Scientific Committee on Consumer Safety

SCCS

OPINION ON

1-Hexyl 4,5-Diamino Pyrazole Sulfate (A163)

The SCCS adopted this opinion at its 10th plenary meeting

on 25 June 2015

Revision of 15 December 2015

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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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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1. BACKGROUND

Submission I on the new hair dye with the chemical name 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate (IUPAC) (No A163) CAS No 1361000-03-4 was transmitted by Cosmetics Europe in May 2014.

The new ingredient 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate (A163) is planned to be used as an oxidative hair colouring agent (precursor) in oxidative hair dye formulations at a maximum on-head concentration of 1.0%.

2. TERMS OF REFERENCE

(1) In light of the data provided, does the SCCS consider 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate (A163) safe when used as an oxidative hair colouring agent (precursor) in oxidative hair dye formulations at a maximum on-head concentration of 1.0%?

(2) Does the SCCS have any further scientific concerns with regard to the use of 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate (A163) in cosmetic products?

3. OPINION

3.1 Chemical and Physical Specifications of 1-Hexyl 4,5-diamino pyrazole sulfate

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

1-Hexyl 4,5-diamino pyrazole sulfate (INCI)

3.1.1.2 Chemical names

1H-Pyrazole-4,5-diamine, 1-hexyl-, sulfate (2:1)
1-Hexyl 4,5-diamino pyrazole sulfate
1-hexyl-1H-pyrazole-4,5-diamino hemisulfate
n-Hexylpyrazole hemisulfate
4-5-Diamino-1-hexyl-1H-pyrazole hemisulfate
C6 Pyrazole hemisulfate

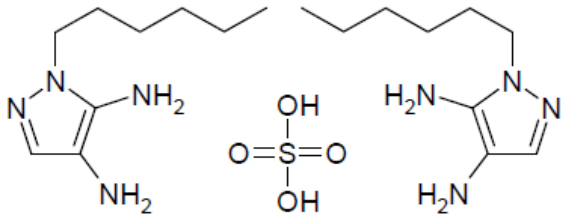
3.1.1.3 Trade names and abbreviations

Trade names: /
COLIPA no: A163

3.1.1.4 CAS / EC number

CAS: 1361000-03-4
EC: /

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula:
C9H18N4 as free base
$C_9H_{18}N_4$. $1/2H_2O_4S$ as hemisulfate

3.1.2 Physical form

White or colourless crystals

3.1.3 Molecular weight

182.27 g/mol as free base
231.31 g/mol as hemisulfate

3.1.4 Purity, composition and substance codes

Batch RD-CRU-096-07/142-01 of 1-Hexyl 4,5-diamino pyrazole sulfate was used in the toxicological testing reported in this dossier. Analytical characterisation of this batch is described below.

Chemical characterisation by 1H NMR spectrum, electrospray MS and elemental analysis
Ref. 5, 8, 9

Relative purity (HPLC % peak area) 210 nm 99.99%, 254 nm 100.00%
Ref. 6

Titration 100.14% (w/w) of hemisulfate by titration with 0.1 M NaOH
100.22% (w/w) of free base by titration with 0.1 M HClO4
Ref. 6

Content by 1H NMR 78.4 % (w/w) free base, 99.5% wt hemisulfate
Ref. 6

Content by 1H NMR 100.1% (w/w) hemisulfate
Ref. 7

SCCS comment
As the UV - Visible spectrum is not provided, it is not certain that the correct wavelength has been chosen for the measurement of the 1-hexyl 4,5-diamino pyrazole sulfate by HPLC/UV.

3.1.5 Impurities / accompanying contaminants

Potential impurity: Dimer
1-hexyl-4-[(E)-(1-hexyl-5-imino-1,5-dihydro-4H-pyrazol-4-ylidene)amino]-1H-pyrazol-5-amine sulfate: Not detected (LOD 10 ppm)
Ref. 6

Metal content Hg, Pb, Cd, As, Sb, Fe, Mn, Co, Cu, Cr: Not detected (LOD 10 ppm)
Ref. 6

3.1.6 Solubility

Water solubility 3.28 g/liter.
Ref. 10

SCCS comment
Water solubility was not determined by EU method A.6.

