Scientific Committee on Consumer Safety

SCCS

OPINION ON

Hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate

COLIPA n° C117

The SCCS adopted this opinion at its 2nd plenary meeting

of 18 June 2013

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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TABLE OF CONTENTS

1. BACKGROUND

Submission I on Hydroxyanthraquinone aminopropyl methyl morpholinium methylsulfate was submitted by COLIPA[1] in June 2003[2].

Submission II was submitted in July 2004 by COLIPA.

The Scientific Committee on Consumer Products (SCCP) adopted at its 3rd plenary meeting on 15 March 2005 an opinion (SCCP/0875/03, final) with the conclusion that:

"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"

Submission III was submitted by COLIPA in July 2005. According to the former submission the substance is used in direct hair dyes formulations at a maximum concentration of 0.5%.

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. *Does the SCCS consider hydroxyanthraquinone aminopropyl methyl morpholinium methylsulfate safe for use in non-oxidative hair dye formulations with a maximum concentration of 0.5% taken into account the new scientific data provided?*

2. *Does the SCCS recommend any further restrictions with regard to the use of hydroxyanthraquinone aminopropyl methyl morpholinium methylsulfate in any non-oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

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

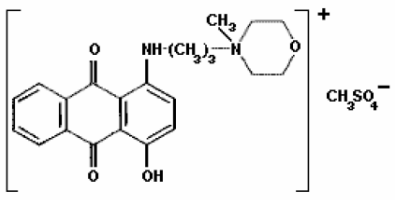
3.1.1.3. Trade names and abbreviations

Imexine BD (Chimex)

3.1.1.4. CAS / EC number

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

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

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

3.1.2. Physical form

Violet powder

3.1.3. Molecular weight

Molecular weight: 492.5 g/mol

3.1.4. Purity, composition and substance codes

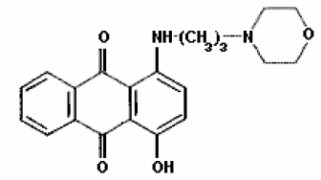
Batch OpT 54[3] was used for all the analytical determinations reported.

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)

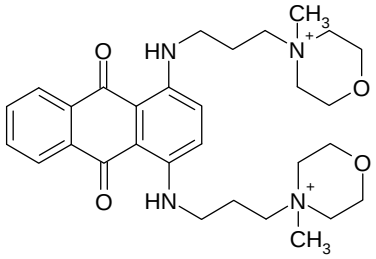
3.1.5. Impurities / accompanying contaminants

Identified Impurities:

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



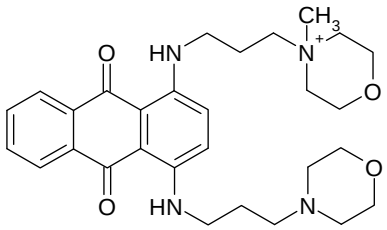
Three other impurities with following proposed chemical structures were identified
Impurity A, content 7% (mole/mole, semi-quantitative)



Exact Mass =522.3

Molecular Formula =$[C_{30}H_{42}N_4O_4]^{2+}$

Impurity B content 2.6% (mole/mole, semiquantitative)

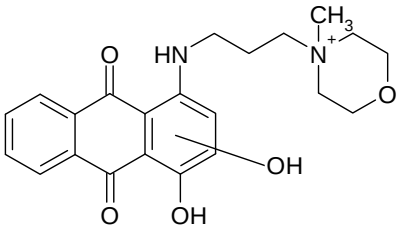


Exact Mass =507.3

Molecular Formula =$[C_{29}H_{39}N_4O_4]^{+}$

Impurity C

[3] *various descriptions were found throughout whole report*



Exact Mass =397.2

Molecular Formula =$[C_{22}H_{25}N_2O_5]^+$

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

Apparent Total Nitroso Content (ATNC) expressed as N-nitroso (NNO)

- Batch 0508813 : 270 - 340 ng/g
- Batch 0506644 : 360 ng/g
- Batch44719/01 : < 50 ng/g
- Batch OpT 54 : 120 ng/g

3.1.6. Solubility

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

Comment
Solubility in water has not been determined by EU Method A.6

3.1.7. Partition coefficient (Log Pow)

Log Pow: 2 (calculated)
Comment:
Log Pow has not been determined by EU method A.8

