Scientific Committee on Consumer Safety

SCCS

OPINION ON

2,3-Diaminodihydroxypyrazolopyrazolone dimethosulfonate

COLIPA n° A159

The SCCS adopted this opinion at its 16th plenary meeting

of 18 September 2012

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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1. BACKGROUND

Submission I for the new hair dye substance 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate (CAS 857035-95-1) was submitted in January 2008 by COLIPA[1].

According to the submission the intended use is as an oxidative hair dye substance with a final concentration on the scalp of up to max 2.0%

2. TERMS OF REFERENCE

1. *Does the SCCS consider 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate safe for use as an oxidative hair dye with a concentration on-head of maximum 2.0 % taken into account the scientific data provided?*

2. *And/or does the SCCS have any further scientific concerns with regard to the use 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate in oxidative 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

2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate (INCI-name)

3.1.1.2. Chemical names

2,3-Diamino-6,7-dihydro-1H,5H-pyrazolo[1,2-a] Pyrazol-1-one dimethanesulfonate
2,3-Diamino-6,7-dihydro-1H,5H-pyrazolo[1,2-a] Pyrazol-1-one dimesylate

3.1.1.3. Trade names and abbreviations

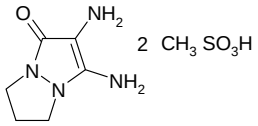
Imexine OBH
R0054002C

3.1.1.4. CAS / EC number

CAS: 857035-95-1
EC: 469-500-8

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_6H_{10}N_4O$, 2 CH_3SO_3H

3.1.2. Physical form

Beige to yellow powder

3.1.3. Molecular weight

Molecular weight: 346.38 g/mol

3.1.4. Purity, composition and substance codes

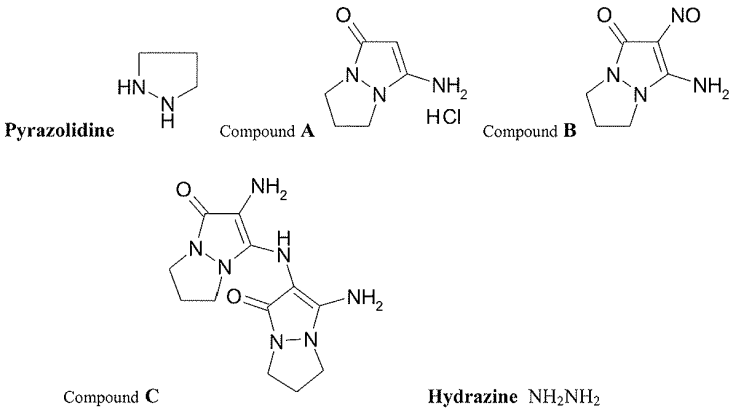
	Imexine OBH		
	009 D 001	**013 L 001**	**013 L 004**
Appearance	Light yellow powder		
Identification			
Infrared spectra	In accordance with the proposed structure		
UV spectra	Compatible with the proposed structure		
1H and 13C NMR spectra	In accordance with the proposed structure		
Mass spectra	Compatible with the proposed structure		
Assays			
Titre by Potentiometry (% w/w) ($HClO_4$ in an acetic anhydride medium)	99.3	99.8	99.8
Titre by HPLC (% w/w) [1]	100	99.5	98.2
HPLC profile UV purity (%) [2]	> 99	> 99	> 99
Impurities content by HPLC			
- Pyrazolidine (step n-3) (µg/g)	< 3 µg/g (D)	< 3 µg/g (ND)	< 3 µg/g (D)
- Compound A (step n-2) [3] **(% w/w)**	< 0.1 (ND)	< 0.1 (D)	< 0.1 (D)
- Compound B (step n-1) [4] **(% w/w)**	< 0.1 (ND)	< 0.1 (ND)	< 0.1 (ND)
- Compound C [5] **(% w/w)**	0.07	0.15	0.24
Hydrazine (µg/g)	< 0.5 µg/g (D)	< 0.5 µg/g (D)	< 0.5 µg/g (D)
Residual solvents by GC			
- Toluene (µg/g)	< 100 µg/g (D)	< 100 µg/g (D)	< 100 µg/g (D)
- Ethanol (% w/w)	< 0.2 (D)	< 0.2 (D)	< 0.2 (D)
Methanesulfonic acid by HPLC (% w/w) (theoretical value: 55.49)	53.4	53.7	55.9
Water content (KF method) (% w/w)	0.03	0.16	0.12
Melting point (DSC)	184.5	184.8	184.4
Ash content (% w/w)	< 0.1	< 0.1	< 0.1
Elemental analysis	Compatible with the proposed structure		

D: detected
ND: not detected
(1) against batch 009 D 001 reference standard considered as pure (100% w/w)
(2) UV detection: UV purity – area%, without response factor.
Irrespective of residual solvents, salts and other non-detectable products
(3) 3-amino-6,7-dihydro-1H,5H-pyrazolo[1,2-a]pyrazol-1-one hydrochloride
(4) 3-amino-2-nitroso-6,7-dihydro-1H,5H-pyrazolo[1,2-a]pyrazol-1-one

(5) 3-amino-2-[(2-amino-1-oxo-6,7-dihydro-1H,5H-pyrazolo[1,2-a]pyrazol-3-yl)amino]-6,7-dihydro-1H,5H-pyrazolo[1,2-a]pyrazol-1-one

Results are expressed in terms of the base.

Compounds A and B: each impurity content was carried out against a reference standard considered as pure. Compound C: C content was carried out against Imexine OBH batch 009 D 001 reference standard considered as pure, UV spectra being similar. Results are expressed in terms of the base.



Comment
No reference standard with known content of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate was used for the quantification of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in the 3 reported batches of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate.

3.1.5. Impurities / accompanying contaminants

See 3.1.4

Metal contents

Imexine OBH Batch 009 D 001
Cr: 1 mg/kg
Fe: 4 mg/kg
Al, As, Ba, Cd, Co, Cu, Mn, Mo, Ni, Pb, Pd, Se, Sn, Ti, V, Zn: Each < 1 mg/kg
Hg: < 0.1 mg/kg

Imexine OBH Batch 013 L 001
Al, As, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Pd, Se, Sn, Ti, V, Zn: Each < 1 mg/kg
Hg: < 0.1 mg/kg

Imexine OBH Batch 013 L 004
Fe: 2 mg/kg
Al, As, Ba, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Pd, Se, Sn, Ti, V, Zn: Each < 1 mg/kg
Hg: < 0.1 mg/kg

3.1.6. Solubility

Water solubility according to EEC method A6: > 603 g/L at 23 °C
Ethanol: S ≤ 1g/100 ml at 23 °C after 24h
DMSO: S ≥ 20g/100 ml at 23 °C after 24h

Comment
Analytical report for water solubility according to EEC method was not submitted

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow}: - 2.8 (experimental, according to EEC method A8)

Comment
Analytical report was not submitted

3.1.8.	Additional physical and chemical specifications

Melting point:	183 – 186 °C
Flash point:	/
Vapour pressure:	/
Boiling point:	/
Density at 20 °C:	/
Viscosity:	/
pKa:	/
UV absorption spectrum:	λmax 238 nm
Refractive index at 20 °C:	/

3.1.9.	Stability

2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in the dosage forms at 20 and 200 mg/mL in purified water was stable over a 6-hour period at room temperature and a 9-day period at +4°C, protected from light and under inert gas atmosphere (deviation from nominal concentration ≤ 8%).

2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in dosage forms at 0.1, 40 and 200 mg/mL in water for injection and at 50 and 180 mg/mL in propylene glycol was stable over a 4-hour period at room temperature, protected from light and under inert gas atmosphere (deviation from nominal concentration ≤ 6%).

General comments on physico-chemical properties

- No reference standard with known content of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate was used for the quantification of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate in the 3 reported batches of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate. The reported content of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in the 3 batches of test item should be considered as semiquantitative measurements.
- Stability of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate in typical hair dye formulations has not been provided.