3.1.7 Partition coefficient (Log P_{ow})

Log Pow at pH 3: 3.045 (OECD TG 117)
Log Pow at pH 7: 1.287 (OECD TG 117)

3.1.8 Additional physical and chemical specifications

Melting point:	84.4 °C
Boiling point:	decomposition at 184.8°C
Flash point:	/
Vapour pressure:	/
Density:	1.31 g/mL
Viscosity:	/
pKa:	
Refractive index:	/
pH:	
UV_Vis spectrum (200-800 nm):	/

3.1.9 Homogeneity and stability

/

SCCS general comments to physico-chemical characterisation
Stability of 1-hexyl 4,5-diamino pyrazole sulfate in typical hair dye formulations has not been reported.

3.2 Function and uses of 1-hexyl 4,5-diamino pyrazole sulfate

1-Hexyl 4,5-diamino pyrazole sulfate is used as an oxidative hair colouring agent (precursor). The intended maximum on-head use concentration is 1.0% in oxidative hair dye formulations. The oxidative colouring agent and the developer are mixed at a ratio of 1+1 to 1+3 (g dye formulation + g developer formulation).

3.3 Chemical and Physical Specifications of 1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride (this salt will not be used commercially)

3.3.1 Chemical identity

3.3.1.1 Primary name and/or INCI name

1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride

3.3.1.2 Chemical names

1H-Pyrazole-4,5-diamine, 1-hexyl-, hydrochloride (1:2) (CA INDEX NAME)

1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride (IUPAC)
4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride
4,5-diamino-1-hexyl-1H-pyrazole, dihydrochloride

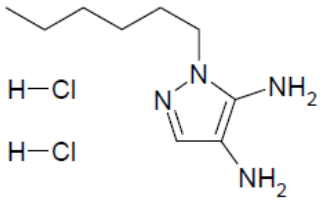
3.3.1.3 Trade names and abbreviations

/

3.3.1.4 CAS / EC number

CAS no.: 1361000-06-7
EC no.: /

3.3.1.5 Structural formula



3.3.1.6 Empirical formula

$C_9H_{18}N_4$ as free base
$C_9H_{18}N_4.2ClH$ as dihydrochloride

3.3.2 Physical form

Light pink powder

3.3.3 Molecular weight

182.27 g/mol as free base
255.19 g/mol as dihydrochloride

3.3.4 Purity, composition and substance codes

Two batches of 1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride were used in the toxicological testing reported in this dossier. Analytical characterisation of these batches is described below.

	1-HEXYL-1H-PYRAZOLE-4,5-DIAMINE DIHYDROCHLORIDE **DTF654/074A-HAY** **Tox batch A**		*1-HEXYL-1H-PYRAZOLE-4,5-DIAMINE DIHYDROCHLORIDE* **RD-CRU 096-07/47-01** **Tox batch B**	
	Result	**Ref**	**Result**	**Ref**
Appearance	light pink powder	1	light pink powder	2
Identification	1H NMR and elemental analysis confirm identity	1	1H NMR and elemental analysis confirm identity	2
Sulfated ash	0.09% wt	1	Not tested	
Relative purity (HPLC area)	210 nm 99.7% 224 nm 99.8% 254 nm 100.0%	1	210 nm 98.76% 224 nm 98.87% 254 nm 99.83% 210 nm 98.90% 224 nm 99.27% 254 nm 99.71%	3 2
Content by ^{1}H NMR	100.2%	1	99.7%	2,4
Water content	0.03% wt	1	Not tested	
Elemental analysis	Theoretical: C, 42.36%; H, 7.90%; N, 21.95%; Cl, 27.79% Found: C, 42.79%; H, 7.80%: N, 22.33%; Cl, 26.76%	1 1	Theoretical: C, 42.36%; H, 7.90%; N, 21.96%; Cl, 27.79% Found C, 42.10%; H, 7.87%; N, 21.73%; Cl, 28.47% Found C, 42.46%; H, 8.13%; N, 22.26%; Cl, 27.98%	2 2 5

3.3.5 Impurities / accompanying contaminants

/

3.3.6 Solubility

1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride RD-CRU 096-07/47-01
>10 % w/v in water without pH adjustment
>10 % w/v in ethanol/water 1:1
>10 % w/v in water (pH 7)