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: /

3.1.9. Homogeneity and Stability

Solution/suspension of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate in water at 10 mg/ml and 160 mg/ml were shown to be homogeneous (Coefficient of Variation of top, middle and bottom concentration was within 3%)

Solution/suspension of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate in water at 10 mg/ml and 160 mg/ml were shown to be stable up to 9 days (Coefficient of Variation of concentrations was within 10%)

General Comments to physico-chemical characterisation

- Hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate is a secondary amine, and thus, prone to nitrosation. The ATNC content (120-360 ppb NNO) in 3 of the 4 batches was higher than 50 ppb NNO. This indicates that the nitrosamine content in hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate may be > 50 ppb.
  - Solubility of Hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate has not been determined by EU Method A.6
  - The Log Pow strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log Pow, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.
- Stability of hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate in typical hair dye formulations is not reported

3.2. Function and uses

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate is used in direct hair dye formulations at a maximum concentration of 0.5%.

3.3. Toxicological Evaluation

Taken from previous opinion (except mutagenicity, 3.3.6)

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:	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, hypo activity, sedation and dyspnoea, one in this group male had a purple-coloured tail from day 2 to day 15 of the study. Sedation and

hypo activity were observed in the males given 1500 mg/kg. Females at both 1500 and 1000 mg/kg showed signs of sedation, hypo activity, 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.

Ref.: 1

SCCS Comment
The acute toxicity in rats is < 2000 mg/kg bw.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

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: OpT 54 ; 87.5% active
Dose: 500 mg
GLP: in compliance
Date: 1995

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

SCCS Comment
An irritant potential of neat Imexine BD could not be excluded due to skin staining.

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: OpT 54 ; 87.5% active
Dose: 100 mg
GLP: in compliance
Date: 1995

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 under the conditions of the study.

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: OpT 54 ; 87.5% active
Dose: Induction:intradermal 1% (0.875% active); epicutaneously 30%
Challenge:10% (8.75% active)
GLP: in compliance
Date: May 1995

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, the 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.

Ref: 4

SCCS Comment
Imexine BD is a strong contact allergen.

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:	OpT 54 ; 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
Date:	July 1995

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 by histopathological examination.

Results
After the first challenge (10%), erythema was observed in one control animal at the 48-hour observation point. Slight (incidence: 5/20) to well defined erythema (incidence: 2/20) was observed in treated animals 24 hours after removal of the test substance. After 48 hours, 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 had 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:	OpT 54 ; purity 100%
Dose:	Induction: 0.5ml of 10% 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
Date:	May-July 1999

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 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 removal 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 rechallenge, 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

SCCS Comment
The study report states that Imexine BD batch OpT 54 was 100% pure. However, other reports indicate that batch OpT 54 (or similarly described) was 87.5% active.

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:	OpT 54 ; 87.5% active
Dose:	Induction: 0.5ml of 5% (4.4% active) on days 1, 8 and 15.
	Challenge: 0.5ml of 1% on day 29
GLP:	in compliance
Date:	Aug-Sept 1999

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 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

SCCS comment on sensitising potential
Imexine BD is a strong skin sensitiser.

3.3.4. Dermal / percutaneous absorption

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

Guideline:	/
Species/strain:	human dermatomed abdominal skin; 465±97 µm
Group size:	4 donors; 2 samples from each donor
Integrity:	Trans epidermal water loss (TEWL)
Chamber:	Flow through diffusion cells
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:	OpT 54 ; purity 87.5 %
Stability in formulation:	0.8% loss after 1 week
Application:	20 mg/cm^2
Receptor fluid:	Physiological saline
Solubility in receptor fluid:	> 71 µg/ml
Detection:	HPLC
GLP:	in compliance
Date:	December 1999

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.

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.

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

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

Ref.: 17

SCCS Comment

As this study was non-guideline, the amount considered absorbed for calculating the MOS is mean + 2SD. This is 1.52% of the applied dose or 1.51 µg/cm².