3.2. Function and uses

2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate is used in oxidative hair dye formulations at a maximum on-head concentration of 2%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 423 (2001)
Species/strain:	rat, Sprague-Dawley Rj: SD (IOPS Han)
No. of animals:	6 females (nulliparous and non-pregnant)
Test substance:	IMEXINE OBH
Batch:	0604070001
Purity:	99.7%
Vehicle:	purified water
Dose:	2000 mg/kg bw
Administration:	oral route (gavage), 10 mL/kg bw
GLP:	in compliance
Study period:	12 September – 3 October 2006

The study was performed according to the "acute toxic class method" (OECD 423). The starting dose level was selected from one of 4 fixed levels (5, 50, 300 or 2000 mg/kg bw), and chosen at 2000 mg/kg bw based on a previous study [14]. On a day designated as day 1, three (3) overnight fasted female SD rats (age about 8 weeks, mean body weight ± SD 192 ± 6 g) received a single oral (gavage) dose of Imexine OBH in distilled water (10 mL/kg bw). Animals were observed regularly on the day of dosing and then at least daily for 14 days. Body weights were recorded on day 1 just before treatment and on days 3, 8 and 15. Animals were killed at the end of the study and subjected to macroscopic examination. No organ/tissue samples were taken. As no death occurred at the dose-level of 2000 mg/kg bw, the results were confirmed in three other females (body weight ± SD 195 ± 1 g).

Results
No mortality occurred at the dose-level of 2000 mg/kg bw. Hypoactivity and piloerection (all the animals) and dyspnea (3/6 animals) were observed within four hours of treatment. No clinical signs persisted thereafter, until the end of the observation period (day 15).
The overall body weight gain of the animals was not affected by treatment with Imexine OBH. There were no macroscopic findings at necropsy.

Conclusion
Under the conditions of this study, the maximal non-lethal dose of Imexine OBH following a single oral gavage to fasted female rats was higher than 2000 mg/kg bw.

Ref.: 1

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402 (1987)
Species/strain:	rat, Sprague-Dawley Rj: SD (IOPS Han)
No. of animals:	10 (5 males and 5 females)
Test substance:	IMEXINE OBH
Batch:	0604070001
Purity:	99.7%
Vehicle:	purified water
Dose:	2000 mg/kg bw
GLP:	in compliance
Study period:	5 – 19 September 2006

Five (5) male and 5 female SD rats (age about 8 weeks, mean body weights ± SD 308 ± 9 g for the males and 219 ± 10 g for the females) received a single topical dose of Imexine OBH in its original form at 2000 mg/kg bw using a moistened compress on an area of the skin representing approximately 10% of the total body surface of the animals. The test site was then covered by a semi-occlusive dressing for 24 hours. On removal of the dressing, any residual test item was wiped off using a moistened cotton pad. Animals were observed immediately after cutaneous application, and then at least once a day throughout the study (day 1 to day 15). Body weights were recorded on day 1 just before treatment and on days 8 and 15. Animals were killed at the end of the study and subjected to macroscopic examination. No organ/tissue samples were taken.

Results
No mortality occurred following cutaneous application at 2000 mg/kg bw in male or female animals. No systemic clinical signs were observed during the study. Yellow discolouration of the skin was noted in all animals throughout the observation period. Crusts were noted in 5/10 animals: from day 5 to day 6 in one animal, from day 5 to day 11 in two animals, from day 5 to day 15 (end of the observation period) in one animal and from day 9 to day 11 in one animal. A dryness of the skin was observed in 2/10 animals: from day 9 to day 11 in one animal and from day 9 to day 15 in one animal.

The overall body weight gain of the animals was not considered to be affected by treatment with Imexine OBH.

There were no macroscopic findings at necropsy.

Conclusion
Under the conditions of this study, the maximum non-lethal dose of Imexine OBH following a single dermal application to rats was higher than 2000 mg/kg bw.

Ref.: 2

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 (2002)
Species/strain:	rabbit, New Zealand White
No. of animals:	3 male
Test substance:	IMEXINE OBH
Batch:	0604070001
Purity:	99.7%
Dose:	500 mg (moistened with purified water)
GLP:	in compliance
Study period:	12 – 17 September 2006

The test item was first applied for periods of 3 minutes, 1 hour and 4 hours to a single male New Zealand White rabbit. Since the test item was neither severely irritant nor corrosive on this first animal, it was then applied for 4 hours to two other animals.
A single dose of 500 mg of the test item in its original form was placed on a gauze pad moistened with purified water, which was then applied to the closely-clipped skin of the rabbits. The test item was held in contact with the skin by means of an adhesive hypoallergenic aerated semi-occlusive dressing and restraining bandage.
Cutaneous reactions were observed approximately 1 hour, 24, 48 and 72 hours after removal of the dressing.

The mean values of the scores for erythema and oedema were calculated for each animal.

Results
No cutaneous reactions were observed. A yellow coloration of the skin was noted all over the study after a 1-hour exposure (one animal) and a 4-hour exposure (three animals).

Conclusion
Under the experimental conditions, the test item IMEXINE OBH was non-irritant when applied topically to rabbits.

Ref.: 3

3.3.2.2. Mucous membrane irritation

Neat substance

Guideline:	OECD 405 (2002)
Species/strain:	rabbit, New Zealand White
No. of animals:	1 male
Test substance:	IMEXINE OBH
Batch:	0604070001
Purity:	99.7%
Dose:	100 mg
GLP:	in compliance
Study period:	19 – 22 September 2006

A single dose of 100 mg of the test item in its original form was introduced into the left conjunctival sac of one New Zealand Whit rabbit. The right eye was not treated and served as control. The eyes were not rinsed after administration of the test item. Ocular reactions were observed approximately 1 hour, 24, 48 and 72 hours after the administration. The mean values of the scores for chemosis, redness of the conjunctiva, iris lesions and corneal opacity were calculated.

Results
A slight chemosis and a slight redness of the conjunctiva were noted on day 1; it increased to a marked chemosis on day 2 and to a severe chemosis on days 3 and 4.
On days 3 and 4, the eyelids of the animal were stuck together. In order to assess the ocular lesions, the eyelids were separated with 0.9% NaCl.
A slight redness of the conjunctiva was observed on day 1. It increased to a severe redness of the conjunctiva until day 4. Clear to whitish purulent discharge was noted from day 1 until day 4. An iritis was noted from day 2 until day 4.
A marked corneal opacity covering one half to three quarters of the corneal area was recorded on day 2. Then, it decreased to a moderate corneal opacity, which covered one quarter to one half of the corneal area on day 2 and the whole area of the cornea on day 4.
As severe irritant effects were observed, the animal was killed on day 4 for ethical reasons.

Conclusion
Under the experimental conditions, the test item IMEXINE OBH was severely irritant when administered by ocular route to rabbits.

Ref.: 4

Diluted substance

Guideline:	OECD 405 (2002)
Species/strain:	rabbit, New Zealand White
No. of animals:	3 males
Test substance:	IMEXINE OBH

Batch: 013 L 001
Purity: 99.7%
Vehicle: purified water
Dose: 5% in purified water
Administration: 0.1 mL
GLP: in compliance
Study period: 18 – 23 October 2006

A single dose of 0.1 mL of the test item at the concentration of 5% (w/w) in purified water was instilled into the left conjunctival sac of New Zealand Whit rabbits. The right eye was not treated and served as control. The eyes were not rinsed after administration of the test item. Ocular reactions were observed approximately 1 hour, 24, 48 and 72 hours after the administration. The mean values of the scores for chemosis, redness of the conjunctiva, iris lesions and corneal opacity were calculated for each animal.

Results
A slight chemosis and a slight redness of the conjunctiva were observed in all the animals on day 1; the slight redness of the conjunctiva persisted on day 2 in one animal.
No other ocular reactions were observed during the study.
Under the experimental conditions, the applicant concluded that the test item 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate at the concentration of 5% in purified water was well-tolerated when administered by ocular route to rabbits.

Ref.: 5

Comment
The SCCS considers 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate diluted at 5% in water slightly irritant to the rabbit eyes.

3.3.3. Skin sensitisation

Local lymph node assay (LLNA)

Guideline: OECD 429 (2002)
Species: CBA/J mouse, nulliparous and non-pregnant females
Group: 28 females (4 animals per group)
Substance: IMEXINE OBH
Batch: 0604070001
Purity: 99.7%
Concentration: 0, 2.5, 5, 10, 15, 18%
Dose: 25 µL
Vehicle: propylene glycol
Negative control: propylene glycol
Positive control: α-hexylcinnamaldehyde (HCA), 25%
GLP: in compliance
Study period: 12 – 25 September 2006

The study of the possible allergic potential of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate was done by the local lymph node assay. Five groups of four female mice were treated with 2.5, 5, 10, 15 and 18 % of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in propylene glycol by topical application at the dorsum of each ear lobe on three consecutive days. A negative control group was treated with the vehicle only and a positive control group was treated with α-hexylcinnamaldehyde at 25%. The test item was solved in propylene glycol due to the unsatisfactory solubility in the recommended vehicles.

