ptyalism and some discolouration at 800 mg/kg bw/d were observed. No changes in food consumption and body weight gain were noted. The resorption rate, mean number of foetuses, mean foetal body weight and the sex ratio was similar to controls. No external foetal anomalies were observed. No substance-related soft tissue anomalies were found. No treatment-related changes in the frequency of variations and abnormalities were registered.
The NOEL for maternal and foetotoxicity as well as teratogenicity was found to be 800 mg/kg bw/d.

Ref.: 16

3.3.9. Toxicokinetics

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3.3.10. Photo-induced toxicity

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3.3.11. Human data

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3.3.12. Special investigations

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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)**	=	**0.89 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**	=	**0.623 mg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	=	**0.010 mg/kg**
No observed effect level (mg/kg) (rat, 13 week, oral)	**NOAEL**	=	**200 mg/kg**
Margin of Safety	**NOAEL / SED**	=	**20000**

3.3.14. Discussion

Purity: 87.5%. Impurities include 1.2% 1-Hydroxy-4-(3-morpholin-4-yl-propylamino)-anthracene-9,10 dione, two other hydroxylated impurities at 7% and 2.6%, water and residual solvents.

Hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content of the dye is not reported.

The acute oral LD_{50} for Imexine BD (87.5% pure) in both sexes of rats was estimated to be between 200 and 2000 mg/kg (175 and 1750 mg/kg active dye; 95% confidence limit).
The NOAEL was 200 mg/kg bw/d in a 13 week sub-chronic oral toxicity study in rats. The NOEL for maternal, foetal toxicity and teratogenicity in rats was 800 mg/kg bw/d.

Because of staining of the skin, evaluation of irritant potential has not been possible. However, it was not irritant to the rabbit eye.

Several studies have shown it to be a contact allergen.

Percutaneous absorption of Imexidine BD, present in a hair dye formulation, has been determined to be 0.89 ± 0.31 µg/cm² in human dermatomed abdominal skin.

IMEXINE BD is mutagenic *in vitro*. It induced gene mutation in bacteria and in cultured mammalian cells. It also induced chromosome aberrations in mammalian cells *in vitro*. The test substance did not induce damage to chromosomes or the mitotic apparatus in the *in vivo* micronucleus test and did not induce DNA damage detectable with the *in vivo* UDS test. Thus, the mutagenic potential of IMEXINE BD seen *in vitro* does not lead to genotoxic or mutagenic effects *in vivo* under appropriate test conditions.

4. CONCLUSION

The SCCP is of the opinion that the information submitted is inadequate to assess the safe use of the substance.

Before any further consideration, the following information is required by July 2005:

* nature/characterisation of the impurities;

* nitrosamine content.

This hair dye, like many other hair dyes, is a skin sensitiser.

5. MINORITY OPINION

Not applicable

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7. ACKNOWLEDGEMENTS

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