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral toxicity

No data submitted

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

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: OpT 54
Purity: 87.5%
Dose: 0, 50, 200, 800 mg/kg bw/day by gavage
GLP: in compliance

The study protocol was similar to the 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
Batch: OpT 54

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 50, 200, 800 mg/kg bw/day for 13 weeks, a further group treated with water 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 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 males 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

SCCS comment
The SCCS considers 50 mg/kg bw/d as the NOAEL due to bw reduction in the middle dose.

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 gene mutation assay

Guideline:	OECD 471 (1994)	
Species/strain:	*S. typhimurium*, TA98, TA100, TA102, TA1535, TA1537; *E. coli*, WP2uvrA	
Replicates:	Triplicates in two independent tests	
Test substance:	IMEXINE BD	
Batch:	op. T54	
Purity:	87.5%	
Solvent:	distilled water	
Concentrations:	experiment I:	312.5, 625, 1250 and 2500 µg/plate without S9-mix 125, 250, 500, 1000 and 2000 µg/plate with S9-mix
	experiment II:	312.5, 625, 1250 and 2500 µg/plate without S9-mix 62.5, 125, 250, 500 and 1000 µg/plate with S9-mix
Treatment:	experiment I:	direct plate incorporation method with 48-72 h incubation without and with S9-mix

	experiment II:	direct plate incorporation method with 48 – 72 h incubation without S9-mix pre-incubation method with 60 minutes pre-incubation and 48 – 72 h incubation with S9-mix
GLP:	in compliance	
Study period:	June 1995	

IMEXINE BD 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. Test concentrations were based on the level of toxicity in a preliminary toxicity test with TA98 and TA100 both without and with S9-mix. Toxicity was evaluated for 6 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of spontaneous revertant colonies and/or clearing of the bacterial background lawn. Experiment I and experiment II without S9-mix was performed with the direct plate incorporation method, experiment II with S9-mix with the pre-incubation method with 60 min pre-incubation. Negative and positive controls were in accordance with the OECD guideline.

Results
In the preliminary toxicity study no substantial toxicity was found 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 a decrease in the number of revertants was observed at concentrations > 1000 µg/plate. Therefore, the maximum concentration chosen was 2000 µg/plate in the first test (direct plate incorporation) and 1000 µg/plate in the second test (preincubation method).
A biologically relevant and concentration-dependent increase in the number of revertants was found in TA100 and TA102 with S9-mix only and in TA1537 and in TA98 both without and with S9-mix. IMEXINE BD did not induce a biologically relevant increase in the number of revertants in experiments with the *E.coli* strain WP2uvrA and S*. typhimurium* strain TA 1535.

Conclusion
Under the experimental conditions used IMEXINE BD was mutagenic in this gene mutation tests in bacteria.

Ref.: 10

Bacterial gene mutation assay

Guideline:	OECD 471 (1997)	
Species/strain:	*S. typhimurium*, TA98, TA100, TA102, TA1535, TA1537	
Replicates:	triplicate cultures in two independent experiments	
Test substance:	hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate	
Batch:	0508813	
Purity:	94.5%	
Solvent:	DMSO	
Concentrations:	experiment I:	0.064, 0.32, 1.6, 8, 40, 200 and 1000 µg/plate without S9-mix 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate with S9-mix
	experiment II	20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation method with 72 h incubation without and with S9-mix	
GLP:	in compliance	
Study period:	11 December 2003 – 30 January 2004	

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate was investigated for the induction of gene mutations in *Salmonella typhimurium* strains (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in toxicity range-finder experiment with TA100 both without and with S9-mix. Toxicity was evaluated for 6 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of spontaneous revertant colonies and/or clearing of the bacterial background lawn. The range finder and both main experiments were performed with the pre-incubation method. The results from the TA100 treatments were included in experiment I. Negative and positive controls were in accordance with the guideline.

Results

In the initial range finder, complete killing of the test bacteria was observed following the top concentration both in the absence and presence of S9-mix. Further evidence of toxicity in the form of a marked decrease in the number of spontaneous revertant colonies was observed after 1000 µg/plate in the absence of S9-mix.

In the experiment I, complete killing was observed in TA98, TA100, TA1537 and TA102 without S9-mix following the top concentration but not in TA1535; with S9-mix in TA98, TA100, TA1537 and TA102 but not in TA1535 and 1537. In experiment II toxicity was observed following the top one or two concentrations in most strains both without (not in TA1535) and with (not in TA1537) S9 metabolic activation.