Five days after the first topical application, the mice were intravenously injected with radio-labelled thymidine. Animals were sacrificed and the draining auricular lymph nodes excised.

The proliferative capacity of pooled lymph node cells were determined by the incorporation of ^{3}H-methyl thymidine measured by β–scintillation counter.

Results

Treatment	Concentration	Stimulation index
2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in propylene glycol	2.5% 5% 10% 15% 18%	0.80 1.74 1.56 2.51 1.84
α-Hexylcinnamaldehyde	25%	5.92

Conclusion
Under the experimental conditions 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate does not induce contact sensitization in the murine Local Lymph Node Assay.

Ref.: 6

Comment
The highest concentration tested was 18% as a homogeneous suspension. No reason was given for not using a higher concentration. No firm conclusion regarding the sensitising potential of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate can be drawn.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (2004)
Tissue:	Human skin, full-thickness from 6 female donors (oxidative condition) and from 5 female donors (non-oxidative condition)
Membranes:	split-thickness membranes, 200-500 µm
Skin integrity:	tritiated water (k_p < 2.5 x 10^{-3} cm/h)
Method:	automated flow-through diffusion cell
Replicate cells:	12 cells
Test substance:	IMEXINE OBH [carbonyl-^{14}C]-Imexine dimesylate (radio-labelled)
Batch:	013 L 001 CFQ14980 Batch 1 (1.18 GBq/mmol, 32 mCi/mmol)
Purity:	99.7% (HPLC) 98.3% (radiochemical purity)
Receptor fluid:	calcium and magnesium free phosphate buffered saline (PBS)
Test formulations:	Formulation containing the primary intermediate R0054002C at 2.94%, (w/w) and the coupler 4-amino-2-hydroxytoluene at circa 1.42%, (w/w) to which [^{14}C]-R0054002C is added. Formulation containing R0054002C at 2.94% (w/w) to which [^{14}C]-R0054002C is added.
Dose applied:	20 mg/cm²
Solubility receptor fluid:	> 603 g/L
Analytical method:	liquid scintillation counting
GLP:	in compliance
Study period:	29 January – 17 May 2007

Dermal absorption of Imexine OBH was tested under oxidative and non-oxidative conditions incorporated into a typical hair dye formulation to give a final concentration of 2% (w/w). [^{14}C]-R0054002C was incorporated into a typical oxidative hair dye formulation at *circa* 4% (w/w) before mixing with developer (1:1, w/w), to give a final concentration of R0054002C

of *circa* 2% (w/w). R0054002C was incorporated into the same typical oxidative hair dye formulation (without coupler) at *circa* 4% (w/w) before being mixed with water (1:1, w/w). Absorption was assessed by collecting receptor fluid (PBS) samples from 0 to 0.5, 0.5 to 1 h and then hourly from 1 to 24 h post dose (flow rate 1.5 mL/h). At 30 min post dose, the skin was washed with water, sodium dodecyl sulphate (SDS) solution (*circa* 2% w/v) and water again. The skin was then dried with tissue paper swabs. At 24 h post dose the underside of the skin was rinsed with receptor fluid. The skin was then removed from the flow-through cells, dried and the stratum corneum removed by tape stripping. All liquid samples were analysed by liquid scintillation counting.

Results
Under the present experimental conditions, for in the oxidative test preparation, most of the applied dose was removed at 30 min post dose (91.21% of the applied dose). At 24 h post dose, a further 0.30% was removed. Therefore, the dislodgeable dose was 91.51% of the applied dose. At 24 h post dose, the absorbed dose and dermal delivery were 0.08% (0.35 µg equiv/cm²) and 0.39% (1.68 µg equiv/cm²) of the applied dose, respectively.
Under the present experimental conditions, in the non-oxidative test preparation, most of the applied dose was removed at 30 min post dose (94.03% of the applied dose). At 24 h post dose, a further 0.33% was removed. Therefore, the dislodgeable dose was 94.36% of the applied dose.

Summary of the mean results:

Formulation / Test Preparation	Oxidative	Non-Oxidative
Target R0054002C Concentration in Formulation (%, w/w)	4.00	4.00
Actual R0054002C Concentration in Formulation (%, w/w)	3.95	4.25
Target R0054002C Concentration in Test Preparation (%, w/w)	2.00	2.00
Actual R0054002C Concentration in Test Preparation (%, w/w)	2.13	2.09
Target Application Rate of Test Preparation (mg/cm^2)	20.00	20.00
Actual Application Rate of Test Preparation (mg/cm^2)	20.06	19.88
R0054002C (% Applied Dose)	(Mean ± SD)	
Dislodgeable Dose	91.51 ± 4.85	94.36 ± 2.92
Unabsorbed Dose *	92.59 ± 4.80	95.62 ± 3.01
Absorbed Dose **	0.08 ± 0.06	0.08 ± 0.06
Dermal Delivery ***	0.39 ± 0.30	0.54 ± 0.49
Mass Balance	92.98 ± 4.64	96.16 ± 2.99
R0054002C (µg equiv./cm^2)	(Mean ± SD)	
Dislodgeable Dose	391.20 ± 22.25	390.92 ± 14.17
Unabsorbed Dose *	395.80 ± 22.15	396.15 ± 14.79
Absorbed Dose **	0.35 ± 0.25	0.34 ± 0.25
Dermal Delivery ***	1.68 ± 1.26	2.23 ± 2.04
Mass Balance	397.48 ± 21.48	398.38 ± 14.82

* Unabsorbed dose = dislodgeable dose + stratum corneum + unexposed skin
** Absorbed dose = receptor fluid + receptor rinse
*** Dermal Delivery = epidermis + dermis + clingfilm + absorbed dose

Oxidative conditions

Table 1: Distribution of Radioactivity (% Applied Dose) at 24 h Post Dose Following Topical Application of [^{14}C]-R0054002C in Oxidative Test Preparation (2%, w/w) to Human Split Thickness Skin

	Cell Number and Donor Number													
	Cell 1 0120	Cell 2 0148	Cell 3 0135	Cell 4 0147	Cell 6 0098	Cell 11 0135	Cell 12 0147	Cell 15 0142	Cell 17 0148	Cell 18 0135	Cell 19 0147	Cell 22 0098	Mean	SD
Skin Wash 0.5h	94.04	80.71	92.06	84.25	92.47	96.98	86.83	86.53	84.73	94.68	92.54	92.35	89.85	5.03
Tissue Swab 0.5 h	2.22	2.15	0.35	0.95	1.64	0.49	0.55	0.48	1.84	1.00	0.53	1.50	1.14	0.70
Pipette Tips 0.5 h	0.20	0.19	0.92	0.06	0.22	0.16	0.14	0.23	0.09	0.13	0.11	0.16	0.22	0.23
Dislodgeable Dose 0.5 h	96.47	83.05	93.33	85.25	94.33	97.62	87.52	87.25	86.65	95.81	93.18	94.01	91.21	4.94
Cell Wash	0.35	0.28	0.07	0.14	0.28	0.06	0.05	0.12	0.44	0.09	0.06	0.17	0.17	0.13
Tissue Swab 24 h	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.05	1.19	0.00	0.00	0.00	0.13	0.34
Total Dislodgeable Dose	97.12	83.33	93.40	85.39	94.61	97.68	87.57	87.42	88.27	95.91	93.24	94.18	91.51	4.85
Stratum Corneum 1-5	0.44	1.48	0.70	0.69	1.90	0.69	0.42	0.46	0.43	0.50	0.34	0.74	0.73	0.48
Stratum Corneum 6-10	0.18	0.42	0.18	0.20	0.43	0.09	0.10	0.12	0.12	0.24	0.20	0.22	0.21	0.11
Stratum Corneum 11-15	0.06	0.16	0.05	0.11	0.13	0.01	0.05	0.07	0.08	0.09	0.07	0.17	0.09	0.05
Stratum Corneum 16-20	0.04	0.07	0.00	0.07	0.00	0.00	0.07	0.05	0.08	0.04	0.03	0.10	0.05	0.03
Stratum Corneum	0.72	2.13	0.93	1.07	2.45	0.79	0.65	0.69	0.71	0.87	0.64	1.23	1.07	0.60
Unexposed Skin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.01
Total Unabsorbed	97.84	85.47	94.33	86.47	97.07	98.47	88.21	88.11	88.99	96.78	93.91	95.42	92.59	4.80
Epidermis	0.19	0.86	0.03	0.27	0.23	0.01	0.32	0.12	0.34	0.10	0.13	0.38	0.25	0.23
Dermis	0.03	0.08	0.01	0.03	0.09	0.00	0.03	0.03	0.26	0.02	0.01	0.07	0.05	0.07
Clingfilm	0.00	0.04	0.00	0.01	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.02	0.01	0.01
Receptor Fluid	0.06	0.09	0.04	0.05	0.22	0.04	0.07	0.10	0.15	0.02	0.05	0.09	0.08	0.06
Receptor Rinse	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total Absorbed	0.06	0.09	0.04	0.05	0.23	0.05	0.07	0.10	0.15	0.02	0.05	0.09	0.08	0.06
Dermal Delivery	0.29	1.06	0.08	0.36	0.55	0.06	0.43	0.24	0.76	0.14	0.19	0.56	0.39	0.30
Mass Balance	98.12	86.53	94.41	86.83	97.61	98.53	88.64	88.35	89.74	96.92	94.10	95.98	92.98	4.64