Ref. 3

3.3.7 Partition coefficient (Log P_{ow})

/

3.3.8 Additional physical and chemical specifications

/

3.3.9 Homogeneity and Stability

Stability in water (10% solution, w/v)
Recovery at t=0h: 99.83%; t=6h: 99.31%; t=2d 99.02%; t=7d 99.39%
Stability in water (correction of pH to 7.0 with NaOH; 10% solution, w/v)
Recovery at t=0h: 99.81%; t=6h: 99.72%; t=2d 99.84%; t=7d 99.74%
Stability in water/ethanol 1:1 (10% solution, w/v)
Recovery at t=0h: 99.80%; t=6h: 99.71%; t=2d 99.81%; t=7d 99.78%

Ref. 3

3.4 Toxicological evaluation

3.4.1 Acute toxicity

3.4.1.1 Acute oral toxicity

No acute oral toxicity studies were performed with 1-hexyl 4,5-diamino pyrazole sulfate. However, no deaths were observed in the sub-chronic (13-week) oral toxicity study or in the developmental toxicity study in rats at dose levels up to 20 mg/kg bw/day (Ref. 22 and Ref. 26).

3.4.1.2 Acute dermal toxicity

/

3.4.1.3 Acute inhalation toxicity

/

3.4.1.4 Acute intraperitoneal toxicity

/

3.4.2 Irritation and corrosivity

3.4.2.1 Skin irritation

***In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance (TER) Test Method**

Guideline:	OECD 430 (April 2004)
Test system:	*Ex vivo* rat dorsal skin discs
Strain:	Wistar (RccHan^{TM}:WIST)
Replicates:	3 discs per condition from 1 female rat
Test substance:	1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1)
Test batch:	RD-CRU-096-07/142-01
Purity:	99.1%
Test item:	Neat (100% powder)
Vehicle:	None, neat substance
Treatment period:	24 hours
Positive control:	10M (~36%) hydrochloric acid

Negative control: Sterile distilled water
GLP: In compliance
Study period: April 2013

One female rat was used for the study. Following an acclimatisation period of two days, the animal was shaved to remove hair from the dorsal surface. The shaved area was washed with an antibiotic wash. After three days, a second antibiotic wash was performed. Three days later, the animal was euthanised by CO_2 asphyxiation followed by cervical dislocation. The dorsal skin was removed from the rat as a single pelt. Excess fat was removed and the pelt mounted, epidermal side uppermost, onto a polytetrafluoroethylene (PTFE) tube. The tissue was secured in place using a rubber "O" ring, excess tissue was trimmed away and the "O" ring/PTFE interface sealed with soft paraffin wax. The tube was supported by a clamp inside a labelled 30 ml glass receptacle containing 10 ml electrolyte solution (154 mM $MgSO_4$). Two skin discs of approximately 0.79 cm^2 were taken from the pelt and the TER measured as a quality control procedure. Each disc had to give a resistance value of greater than 10 kΩ in order for the remainder of the pelt to be used in the assay. If the value for either disc fell below the 10 kΩ threshold, the pelt was discarded. The quality control discs were then discarded and new discs from the acceptable pelt were mounted on the PTFE tubes. 1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) was applied neat to the epidermal surface of three skin discs for a contact period of 24 hours. Sufficient test material was applied evenly to the skin discs to ensure that the whole surface of the epidermis was covered. A volume of 150 µl distilled water was applied to ensure good contact with the skin. At the end of the exposure period, the test material was removed by washing the skin disc with a jet of warm tap water until no further test material could be removed. Three positive (hydrochloric acid 10 M (~36%)) and negative control (sterile distilled water) discs were also assayed for a contact period of 24 hours. The TER was measured using a Wheatstone Bridge with a "low voltage alternating current" and the value, expressed in Ω or kΩ per skin disc, was determined. Prior to measurement, the surface tension of the skin disc was reduced by adding a sufficient volume of 70% ethanol to cover the epidermis. The ethanol was removed by inverting the tube after approximately 3 seconds. The mean TER for the skin discs was calculated.

Results
The mean TER after a contact period of 24 hours with 1-hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) was 28.9 ± 5.6 kΩ. The quality criteria for acceptance of the results were satisfied based on a TER for the positive control of 873.5 ± 71.4 Ω and 17.4 ± 1.4 kΩ for the negative control. The reading of one skin disc treated with positive control was not measureable due to perforation of the tissue resulting from the severe corrosive nature of this acid. This was not considered to affect the purpose or integrity of the study.