Concentration dependent and statistically significant increases in the number of revertants were found in TA102 (experiment I only) and TA1537 without S9-mix and in TA98, TA100 (experiment II only) and TA102 with S9-mix.

Conclusion

Under the experimental conditions used, hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate was mutagenic in this gene mutation tests in bacteria.

Ref.: 2 Subm II

Gene mutation test in mammalian cells (*tk* locus)

Guideline:	OECD 476
Cells:	L5178Y mouse lymphoma cells $tk^{+/-}$
Replicates:	duplicate cultures in two independent tests
Test substance:	IMEXINE BD
Batch:	opT 54
Solvent:	distilled water
Purity:	87.5%
Concentrations:	500, 1000, 2000, 3000 and 4000 µg/ml in experiment I without and with S9-mix and in experiment II without S9-mix. 187.5, 375, 750, 1500 and 300 µg/ml in experiment II with S9-mix
Treatment	3 h both without and with S9 mix; expression period 2 days and a selection period of 10 ± 1 days.
GLP:	in compliance
Study period:	13 April 1995 – 5 September 1995

IMEXINE BD 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 Aroclor 1254-induced rats was used as the exogenous metabolic activation system. Test concentrations were based on the results of a preliminary toxicity test with 6 concentrations up to the prescribed maximum concentration of 5000 µg/ml measuring survival relative to the concurrent vehicle control cell cultures.

In the main test, cells were treated for 3 h followed by an expression period of 2 days to fix the DNA damage into a stable *tk* mutation. To discriminate between large (indicative for

mutagenic effects) and small colonies (indicative for a clastogenic effect) colony seizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
Both in the absence and presence of S9-mix the appropriate level of toxicity (10-20% survival after the highest dose) was not reached.
In the first experiment without S9-mix a biological increase in the relative mutant frequency was not observed despite an increase in the absolute mutant frequency, obviously due to a reduced cloning efficiency in the vehicle control. In the second experiment without S9-mix, a statistically significant and concentration-related increase in the mutant frequency was measured. In the first test with S9-mix a statistically significant and concentration-dependent increase in the mutant frequency was obtained while in the second experiment the increase in mutant frequency was less obvious and not higher than a doubling of the control value. An increased number of small colonies was observed in all experiments.

Conclusion
Under the experimental conditions used, IMEXINE BD was mutagenic in this mouse lymphoma assay at the *tk* locus.

Ref.: 12

SCCS Comment
The finding of an increased number of small colonies in all experiments may indicate to a clastogenic next to a mutagenic effect of IMEXINE BD in this mouse lymphoma assay.

Gene mutation test in mammalian cells (*hprt* locus*)*

Guideline:	OECD 476 (1997)	
Cells:	L5178Y mouse lymphoma cells	
Replicates:	duplicate cultures in two independent tests	
Test substance:	hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate	
Batch:	0508813	
Solvent:	DMSO	
Purity:	94.5%	
Concentrations:	experiment I:	250, 500, 600, 700, 800, 900, 1000 and 1100 µg/ml without and with S9-mix
	experiment II	100, 250, 400, 500, 600, 700, 800, 900, 1000 and 1100 µg/ml without and with S9-mix
Treatment	3 h both without and with S9-mix; expression period 7 days and a selection period of 11-12 days.	
GLP:	in compliance	
Study period:	4 December 2003 – 26 January 2004	

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate was assayed for gene mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. Liver S9 fraction from Arachlor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a cytotoxicity range-finding experiment measuring relative survival with 6 concentrations up to the maximum concentration of 2000 µg/ml. In the main tests, cells were treated for 3 h followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured as percentage survival of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the cytotoxicity range-finder experiment, precipitation and extreme cytotoxicity (10% relative survival) was observed at the two highest concentrations (1000 and 2000 µg/ml) both without and with S9-mix after the 3 h exposure period. The highest concentration to

give > 10% relative survival (500 µg/ml) yielded 44% and 46% relative survival without and with S9-mix, respectively. In experiment I the highest concentrations analysed were 900 µg/ml without S9-mix and 1000 µg/ml with S9-mix giving 14 and 13% relative survival, respectively; in experiment II 1000 µg/ml without S9-mix and 1100 µg/ml with S9-mix giving 13 and 16% relative survival.
In experiment I in the presence of S9-mix occasionally statistically significant increases in the mutant frequency were observed. However, the mutant frequencies were predominantly within the range of the historical controls. As a biologically relevant increase in the mutant frequency was not found in experiment II in the presence of S9-mix, the positive results could of experiment I could not be reproduced in experiment II and, consequently, were considered as not biologically relevant.
No biological relevant increases in mutant frequencies were observed following treatment with hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate at any dose level tested, in the absence of S9-mix in both experiments.