Δ = Results calculated from data containing N.S. samples
* = Results calculated from data less than 30 d.p.m. above background
° = Mean includes results calculated from data less than 30 d.p.m. above background

Table 2: Distribution of [14C]-R0054002C (µg equiv./cm²) at 24 h Post Dose Following Topical Application of the Oxidative Test Preparation (2%, w/w) to Human Split-Thickness Skin

	Cell Number and Donor Number													
	Cell 1 0120	Cell 2 0148	Cell 3 0135	Cell 4 0147	Cell 6 0098	Cell 11 0135	Cell 12 0147	Cell 15 0142	Cell 17 0148	Cell 18 0135	Cell 19 0147	Cell 22 0098	Mean	SD
Skin Wash 0.5h	396.60	340.36	388.23	355.28	404.53	424.25	379.87	378.57	358.55	400.69	391.64	390.81	384.12	23.40
Tissue Swab 0.5 h	9.38	9.08	1.49	3.99	7.19	2.13	2.41	2.10	7.77	4.24	2.25	6.35	4.87	2.93
Pipette Tips 0.5 h	0.84	0.82	3.89	0.26	0.95	0.70	0.61	1.02	0.38	0.54	0.45	0.68	0.93	0.96
Dislodgeable Dose 0.5 h	406.83	350.26	393.61	359.53	412.68	427.08	382.89	381.69	366.70	405.47	394.33	397.84	389.91	22.71
Cell Wash	1.47	1.17	0.28	0.59	1.22	0.24	0.20	0.52	1.85	0.39	0.26	0.73	0.74	0.55
Tissue Swab 24 h	1.26	0.00	0.00	0.00	0.01	0.00	0.00	0.22	5.02	0.01	0.00	0.01	0.54	1.45
Total Dislodgeable Dose	409.56	351.43	393.90	360.12	413.91	427.32	383.09	382.43	373.56	405.88	394.60	398.58	391.20	22.25
Stratum Corneum 1-5	1.85	6.23	2.94	2.93	8.31	3.03	1.83	2.01	1.81	2.13	1.43	3.11	3.13	2.06
Stratum Corneum 6-10	0.75	1.78	0.77	0.85	1.86	0.40	0.45	0.51	0.51	1.03	0.86	0.94	0.89	0.48
Stratum Corneum 11-15	0.25	0.69	0.20	0.45	0.56	0.04	0.23	0.29	0.33	0.37	0.29	0.72	0.37	0.20
Stratum Corneum 16-20	0.17	0.30	0.01	0.30	0.00	0.00	0.31	0.22	0.34	0.16	0.15	0.44	0.20	0.14
Stratum Corneum	3.03	8.99	3.93	4.52	10.73	3.47	2.82	3.03	3.00	3.69	2.72	5.21	4.60	2.59
Unexposed Skin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.09	0.00	0.01	0.02
Total Unabsorbed	412.59	360.43	397.82	364.65	424.65	430.79	385.92	385.46	376.58	409.57	397.41	403.79	395.80	22.15
Epidermis	0.80	3.64	0.12	1.13	0.99	0.05	1.39	0.50	1.42	0.43	0.55	1.63	1.05	0.96
Dermis	0.15	0.32	0.03	0.12	0.37	0.02	0.12	0.13	1.09	0.07	0.05	0.30	0.23	0.30
Clingfilm	0.02	0.15	0.01	0.04	0.04	0.00	0.05	0.01	0.04	0.01	0.01	0.06	0.04	0.04
Receptor Fluid	0.24	0.36	0.19	0.23	0.98	0.20	0.30	0.42	0.65	0.07	0.21	0.38	0.35	0.25
Receptor Rinse	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total Absorbed	0.24	0.37	0.19	0.23	0.99	0.20	0.30	0.43	0.65	0.07	0.21	0.39	0.35	0.25
Dermal Delivery	1.21	4.48	0.34	1.52	2.39	0.27	1.86	1.07	3.20	0.58	0.82	2.38	1.68	1.26
Mass Balance	413.80	364.91	398.17	366.17	427.04	431.06	387.78	386.53	379.77	410.15	398.23	406.17	397.48	21.48

Non-oxidative conditions

Table 3: Distribution of [14C]-R0054002C (µg equiv./cm²) at 24h Post Dose Following Topical Application of the Non-Oxidative Test Preparation (2%, w/w) to Human Split-Thickness Skin

	Cell Number and Donor Number													
	Cell 26 0148	Cell 27 0135	Cell 29 0142	Cell 30 0098	Cell 31 0147	Cell 33 0148	Cell 35 0135	Cell 38 0147	Cell 39 0098	Cell 40 0148	*Cell 42 0135*	Cell 43 0142	Mean	SD
Skin Wash 0.5h	352.47	365.24	380.11	391.26	397.83	377.37	391.80	387.42	405.21	370.08	*329.80*	389.48	382.57	15.40
Tissue Swab 0.5 h	7.96	1.23	5.06	4.75	6.19	16.32	4.42	4.04	2.65	12.74	*9.51*	5.09	6.40	4.44
Pipette Tips 0.5 h	0.90	0.40	0.61	0.28	0.26	0.64	1.25	0.60	0.37	0.25	*0.50*	0.74	0.57	0.31
Dislodgeable Dose 0.5 h	361.33	366.87	385.78	396.29	404.27	394.33	397.46	392.06	408.23	383.07	*339.81*	395.31	389.54	14.50
Cell Wash	1.87	0.07	1.06	0.78	0.78	1.07	0.80	0.93	0.26	6.03	*0.76*	0.42	1.28	1.65
Tissue Swab 24 h	0.01	0.00	0.39	0.10	0.02	0.01	0.01	0.01	0.00	0.44	*0.01*	0.01	0.09	0.16
Total Dislodgeable Dose	363.21	366.95	387.23	397.16	405.07	395.40	398.27	393.00	408.50	389.54	*340.57*	395.73	390.92	14.17
Stratum Corneum 1-5	1.78	2.01	3.20	2.36	3.12	1.82	3.11	2.28	1.38	10.40	*4.05*	1.87	3.03	2.52
Stratum Corneum 6-10	0.84	0.73	1.12	1.26	1.36	1.12	0.71	0.80	0.65	2.84	*1.05*	0.85	1.12	0.62
Stratum Corneum 11-15	0.57	0.13	0.64	0.88	0.70	0.70	0.00	0.73	0.33	1.83	*0.26*	0.49	0.64	0.48
Stratum Corneum 16-20	0.63	0.04	0.22	0.75	0.73	0.59	0.00	0.45	0.22	1.33	*0.15*	0.00	0.45	0.41
Stratum Corneum	3.82	2.91	5.17	5.24	5.90	4.22	3.83	4.26	2.57	16.40	*5.51*	3.21	5.23	3.84
Unexposed Skin	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Total Unabsorbed	367.03	369.86	392.40	402.41	410.98	399.62	402.11	397.27	411.07	405.94	*346.08*	398.95	396.15	14.79
Epidermis	3.16	0.09	1.67	1.02	0.74	1.53	0.46	0.61	0.25	4.73	*0.21*	0.73	1.36	1.41
Dermis	0.59	0.08	0.43	0.46	0.07	0.42	0.27	0.06	0.38	1.71	*0.18*	0.19	0.42	0.46
Clingfilm	0.13	0.00	0.10	0.05	0.04	0.05	0.04	0.02	0.01	0.57	*0.01*	0.07	0.10	0.16
Receptor Fluid	0.74	0.18	0.37	0.30	0.15	0.17	0.18	0.16	0.88	0.26	*0.14*	0.31	0.34	0.25
Receptor Rinse	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.01	*0.00*	0.00	0.00	0.00
Total Absorbed	0.75	0.18	0.38	0.31	0.16	0.17	0.19	0.16	0.88	0.26	*0.14*	0.31	0.34	0.25
Dermal Delivery	4.64	0.36	2.58	1.84	1.00	2.17	0.96	0.85	1.53	7.28	*0.55*	1.31	2.23	2.04
Mass Balance	371.66	370.21	394.98	404.24	411.98	401.79	403.06	398.12	412.59	413.22	*346.63*	400.26	398.38	14.82