Conclusion
1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) as neat substance is considered unlikely to have the potential to cause skin corrosion *in vivo*.

Ref. 11

***In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method**

Guideline:	OECD 439 (July 2010)
Test system:	Episkin™ Reconstructed Human Epidermis Model
Replicates:	3 tissues per condition
Test substance:	1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1)
Test batch:	RD-CRU-096-07/142-01
Purity:	99.1%
Test item:	Neat (100% powder)
Vehicle:	None, neat substance

Dose level:	10 mg
Treatment period:	15 minutes
Post-treatment incubation time:	42 hours
Positive control:	5% (w/v) aqueous solution of sodium dodecyl sulphate
Negative control:	Sterile water
Direct interaction with MTT:	Positive
Colouring of epidermis:	Positive
GLP:	In compliance
Study period:	23 April 2013 - 24 June 2013

Nine tissues were treated with the test item for an exposure period of 15 minutes. The test item was applied topically to the corresponding tissues ensuring uniform covering. Approximately 10 mg of the test item was applied to the epidermis surface. The epidermis surface had previously been moistened with 5 µl of sterile distilled water to improve contact between the solid test item and the epidermis. Nine tissues treated with 10 µl of sterile water served as the negative controls and six tissues treated with 10 µl of SDS 5% w/v served as the positive controls. The plates were kept in the biological safety cabinet at room temperature for 15 minutes. At the end of the exposure period, each tissue was rinsed with PBS. The rinsed tissues were transferred to the appropriate wells of a 12-well plate previously prepared with 2 ml of maintenance medium. The rinsed tissues were incubated at 37°C and 5% CO_2 for 42 ± 1 hours. The negative and positive control groups were rinsed using the same process. Following the 42-hour post-treatment incubation period, three tissues for each treatment group, including negative and positive controls, were transferred to 12-well plates containing 2 ml of 0.3 mg/ml MTT solution, freshly prepared in assay medium. The tissues were incubated for 3 hours at 37.0°C, 5% CO_2 in air. At the end of the 3-hour incubation period, the tissues were examined and the degree of MTT staining evaluated. Following qualitative evaluation of tissue viability a total biopsy of the epidermis was made. The epidermis was carefully separated from the collagen matrix and both parts placed into micro tubes containing 500 µl of acidified isopropanol. Each tube was plugged to prevent evaporation and mixed thoroughly on a vortex mixer. The tubes were refrigerated at 1-10°C until day 6 of the experiment allowing extraction of formazan crystals out of the MTT-loaded tissues. Since it is possible that coloured test items may stain the tissues, three test item treated tissues for each group were used for MTT correction purposes. The tissues were transferred to 12-well plates containing 2 ml of assay medium. Similarly three tissues treated with the negative control item were also transferred to 12-well plates containing 2 ml of assay medium for determining and correcting background colour in the tissues. These tissues were then treated identically to the tissues placed in the MTT solution. The final three tissues for each treatment group were retained for histopathology. At the end of the formazan extraction period, the optical density was measured at 540 nm versus acidified isopropanol as blank and the % cell viability was calculated.

Results
1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) did not induce a decrease in cell viability in the MTT assay. Compared to the negative control tissues, the MTT relative viability of the test item treated tissues was 119.4 ± 10.6%. The test item was determined to have the ability to directly reduce MTT. Therefore, the additional control procedure using water-killed tissues was performed to enable quantitative correction for possible direct reduction of MTT by the test substance. The results obtained showed a negligible degree of direct reduction of MTT occurred. Consequently, the test item was considered not to have caused any colour interference and therefore quantitative correction of the results for colour interference was unnecessary.
Histological evaluation of the treated tissues showed minimal epidermal effects in the treated cultures in comparison to the negative control cultures confirming an absence of meaningful epidermal effects in histopathology for 1-hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) tested neat.
The negative, positive and vehicle controls confirmed the validity of the assay. For the negative control, the mean OD_{540} values of 0.712 ± 0.033 were within the accepted range

(≥ 0.6). The standard deviation (4.6) of the percentage viability was within the accepted tolerance interval (≤18%) and intact histological tissue structures were observed. For the positive control the relative mean viability (12.1) was within the accepted range (≤40%) relative to the negative control treated tissues and the standard deviation of the percentage viability (3.5) was within accepted tolerance interval (≤18%). The histological evaluation identified marked necrosis/disruption with some cell layers intact or necrosis/disruption with some cell layers.