Conclusion
Under the experimental conditions used, hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate was considered not mutagenic in this gene mutation test in mammalian cells at the *hprt* locus.

Ref.: 3 Subm II

Chromosome aberration test in mammalian cells

Guideline:	OECD 473 (1994)	
Cells:	Chinese hamster ovary (CHO) cells	
Replicates:	duplicate cultures in 2 independent tests	
Test substance:	IMEXINE BD	
Batch:	op T54	
Solvent:	distilled water	
Purity:	87.5%	
Concentrations:	experiment I:	50, 150, and 500 µg/ml without S9-mix
		500, 1500 and 5000 µg/ml with S9-mix
	experiment II	125, 250 and 500 µg/ml without S9-mix (20 h)
		1250, 2500 and 5000 µg/ml with S9-mix (20 h)
		125, 250 and 375 µg/ml without S9-mix (44 h)
		1250, 2500 and 5000 µg/ml with S9-mix (44 h)
Treatment:	experiment I:	20 h treatment and harvest time 20 h after start of treatment without S9-mix
		3 h treatment and harvest time 20 h after start of treatment without S9-mix
	experiment II:	20 h treatment and harvest time 20 h after start of treatment without S9-mix
		3 h treatment and harvest time 20 h after start of treatment without S9-mix
		44 h treatment and harvest time 44 h after start of treatment without S9-mix
		3 h treatment and harvest time 44 h after start of treatment without S9-mix
GLP:	in compliance	
Study period:	24 January 1995 – 19 April 1995	

IMEXINE BD has been investigated for induction of chromosomal aberrations in CHO cells. Liver S9 fraction from Aroclor1254-induced rats was used as the exogenous metabolic activation system. IMEXINE BD was freely soluble in distilled water at 150 mg/ml expressed in active material. Up to the prescribed maximum concentration of 5000 µg/ml, no

precipitation was observed. Therefore, in the first experiment both with and without S9-mix 6 concentrations were used up to 5000 µg/ml. Next to 2 lower concentrations, the top concentrations for scoring were based upon a 38-65% reduction in the mitotic index. In the different experiments, cells were treated continuously for 20 or 44 h and harvested immediately after the end of treatment or for 3 h and harvested 20 or 44 h after the start of treatment. Approximately 1.5 h before harvest, each culture was treated with colcemid to block cells at metaphase of mitosis. Negative and positive controls were in accordance with the OECD guideline.

Results

In the experiments with a harvest time of 20 h the required reduction in mitotic index was found but not in the experiments with a harvest time of 44 h.

The test substance induced a statistically significant and concentration dependent increase in the number of cells with chromosome aberrations in all experiments with and without S9-mix at both harvest times.

Conclusion

Under the experimental conditions used, IMEXINE BD was considered genotoxic (clastogenic) in this chromosome aberration test in mammalian cells (CHO cells).

Ref.: 11

Chromosome aberration test in human peripheral blood lymphocytes

Guideline:	OECD 473 (1997)	
Species/Strain:	Human peripheral lymphocytes from three healthy female donors	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate	
Batch:	0508813	
Solvent:	DMSO	
Purity:	94.5%	
Concentrations:	experiment 1:	103.1, 161.1 and 251.7 µg/ml without S9-mix
		52.79, 103.1 and 251.7 µg/ml with S9-mix
	experiment 2:	72.54, 100.4 and 118.1 µg/ml without S9-mix
		118.1, 139.0 and 226.3 µg/ml with S9-mix
Treatment	experiment 1:	3 h treatment without and with S9-mix; harvest time 20 h after the start of treatment
	experiment 2:	20 h treatment without S9-mix; harvest time 20 h after start of treatment.
		3 h treatment with S9-mix; harvest time 20 h after start of treatment.
GLP:	in compliance	
Study period:	9 December 2003 – 4 February 2004	