Cell 42 rejected form mean and SD due to low mass balance (<85%)

Table 4: Distribution of Radioactivity (% Applied Dose) at 24 h Post Dose Following Topical Application of [14C]-R0054002C in Non-Oxidative Test Preparation (2%, w/w) to Human Split Thickness Skin

	Cell Number and Donor Number													
	Cell 26 0148	Cell 27 0135	Cell 29 0142	Cell 30 0098	Cell 31 0147	Cell 33 0148	Cell 35 0135	Cell 38 0147	Cell 39 0098	Cell 40 0148	*Cell 42 0135*	Cell 43 0142	Mean	SD
Skin Wash 0.5h	86.00	89.12	92.75	94.20	95.78	90.85	94.33	94.53	96.81	88.42	*78.79*	93.05	92.35	3.36
Tissue Swab 0.5 h	1.94	0.30	1.24	1.14	1.49	3.93	1.06	0.99	0.63	3.04	*2.27*	1.22	1.54	1.07
Pipette Tips 0.5 h	0.22	0.10	0.15	0.07	0.06	0.15	0.30	0.15	0.09	0.06	*0.12*	0.18	0.14	0.07
Dislodgeable Dose 0.5 h	88.16	89.52	94.13	95.41	97.33	94.94	95.69	95.66	97.53	91.52	*81.18*	94.44	94.03	3.04
Cell Wash	0.46	0.02	0.26	0.19	0.19	0.26	0.19	0.23	0.06	1.44	*0.18*	0.10	0.31	0.39
Tissue Swab 24 h	0.00	0.00	0.10	0.02	0.00	0.00	0.00	0.00	0.00	0.11	*0.00*	0.00	0.02	0.04
Total Dislodgeable Dose	88.62	89.53	94.48	95.62	97.52	95.19	95.88	95.89	97.59	93.06	*81.37*	94.54	94.36	2.92
Stratum Corneum 1-5	0.44	0.49	0.78	0.57	0.75	0.44	0.75	0.56	0.33	2.49	*0.97*	0.45	0.73	0.60
Stratum Corneum 6-10	0.21	0.18	0.27	0.30	0.33	0.27	Δ0.17	0.20	0.16	0.68	*0.25*	0.20	0.27	0.15
Stratum Corneum 11-15	0.14	0.03	0.16	0.21	0.17	0.17	Δ0.00	0.18	0.08	0.44	*0.06*	Δ0.12	0.15	0.11
Stratum Corneum 16-20	0.15	0.01	0.05	0.18	0.18	0.14	Δ0.00	0.11	0.05	0.32	*0.04*	Δ0.00	0.11	0.10
Stratum Corneum	0.93	0.71	1.26	1.26	1.42	1.02	0.92	1.04	0.61	3.92	*1.32*	0.77	1.26	0.92
Unexposed Skin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Total Unabsorbed	89.55	90.24	95.75	96.88	98.94	96.21	96.81	96.93	98.21	96.98	*82.68*	95.31	95.62	3.01
Epidermis	0.77	0.02	0.41	0.25	0.18	0.37	0.11	0.15	0.06	1.13	*0.05*	0.18	0.33	0.34
Dermis	0.14	0.02	0.10	0.11	0.02	0.10	0.06	0.02	0.09	0.41	*0.04*	0.05	0.10	0.11
Clingfilm	0.03	0.00	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.14	*0.00*	0.02	0.02	0.04
Receptor Fluid	0.18	0.04	0.09	0.07	0.04	0.04	0.04	0.04	0.21	0.06	*0.03*	0.07	0.08	0.06
Receptor Rinse	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Total Absorbed	0.18	0.04	0.09	0.07	0.04	0.04	0.04	0.04	0.21	0.06	*0.03*	0.08	0.08	0.06
Dermal Delivery	1.13	0.09	0.63	0.44	0.24	0.52	0.23	0.21	0.36	1.74	*0.13*	0.31	0.54	0.49
Mass Balance	90.69	90.33	96.37	97.32	99.18	96.73	97.04	97.14	98.57	98.72	*82.81*	95.62	96.16	2.99

Δ = Results calculated from data containing N.S. samples
Cell 42 rejected form mean and SD due to low mass balance (<85%)

The dermal absorption was 1.68 ± 1.26 µg /cm^2 for oxidative condition and 2.23 ± 2.04 µg/cm² for non-oxidative conditions.

Conclusion

The study authors concluded that the dermal absorption figures to be taken into consideration for the calculation of the margin of safety were 1.68 µg /cm^2 for the oxidative conditions and 2.23 µg /cm^2 for the non-oxidative conditions.

Ref.: 12

Comment
The dermal absorption figure to be taken into consideration for the calculation of the margin of safety is the Mean + SD (1.68 + 1.26) = 2.94 µg/cm^2 for the oxidative conditions.

3.3.5. Repeated dose toxicity

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

No data submitted

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

Guideline:	OECD 408 (1998)
Species/strain:	Sprague-Dawley rat, Rj Han: SD, Han (IOPS Han)
Group size:	10 males and 10 females Recovery animals: 6 males and 6 females in control and high dose group Satellite animals for the toxicokinetic study: 2 males and 2 females in the control group; 6 males and 6 females each for the test item groups
Test substance:	IMEXINE OBH (R0054002C)
Batch:	0604070001
Purity:	99.7%
Vehicle:	purified water
Dose levels:	0, 100, 300 and 1000 mg/kg bw/day
Dosage volume:	5 mL/kg bw/day
Route:	oral (gavage)
GLP statement:	Yes
Study period:	19 September 2006 – 31 January 2007 (experimental phase) Study director's approval of the study plan 28 Sept 2006 Study completion date 6 Nov 2007

The subchronic toxicity of Imexine OBH was investigated in SD rats (10/sex/group) after daily oral gavage at 0, 100, 300 or 1000 mg/kg bw/day in 5 mL/kg purified water for 13 weeks. These dose levels were selected on the basis of the results from a preliminary 2-week oral toxicity study performed in rats at 100, 300 or 1000 mg/kg bw/day, in which no significant toxic effects were observed [15].
Recovery animals (6/sex/group) were added to the control and high-dose groups and were held for a further 4-week treatment-free period. Satellite animals (2/sex for the control group and 6/sex for each Imexine OBH group) were added to the different groups and were assigned to toxicokinetic evaluation performed after single dosing (Day 1) and on day 90 (week 13).
Evaluations and measurements included daily morbidity/mortality checks, daily clinical examinations, weekly detailed clinical examination, weekly body weight and food consumption, and ophthalmological examination (during the acclimation period for all animals and in week 13 for control and high dose groups). At the end of the treatment period, a neurotoxicology evaluation, using a Functional Observation Battery (FOB) and a detailed clinical examination was carried out on each animal. Motor activity was also evaluated at the end of the recovery period for the females. Haematological, blood biochemical investigations and urinalysis were performed at the end of the treatment period. At the end of the recovery period, blood clotting parameters, blood biochemistry and urinalysis parameters were evaluated.

At the end of treatment (week 13) and recovery (week 17) periods, designated animals were killed and subjected to macroscopic examination; selected organs were weighed, and a wide range of organs/tissues were preserved. Microscopic examination was performed for specified tissues/organs from control and high-dose rats, as well as for any macroscopic lesions observed in animals of the low- and intermediate-dose groups.

Results
The chemical analysis of the weekly dose preparations administered during the study showed that achieved concentrations were in agreement with the intended values (range [-5%; +10%]). Stability of Imexine OBH in purified water at the low and high concentrations was demonstrated over 1-, 2-, 4- and 6-hour storage period at ambient temperature, protected from light and under nitrogen gas, and over 4- and 9-day storage period at +4°C, protected from light and under nitrogen gas [13].
One male in the main high-dose group (week 10, day 65) was found dead. Another male in the main high-dose group (week 13, day 86) was prematurely killed. Signs of poor clinical conditions and body weight loss were observed for these high-dose males during the week preceding death. At *ante mortem* clinical pathology, most of the parameters were affected reflecting their moribund state. At necropsy no findings which could have clearly contributed to the death or moribund state were noted. According to the study authors, the above mentioned death and moribund state were attributed to the test item treatment. At 1000 mg/kg bw/day, one female was prematurely killed (week 9). This animal showed many clinical signs which were consecutive to an accidental procedure at gavage (as confirmed by the necropsy findings). This death was thus considered not to be test item treatment-related but rather accidental due to misdosing. One satellite male given 300 mg/kg bw/day group were satellite animal was found dead without any prior clinical signs or abnormal findings at necropsy (week 2). Accordingly, this death was considered to bear no relationship with treatment with Imexine OBH.

All Imexine OBH-treated animals showed red-orange coloured litter from week 1 until the end of the dosing period, which was indicative of urinary excretion of the test material.
At 1000 mg/kg bw/day, excessive salivation was observed in all animals from week one until the end of the dosing period. In addition, yellow-orange coloured urogenital region was sporadically observed during the dosing and recovery period. In this dose group, loud breathing was noted in a few animals by the end of the dosing period and in one male and one female during the recovery period. This sign was also observed (week 6 or 8) in one male and one female associated with other signs of poor clinical condition. Abdominal breathing or dyspnea was noted in single animals by the end of the dosing period as well.
At 300 and 100 mg/kg bw/day none of these or other clinical signs were noted.

No Imexine OBH-related effects were observed during the detailed clinical observation (during the Functional Observation Battery) or at motor activity, landing foot splay, rectal temperature or forelimb strength measurements in males at all dose-levels and in females at 100 and 300 mg/kg bw/day. At 1000 mg/kg bw/day, loud breathing and piloerection were noted in one female. Additionally, at motor activity measurement performed at the end of the dosing period, when compared to controls, females given 1000 mg/kg bw/day showed a slightly lower mean number of horizontal and rearing movements which returned to within the control range at the end of the treatment-free period. The other parameters monitored within the Functional Observation Battery (i.e., landing foot splay, rectal temperature and forelimb grip strength) were not affected.

There were no treatment-related effects on body weight, body weight gain and food consumption at any dose-level. There were no ophthalmological findings.
At 1000 mg/kg bw/day, when compared to controls, males had a slightly lower mean activated partial thromboplastin time (13.7s *vs* 16.6s, $p<0.01$) and females showed a slightly higher mean blood fibrinogen concentration (2.96 g/L *vs* 2.42 g/L, $p<0.01$). These isolated changes of minimal to slight amplitude were of low toxicological significance, but a

relationship to treatment was not excluded. On completion of the treatment-free period, all mean values from recovery animals were similar to control.

Males given Imexine OBH at all dose-levels showed a slightly higher non dose-related blood urea concentration when compared to controls (between 6.2 to 6.6 mmol/L *vs* 5.2 mmol/L, $p<0.01$). At 1000 mg/kg bw/day, marginally but statistically significantly low chloride (both sexes), creatinine (females) and protein (males) concentrations were noted. In high-dose males, alkaline phosphatase activity was lower than controls (210 IU/L *vs* 257 IU/L, $p<0.01$). These changes of minimal to slight magnitude, observed in a single sex and/or without any dose relationship were all reversible and considered to be of no toxicological significance in the absence of any corroborating macroscopic or microscopic findings.

At 1000 mg/kg bw/day, a reversible low urinary pH was noted at the end of the treatment-period in both males and females confirming similar findings in a 15-day repeated-dose toxicity study earlier conducted in the same laboratory (study not available). Presence of proteins (females), nitrites (both sexes), bilirubin (females) and glucose (both sexes) were noted with a similar incidence at 1000 and 300 mg/kg bw/day. Trace levels of proteins (3/10 females) and nitrites were observed in the urine at 100 mg/kg bw/day. Marked urine colour was observed in almost all animals given 1000 or 300 mg/kg bw/day and in approximately half the animals given 100 mg/kg bw/day. All these changes were reversible after a 4-week treatment-free period. A relationship to treatment was not excluded but these changes were considered of low toxicological significance as they were isolated and did not correlate with any variations at clinical pathology and/or physiopathology. Moreover, some of these changes (i.e., nitrites, bilirubin) were considered to be related to urinary excretion of Imexine OBH and the subsequent marked urine discolouration.

At necropsy, no relevant changes in absolute and relative organ weight were noted. There were no macroscopic or microscopic findings attributed to treatment with Imexine OBH at any dose-level.

Conclusion
Under the experimental conditions of this study, and based on the findings noted at urinalysis (few animals concerned, reversibility after a treatment-free period, and no correlation of these findings with any clinical pathology or histopathology changes), it was considered that the No Observed Adverse Effect Level (NOAEL) was 300 mg/kg bw/day.

Ref.: 7

Comments
The results of the toxicokinetic study are not included in the study report due to the instability of the test item within the plasma matrix during the validation phase of the method and the absence of a validated method.

The slightly higher non dose-related blood urea concentrations in treated males when compared to controls (between 6.2 to 6.6 mmol/L *vs* 5.2 mmol/L, $p<0.01$) may be explained by an unusually low mean value of the male controls, as the female values including the control value were in the range between 6.1 and 6.6 mmol/L.

A more detailed analysis of the urinary parameters discussed above revealed dose-related increases for the bilirubin concentrations in females and dose-related increases for the glucose and nitrite concentrations in males and females above 100 mg/kg bw/day. No hyperbilirubinaemia or hyperglycaemia was found. It is agreed that the nitrite determinations might be influenced by the highly coloured urine samples at mid and high dose and thus appear questionable. The positive (reversible) bilirubin and glucose findings are not considered adverse. Thus, a NOAEL of 300 mg/kg bw/day is derived.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial reverse mutation test

Guideline:	OECD 471 (1997)		
Species/strain:	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 and TA102		
Replicates:	three plates/dose, two independent experiments		
Test substance:	IMEXINE OBH		
Batch:	0604070001		
Purity:	99.7%		
Vehicle:	water for injections DMSO (positive controls except mitomycin C = distilled water)		
Concentrations:	without S9-mix - 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate for TA 1537, TA 98, TA 102 in both experiments - 78.13, 156.3, 312.5, 625, 1250 and 2500 µg/plate for TA 1535 in the first experiment and for TA 100 in both experiments, - 39.06, 78.13, 156.3, 312.5, 625 and 1250 µg/plate, for TA 1535 in the second experiment with S9-mix - 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate, for all tester strains in both experiments		
Treatment:	direct plate incorporation (experiment 1, without and with S9-mix; experiment 2 without S9-mix) Preincubation method (experiment 2, with S9-mix)		
Positive controls:	without S9-mix:	sodium azide	TA1535, TA100
		9-aminoacridine	TA1537
		2-nitrofluorene	TA98
		Mitomycin C	TA102
	With S9-mix:	2-anthramine	TA1535, TA1537, TA98, TA100, TA102
GLP:	in compliance		
Study period:	15 September – 10 October 2006		

Imexine OBH was evaluated for the induction of gene mutations in two independent experiments with five *Salmonella* tester strains both in the absence and the presence of Aroclor induced rat liver S9-mix. Test concentrations were based on the results of a preliminary toxicity test with strains TA98, TA100 and TA102. 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 revertant colonies and/or thinning of the bacterial background lawn. Positive and negative controls were included according to OECD guidelines.

Results
In the absence of S9-mix, no precipitate was observed in the Petri plates at any concentration tested. A moderate toxicity was noted at 5000 µg/plate in TA 1537 and TA 102 strains, at 2500 µg/plate in TA 100 strain and at concentration levels ≥ 1250 µg/plate in TA 1535 strain. In the presence of S9-mix, no precipitate was observed at any concentration tested. A moderate toxicity was noted at 5000 µg/plate in TA 1535 and TA

1537 strains in the first experiment (direct plate incorporation method). Imexine OBH did not induce any significant increase in the number of revertants in any of the five strains, either in the absence or the presence of S9 mix.