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate has been investigated for the induction of chromosomal aberrations in human lymphocytes of 3 healthy non-smoking female donors both in the absence and presence of metabolic activation. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. In both experiments for both harvest times and in the absence and presence of S9-mix, human lymphocytes were exposed to various concentrations of hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate. The concentrations for chromosome analysis were selected on the basis of the effect of hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate on the mitotic index. Chromosome aberrations were analysed at 3 concentrations, the highest concentration inducing approximately 55% mitotic inhibition.

Cells were treated for 3 h (without and with S9-mix) or 20 h (without S9-mix) and harvested 20 h after the start of treatment. Approximately 2 h before harvest, each culture

was treated with colcemid (1 µg/ml culture medium) to block cells at metaphase of mitosis. Negative and positive controls were in accordance with the OECD guideline.

Results

Although after 3 h treatment in the absence S9-mix an increase in the number of cells with chromosomal aberrations was found at the highest concentration tested, a biologically relevant and concentration dependent increase in the number of cells with chromosome aberrations was not found.

Statistically significant and concentration dependent increases in the number of cells with chromosomal aberrations compared to the concurrent negative controls and the historical negative control range were found for both experiments with S9-mix and after 20 h exposure without S9-mix.

Increases in the number of cells with numerical aberrations, that exceeded the concurrent controls and the historical negative control range, were found in experiment 1 in the presence of S9-mix at the highest concentration tested. As these findings were not reproduced in experiment 2 and not found in the experiments without S9-mix, they were considered not biologically relevant.

Conclusion

Under the experimental conditions used, hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate was genotoxic (clastogenic) in this chromosome aberration test in human lymphocytes.

Ref.: 4 Subm II

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474
Species/strain:	Mouse, Swiss OF1/ICO:OF1 (IOPS Caw)
Group size:	5 mice/sex/group
Test substance:	IMEXINE BD
Batch:	op. T54
Purity:	87.5%
Vehicle:	distilled water
Dose levels:	0, 500, 1000 and 2000 mg/kg bw/day
Route:	orally, twice at 24h interval
Sacrifice times:	24 h after treatment the last treatment
GLP:	in compliance
Study period:	6 November 1995 – 30 April 1996

IMEXINE BD has been investigated for induction of micronuclei in bone marrow cells of mice. Test doses were based on the results of a preliminary toxicity test on a group of 3 male and 3 female mice recording clinical signs and mortality for a period of 48 h performed under identical conditions as in the main study.

In the main experiment male and female mice were exposed orally twice at 24 h intervals to 0, 500, 1000, 2000 mg/kg bw/day. The mice were examined for acute toxic symptoms and/or mortality. Bone marrow cells were collected 24 h after the last treatment. For each mouse the percentage of polychromatic erythrocytes with a micronucleus was counted in 2000 polychromatic erythrocytes. In addition, for each mouse of the vehicle control and the highest dose group an additional 2000 polychromatic erythrocytes will be counted. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Negative and positive controls were in accordance with the OECD guideline.

Results
Since in the preliminary toxicity tests no toxic effects were observed, 2000 mg/kg bw was selected as the top dose-level.
One female mouse of the 2000 mg/kg bw/day group was found dead 2 h after the last treatment. No other mortality was observed. No clinical signs were observed in the mice of both sexes in any group. 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.
In the groups treated with 500 and 2000 mg/kg bw/day an increase in the number of polychromatic erythrocytes with micronuclei was observed compared to the untreated control group. However, the mean MNPCE frequencies were not statistically significantly increased in any of the groups treated with the test substance. Moreover, the findings were always within the range of the historical negative control values.

Conclusions
Under the experimental conditions used IMEXINE BD did not induce an increase in the number of bone marrow cells with micronuclei and, consequently, IMEXINE BD is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 13

SCCS Comment
Only the average numbers of bone marrow with micronuclei per group are reported and not the individual data per mouse. The lack of the individual data per mouse diminishes the value of the test.