Conclusion
Under the conditions of the study, IMEXINE OBH did not show any mutagenic activity in the bacterial reverse mutation test with *Salmonella typhimurium* either in the absence or the presence of metabolic activation.

Ref.: 8

In vitro mammalian cell gene mutation test

Guideline:	OECD 476 (1997)	
Species/strain:	L5178Y $TK^{+/-}$ mouse lymphoma cells	
Replicates:	2 independent experiments, without and with S9-mix	
Test substance:	IMEXINE OBH	
Batch:	013 L 001	
Purity:	99.7%	
Vehicle:	purified water	
Concentrations:	experiment 1, without S9-mix:	500, 1000, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3464 µg/mL
	experiment 1, with S9-mix:	500, 1000, 1500, 2000, 2750, 3464 µg/mL
	experiment 2, without S9-mix:	500, 1000, 1200, 1400, 1600, 1800, 2000, 2250, 2500 µg/mL
	experiment 2, with S9-mix:	500, 1000, 1500, 2000, 2750, 3464 µg/mL
Treatment:	3h treatment incubation period	
Positive control:	4-nitroquinoline-1-oxide (without S9-mix) benzo(a)pyrene (with S9-mix)	
GLP:	in compliance	
Study period:	13 September – 13 November 2006	

Imexine OBH was assayed for induction of mutations at the *hprt* locus in mouse lymphoma cells using a fluctuation protocol. The study consisted of a cytotoxicity range-finding experiment followed by two independent experiments, each conducted in the absence and presence of metabolic activation by an Aroclor induced rat liver S9-mix

Test concentrations were based on the results of a cytotoxic range-finding experiment with six test concentrations ranging from 108.3 to 3464 µg/ml (equivalent to 10 mM), both without and with S9-mix measuring raw plate counts and relative survival. In the main test, 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 relative survival to the survival of the vehicle control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
The maximum concentrations evaluated for mutations were based on toxicity. In experiment 1 without S9-mix, the five highest concentrations (2250-3464 µg/mL) tested were too toxic for evaluation and 2000 µg/mL in the absence of S9-mix, with a relative survival (RS) of 13.63% was later excluded from statistical analysis due to excessive heterogeneity in mutants between cultures (Mutant frequency (MF) = 4.81 in culture one and 25.01 in culture 2, compared to 4.82 and 3.62 in the negative control cultures). The highest concentrations selected for mutant analysis were 1750 µg/mL in the absence of S9-mix (RS = 31.89%) and 3464 µg/mL (= 10mM) in the presence of S9-mix (RS = 72.6%). In experiment 2, nine concentrations, ranging from 500 to 2500 µg/mL, were tested in the

absence of S9-mix and six concentrations, ranging from 500 to 3464 µg/mL, were tested in the presence of S9-mix. The highest three concentrations tested in the absence of S9-mix (2000-2500 µg/mL) were considered too toxic for selection to determine viability and mutant frequency. The highest concentrations analysed were 1800 µg/mL in the absence of S9-mix and 3464 µg/mL in the presence of S9-mix, which yielded 13.11% and 59.58% RS, respectively.
There were no statistically significant increases in mutant frequency following treatment with Imexine OBH at any concentration analysed in the absence or presence of S9-mix in the two experiments. Small increases in mutant frequency were observed in the absence of S9-mix at 2000 µg/mL in experiment 1 and at 1800 µg/mL in experiment 2. In one of the cultures treated at 2000 µg/mL (giving 13.63% RS) in experiment 1, there was an increase in mutant frequency but not in the other culture, which could be explained by severe toxicity. In experiment 2, 2000 µg/mL was too toxic to evaluate. Also the increase in experiment 2 at the highest tested concentration (1800 µg/ml) was observed in only one of the two replicate cultures and the mean mutant frequency values at these concentrations fell within the historical control range for mutant frequency based on recent experiments. A weak linear trend was observed in the absence of S9-mix in experiment 2, but there were no significant increases in mutant frequency at any concentration analysed in this experiment. Therefore, the increases in mutant frequency at the highest tested concentrations without S9-mix are not considered of biological relevance.

Conclusion
It is concluded that Imexine OBH did not induce mutation at the *hprt* locus of L5178Y mouse lymphoma cells when tested under the conditions employed in this study.

Ref.: 9

In vitro mammalian chromosome aberration test

Guideline:	OECD 473 (1997)
Cells:	cultured human lymphocytes
Replicates:	duplicate cultures in 2 independent experiments, without and with S9-mix
Test substance:	IMEXINE OBH
Batch:	0604070001
Purity:	99.7%
Vehicle:	water for injections
Treatment:	1st experiment (without and with S9-mix): 3h treatment, 20h harvest 2nd experiment (without S9-mix): continuous exposure until harvest (20h) 2nd experiment (with S9-mix): 3h treatment, 20h harvest
Treatment volume:	100 µL/5.5 mL culture medium
Concentrations:	1st experiment, without and with S9-mix: 0, 5, 7.5, 10 mM 2nd experiment, without S9-mix: 0, 1.25, 2.5, 3 mM 2nd experiment, with S9-mix: 0, 5, 7.5, 10 mM
Positive control:	without S9-mix: mitomycin (3 µg/mL (3h treatment; 0.2 µg/mL (continuous treatment) with S9-mix: cyclophosphamide (12.5 or 25 µg/mL)
GLP:	in compliance
Study period:	13 September – 27 November 2006

Imexine OBH has been investigated for induction of chromosome aberrations in cultured human lymphocytes in the absence and presence of Aroclor activated rat liver S9-mix. After isolation the lymphocytes were stimulated to divide with 3.6% phytohaemagglutinin for 48 h.
The highest concentrations of Imexine OBH for metaphase analysis were selected on the basis of pH, osmolarity and cytotoxicity criteria. Reduction in the mitotic index was taken as

a measure for cytotoxicity. The highest concentration tested in the first experiment without S9-mix was 10 mM (corresponding to 3464 µg/mL) inducing a slight decrease (32%) in mitotic index. The highest concentration tested in the second experiment without S9-mix was 3mM inducing a 63% decrease of mitotic index With S9-mix the 10mM was the highest concentration tested in both experiments inducing slight to moderate decreases (41-52%) in mitotic index.
Cells were harvested 20 hours after the beginning of treatment (corresponding to approximately 1.5 normal cell cycles) in both experiments. One and a half hour prior to harvest, cell cultures were treated with a colcemid solution (10 µg/mL) to block cells in metaphase. Chromosome preparations were stained and examined microscopically for mitotic index and for aberrations when selected. Two hundred well spread metaphases per concentration (100 per culture) were evaluated blind. Negative and positive controls were in accordance with the OECD guideline.

Results
In experiments without S9-mix, a slight decrease in mitotic index was observed at concentrations ≥7.5 mM (32-33% decrease) after 3 h treatment. After 20 h treatment without S9-mix, a slight to strong decrease in mitotic index was noted at concentrations ≥0.63 mM (33-81%)
No significant increase of cells with structural chromosomal aberrations without S9-mix at any of the treatment times was noted.
In experiments with S9-mix a moderate decrease was observed in mitotic index (41 and 52% in the two experiments respectively) at the highest tested concentration (10mM).
No significant increase in cells with structural chromosomal aberrations was noted in either experiment.

Conclusion
Under the experimental conditions used Imexine OBH did not induce structural chromosomal aberrations in cultured human lymphocytes when tested up to the highest recommended concentration (10mM) or severe toxicity (81 % reduction in mitotic index).
Ref.: 10

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

No data submitted

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

Prenatal development toxicity study

Guideline:	OECD 414 (2001)
Species/strain:	Sprague-Dawley rat, Rj Han: SD, Han (*IOPS* Han)
Group size:	24 mated female rats per dose group
Test substance:	IMEXINE OBH (R0054002C)
Batch:	013 L 001
Purity:	99.7%

Vehicle:	purified water
Dose levels:	0, 100, 300 and 1000 mg/kg bw/day
Dosage volume:	5 mL/kg bw
Treatment:	gavage, daily from day 6 to day 20 *post-coitum*
GLP statement:	in compliance
Study period:	13 September – 9 October 2006

The potential effects of R0054002C on pregnant rats and embryo-foetal development were evaluated through daily oral gavage at 0, 100, 300 or 1000 mg/kg bw/day to mated SD rats (24/group) from day 6 through day 20 of gestation [the day of mating was designated as Gestation Day 0 (GD 0)]. The test item R0054002C was dissolved in purified water and given at 5 mL/kg bw. These dose levels were selected on the basis of the results of a preliminary study at the same dose levels where no maternal or developmental effects were observed [16].

Maternal evaluations and measurements included daily mortality/morbidity, daily clinical signs and body weight/food consumption at designated intervals during gestation.
The dams were killed on GD 21 and subjected to macroscopic evaluation; gravid uterus weight was recorded and foetuses were removed. The following litter parameters were recorded: number of corpora lutea, number and distribution of implantation sites, of early and late resorptions, of uterine scars and dead and live foetuses. Placentas were examined grossly. All foetuses were weighed and sexed. Foetuses from the first 20 litters were submitted to external examination, and were examined for visceral abnormalities, and the remaining foetuses were examined for skeletal abnormalities. Calculations were made for pre- and post-implantation loss rates, as well as for foetal or litter incidences and mean numbers of litters within each group containing foetuses with a particular observation.

Results
The chemical analysis of the weekly dose preparations administered during the study showed that achieved concentrations were in agreement with the intended values (range [-7%; +3%]. Stability of R0054002C in purified water at the lowest and highest concentrations was demonstrated over 1-, 2-, 4- and 6-hour storage period at ambient temperature, protected from light and under nitrogen gas, and over 4- and 9-day storage period at +4°C, protected from light and under nitrogen gas [13].
No unscheduled deaths occurred during the study. There were no effects of R00R4002C on body weight, body weight change or food consumption at any dose level.
All females given R0054002C showed reddish coloured bedding from the first 5 days of the dosing period until sacrifice, indicative of urinary excretion of R0054002C and thus systemic exposure following oral dosing with this hair dye ingredient. At 1000 mg/kg bw/day, one female had loud and abdominal breathing and excessive salivation as well, and another had excessive salivation; these clinical symptoms were observed from days 17 p.c. to day 20 p.c. A relationship to treatment was not excluded.
All females were pregnant and had viable foetuses at term. Caesarean data, including gravid uterus weight, number of corpora lutea, pre- and post- implantation losses, did not show any treatment-related changes.
Lumbar vertebrae were missing in one male foetus of the high dose group. There were no other effects of treatment on foetal sex ratio, foetal body weight or foetal development. Examination of the foetuses did not reveal any treatment-related external, visceral or skeletal (bone and cartilage) variation or malformations.

Conclusion
Under the conditions of the study, there were no embryo-foetal and teratogenic effects. Accordingly, 1000 mg/kg bw/day was identified as the No Adverse Effect Level (NOAEL) for maternal toxicity, and as the No Effect Level (NOEL) for the developmental toxicity.

Ref.: 11

Comment

The clinical symptoms reported for two females in the high dose group are considered adverse, as there were similar clinical symptoms observed in the high dose group at the same dose in the 90-day repeated dose toxicity study [7]. Therefore, the NOAEL for maternal toxicity is considered 300 mg/kg bw/day. Lumbar vertebrae absent in one foetus of the high dose group were considered an incidental malformation by the study authors but not treatment-related. The NOAEL for the developmental toxicity is considered 1000 mg/kg bw/day.

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
Oxidative conditions

2,3-diaminodihydroxypyrazolopyrazolone dimethosulfonate

Absorption through the skin	**A**	**=**	**2.94 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.71 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.03 mg/kg bw/d**
No Observed Adverse Effect Level (90 day study, oral, rat)	**NOAEL**	**=**	**300 mg/kg bw/d**
50% bio-availability*		**=**	**150 mg**
MOS		**=**	**5000**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14. Discussion

Physico-chemical specifications
2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate is used in oxidative hair dye formulations at a maximum on-head concentration of 2%.
No reference standard with known content of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate was used for the quantification of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in the 3 reported batches of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate. The reported content of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate in the 3 batches of test item should be considered as semiquantitative measurements.
Stability of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate in typical hair dye formulations has not been provided.

General toxicity
In an acute oral toxicity study in rats conducted with a single dose of 2000 mg/kg bw no mortality and no gross macroscopic findings were observed. Clinical signs disappeared and overall body weight gain of the animals was not affected up to the end of the observation period (day 15). In an acute dermal toxicity study in rats, no mortality or systemic clinical symptoms but only local effects on the skin (crusts, dryness) were observed up to day 15.

A sub-chronic (90 days) oral toxicity study in rats was conducted with doses of 0, 100, 300 and 1000 mg/kg bw/day. Recovery animals were added to the control and high-dose groups and were held for a further 4-week treatment-free period. Two males in the high dose group died or were found moribund (weeks 10 and 13). The deaths were considered test item treatment-related. Main clinical signs, excessive salivation in all animals from week one until the end of the dosing period and loud or abdominal breathing in some animals were only observed in the high dose group. The urinalysis revealed dose-related enhanced bilirubin and glucose concentrations in the mid and high dose group (not accompanied by bilirubinaemia or glycemia). These effects are not considered adverse. The NOAEL of this study is considered 300 mg/kg bw/day.

The potential effects of 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate on embryo-foetal development were evaluated in an oral (gavage) study where pregnant rats were given 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate at 100, 300 or 1000 mg/kg bw/day during the sensitive period of organogenesis. At 1000 mg/kg bw/day, a female showed loud and abdominal breathing and another excessive salivation.
There were no signs of toxicity on embryo-foetal development or on the growth in utero. Lumbar vertebrae absent in one foetus of the high dose group were considered a malformation by the study authors but not treatment-related. 2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate is regarded to have no embryotoxic or teratogenic potential under the conditions of the study. Based on the clinical symptoms of two females in the high dose group, which were similar to the clinical symptoms at the same dose in the 90-day toxicity study, a dose of 300 mg/kg bw/day is considered as the NOAEL for maternal toxicity. The NOAEL for the developmental toxicity is considered 1000 mg/kg bw/day.

Irritation, sensitisation
Under the experimental conditions, the test item was non-irritant when applied topically to rabbit skin. It was severely irritant when applied neat to rabbit eye. A 5% dilution was slightly irritant to the rabbit eyes.

Under the experimental conditions, the test item did not induce contact sensitization in the murine Local Lymph Node Assay. No firm conclusion regarding the sensitising potential of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate can be drawn, since the highest concentration tested was 18%.

Dermal absorption
The dermal absorption figure to be taken into consideration for the calculation of the margin of safety is the Mean + SD or 1.68 + 1.26 = 2.94 µg /cm^2 for the oxidative conditions.

Mutagenicity
2,3-Diaminodihydropyrazolopyrazolone dimethosulfonate was tested in 3 *in vitro* mutagenicity tests for induction of gene mutations and structural chromosomal aberrations. The test substance did not induce gene mutations either in bacteria or in mammalian cells and did not induce structural chromosomal aberration in mammalian cells. It is therefore concluded that the substance has no mutagenic or clastogenic potential. It has not been tested specifically for aneuploidy but there were no indications for any aneugenic potential in the chromosome aberration test.

Carcinogenicity

No data submitted

4. CONCLUSION

The SCCS is of the opinion that the use of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate with a maximum on-head concentration of 2.0% in oxidative hair dye formulations does not pose a risk to the health of the consumer.

A sensitising potential of 2,3-diaminodihydropyrazolopyrazolone dimethosulfonate cannot be excluded.

5. MINORITY OPINION

Not applicable

6. REFERENCES

References in italics are not submitted as full reports in the present dossier [14-16]. They consist of reports for preliminary toxicity studies and can be provided upon request. Appropriate data bases were searched for relevant safety data on Imexine OBH. No reports were identified in the literature that provided new information which is reasonably expected to substantially alter the human risk assessment performed in the present submission.

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6. Pelcot, C. Imexine OBH - Evaluation of Skin Sensitization Potential in Mice Using the Local Lymph Node Assay (LLNA). CIT Study No. 32047 TSS, 2007.
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13. Mathieu, D. R0054002C - Validation of the analytical method and determination of stability of dosage forms. CIT Study No. 32312 AHS, 2007.
14. *Sire, G. R0054002C - Acute Oral Toxicity in Rats "Fixed Dose Method". CIT Study No. 30168 TAR, 2006.*
15. *Désert, P. R0054002C - 2-week toxicity study by oral route (gavage) in rats. CIT Study No. 30156 TSR, 2006.*
16. *Désert, P. R0054002C - Preliminary study of prenatal developmental toxicity by oral route (gavage) in rats. CIT Study No. 30157 RSR, 2006*