Unscheduled DNA Synthesis (UDS) Test

Guideline:	draft OECD 486 (1991)
Species/strain:	rat, Wistar HanIbm: WIST (SPF)
Group size:	4 male rats/group
Test substance:	Imexine BD in
Batch No.:	Op T54
Purity:	87.5%
Vehicle:	deionised water
Dose levels:	0, 200 and 2000 mg/kg bw
Route:	orally by gavage
Sacrifice times:	2 (high dose group only) and 16 hours
GLP:	in compliance
Study period:	10 July 1997 – 7 October 1997

Imexine BD was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test doses were based on a pre-experiment for toxicity, using the same conditions as in the UDS test, measuring acute toxic symptoms at intervals of 1 h and 24 h after oral administration of 2000 mg/kg bw. In the main experiment the rats were treated with 0, 200 and 2000 mg/kg bw once by oral gavage. The animals were starved before treatment.
Hepatocytes for UDS analysis were collected by perfusion with 0.05% w/v collagenase approximately 2 h (high dose only) and 16 h after administration of Imexine BD. The quality of the actual performed perfusion was determined by the trypan blue dye exclusion method.
At least 3 cultures were established for each animal. At least 90 minutes after plating the cells were incubated for 4 h with 5 µCi/ml ^{3}H-thymidine (specific activity 20 Ci/mmol) followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 15 days.
The number of grains in a nuclear area and the number in one nuclear-sized cytoplasmic area adjacent to this nucleus was counted. At least 2 slides per rat and 50 cells per slide were evaluated. The mean nuclear and cytoplasmic grain counts as well as the mean net grain counts (nuclear minus cytoplasmic grain count) were reported separately.

Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment for toxicity at 2000 mg/kg bw both rats showed apathy 1 h and excitement 24 h after treatment. For both rats violet colored urine was reported at 24 h.
The viability of the hepatocytes determined by means of the trypan blue dye exclusion assay was not substantially affected by the treatment and was in the range of the historical laboratory control data.
A biological relevant increase in mean net nuclear grain count as compared to the untreated control was not found in hepatocytes of any treated animal both for the 2 h and the 16 h treatment time.

Conclusions
Under the experimental conditions used, Imexine BD did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 14

Unscheduled DNA Synthesis (UDS) Test

Guideline:	draft OECD 486
Species/strain:	rat, Crl:CD®(SD)IGS BR
Group size:	4 male rats/group
Test substance:	hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate
Batch:	0508813
Vehicle:	water
Purity:	94.5%
Dose level:	0, 500, 1000 and 2000 mg/kg bw
Route:	oral gavage
Sacrifice times:	2- 4 h and 14-16 h after dosing
GLP:	in compliance
Study period:	18 November 2003 – 4 February 2004

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test doses were based on a dose range finding study for toxic symptoms and/or mortality. Five groups of 3 rats were treated orally with doses ranging from 270 up to 2160 mg/kg bw and were observed at intervals of 1, 2 and 4 h and daily after treatment. In the main experiment the rats were treated with 0, 500, 1000 and 2000 mg/kg bw once by oral gavage.
Hepatocytes for UDS analysis were collected by perfusion with HBBS/EGTA followed by WMEC. 2-4 and 14-16 h after administration of hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate. The hepatocytes were obtained by mechanical dispersion of excised liver tissue. After an attachment period of1.5 to 2 h after plating the cells were incubated for 4 h with 10 µCi/ml ^{3}H-thymidine (specific activity 40-60 Ci/mmol) followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 8 days.
UDS was reported as net grains per nucleus: the nuclear grain count subtracted with the average number of grains of 3 nuclear-sized areas adjacent to each nucleus. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 3 replicate slides per rat. Negative and positive controls were in accordance with the OECD guideline.

Results
In the dose range finder study for toxicity, 2 rats showed purple stain at the front feet (540 mg/kg bw), 3 rats showed purple stain at all feet (1080 and 2160 mg/kg bw) and all rats treated with doses of 1080 mg/kg bw and above showed discoloured black faeces. In the rats which were sacrificed 2-4 h after treatment, all rats showed purple stain at the front feet. The rats of the 14-16 sacrifice groups had purple stain at the front or front feet (500

and 2000 mg/kg bw), discoloured black faeces (500 and 1000 mg/kg bw) or soft faeces, dark orange genital discharge of black faeces (2000 mg/kg bw). The viability of the isolated hepatocytes ranged from 70 – 100%.
Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate did not cause any biological relevant of statistically significant changes in the degree of nuclear labelling of cultured hepatocytes after treatment of male rats, whether assayed at 2-4 or 14-16 h after treatment.

Conclusions
Under the experimental conditions used, hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test. Ref.: 14

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

Preliminary study

Guideline:	/
Species/strain:	Sprague-Dawley rats Crl CD (SD) BR
Group size:	7 mated females
Test substance:	Imexine BD suspended in water
Batch:	OpT 54
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 checked daily. 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 in the 800 mg/kg bw dose group. 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
Batch:	OpT 54
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 NOAEL for maternal and foetotoxicity as well as teratogenicity was found to be 800 mg/kg bw/d.

Ref.: 16

3.3.9. Toxicokinetics

No data submitted

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

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate

Absorption through the skin	**A**	**=**	**1.51 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.0.876 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.015 mg/kg bw/d**
No Observed Adverse Effect Level (13-week, oral route, rat)	**NOAEL**	**=**	**50 mg/kg bw/d**
Bioavailability 50%		**=**	**100**
MOS		**=**	**1667**

3.3.14. Discussion

Physico-Chemical Properties

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate is used in direct hair dye formulations at a maximum concentration of 0.5%.

Purity: 87.5%. Impurities include 1.2% 1-Hydroxy-4-(3-morpholin-4-yl-propylamino)-anthracene-9,10 dione, three other impurities with proposed tentative structures water and residual solvents.Hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate is a secondary amine, and thus, it is prone to nitrosation. ATNC (Apparent Total Nitroso Content expressed as N-nitroso (NNO)) content in 3 of the 4 batches was 120-360 ppb NNO, indicating that nitrosamine content in Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate may be over 50 ppb. The nitrosamine content must be below 50 ppb, and the hairdye should not be used together with nitrosating agents in a hairdye formulation. Solubility of Hydroxyanthraquinone-aminopropyl methyl morpholinium methosulfate has not been determined by EU Method A.6. The Log Pow strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log Pow, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies. Stability of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate in typical hair dye formulations has not been reported.

General Toxicity

The acute oral toxicity of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate (87.5% pure) in both sexes of rats was estimated to be < 2000 mg/kg. The NOAEL was 50 mg/kg bw/d in a 13 week sub-chronic oral toxicity study in rats. The NOAEL for maternal, foetal toxicity and teratogenicity in rats was 800 mg/kg bw/d.

Irritation/Sensitisation

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

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate is a strong contact allergen.

Percutaneous absorption

Percutaneous absorption of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate, present in a hair dye formulation, has been determined to be 0.89 ± 0.31

µg/cm² in human dermatomed abdominal skin. As this study was non-guideline, the amount considered absorbed for calculating the MOS is mean + 2SD. This is 1.52% of the applied does or 1.51 µg/cm².

Mutagenicity/Genotoxicity

Overall, the genotoxicity of hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy.

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate induced gene mutations both in two gene mutation tests in bacteria and in a mouse lymphoma assay at the *tk* locus. In the latter test, next to large colonies, also the number of small colonies increased which may indicate to a clastogenic next to a mutagenic effect of. Two *in vitro* chromosome aberration tests were positive confirming the clastogenic potential found in the mouse lymphoma assay. A gene mutation test in mammalian cells using the *hprt* locus was negative.

The positive findings from the *in vitro* tests for both gene mutations and chromosome aberrations were not confirmed in *in vivo* tests. An *in vivo* micronucleus test in mice and two unscheduled DNA synthesis tests were negative.

Consequently, on the basis of these tests, hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate can be considered to have no genotoxic potential and additional tests are unnecessary.

4. CONCLUSION

The SCCS is of the opinion that the use of Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate with a maximum concentration of 0.5% in non-oxidative hair dye formulations does not pose a risk to the health of the consumer, apart from its sensitising potential.

Hydroxyanthraquinone aminopropyl methyl morpholinium methosulfate is a secondary amine, and thus it is prone to nitrosation. It should not be used together with nitrosating agents. The nitrosamine content should be <50 ppb.

This hair dye is a strong skin sensitiser.

5. MINORITY OPINION

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