Conclusion
1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) is considered to be non-irritant (MTT viability > 50%), when applied as neat test item. This result was confirmed by histological examinations. Therefore, the test item 1-hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) was not required to be classified as irritant to the skin.

Ref. 12

SCCS comment
HPLC method should have been used for separating MTT derived colour from that of the test substance.

SCCS overall conclusion on skin irritation
1-Hexyl 4,5-diamine pyrazole sulfate tested as neat substance in state-of-the-art *in vitro* test methods for skin corrosion (TER assay) and skin irritation (EpiSkin™ RHE method), showed that 1-hexyl 4,5-diamine pyrazole sulfate is non-corrosive and non-irritant.

3.4.2.2 Eye irritation

Isolated Chicken Eye Test Method

Guideline:	OECD 438 (September 2009)
Test system:	Isolated chicken eyes
Strain:	ROSS, spring chickens, 7 weeks old male and female chickens
Number of replicates:	3 eyes per condition
Test substance:	1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1)
Test batch:	RD-CRU-096-07/142-01
Purity:	99.9% (at 210 nm)
Test item:	Neat (100% powder), 5% (w/w) in aqueous vehicle containing 0.9% (w/w) sodium hydroxide / 0.1% (w/w) ascorbic acid, 1.5% (w/w) in aqueous vehicle containing 0.28% (w/w) sodium hydroxide / 0.1% (w/w) ascorbic acid
Dose level:	30 mg of neat substance or 30 µl of 5% (w/w) and 1.5% (w/w) liquid test items
Treatment period:	10 seconds
Positive control:	Sodium hydroxide (neat)
Negative control:	Physiological saline 0.9%
GLP:	In compliance
Study period:	March-April 2013

A total of 14 eyes were carefully selected for testing: 9 for the test item, 3 for the positive and 2 for the negative control. Eyes with a corneal thickness deviating more than 10%, eyes that showed opacity (score higher than 0.5) or any signs of other damage were rejected. After an equilibration period of 45-60 minutes, the corneal thickness of the eyes was measured once more to determine the zero reference value for corneal swelling calculations. Immediately thereafter, the clamp holding the test eye was placed on paper

tissues outside the chamber with the cornea facing upwards. Next, three corneas were treated with 30 mg of neat, 30 µl of 1.5% (w/w) or 30 µl of 5% (w/w) 1-hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) or control substances. After an exposure period of 10 seconds, the corneal surface was rinsed thoroughly with 20 ml of isotonic saline at ambient temperature. After rinsing, each eye in the holder was returned to its chamber and examined at 0, 30, 75, 120, 180 and 240 minutes after treatment. Fluorescein retention was scored only at 30 minutes after treatment. After the final examination, the test and control eyes were preserved in a neutral aqueous phosphate-buffered solution of 4% formaldehyde and prepared for histopathology examination. Ocular effects were evaluated using the endpoints of corneal thickness (swelling), corneal opacity and fluorescein retention.

Results
1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) tested neat caused very slight corneal effects in that there was very slight swelling (1%), slight opacity (1.0) and very slight fluorescein retention (0.5). The calculated Irritation Index was 31. Microscopic examination of the treated corneas revealed no abnormalities of the epithelium, stroma or endothelium.

1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) at 1.5% (w/w) caused very slight corneal effects with very slight swelling (3%), very slight opacity (0.5) and no fluorescein retention (0.0) was observed. The calculated Irritation Index was 13. Microscopic examination of the treated corneas revealed no abnormalities of the epithelium, stroma or endothelium.

1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) tested at 5% (w/w) caused slight to moderate corneal effects with slight swelling (15%), slight opacity (1.0) and slight to moderate fluorescein retention (1.5) was observed. The calculated Irritation Index was 65. Microscopic examination of the treated corneas revealed very slight or slight erosion and very slight vacuolation of the epithelium. No abnormalities of the stroma or endothelium were observed.

The results of the positive and negative controls confirmed the validity of the study. The positive control (neat sodium hydroxide) caused severe corneal effects with severe swelling (49%), severe opacity (4.0) and severe fluorescein retention (3.0) observed. The calculated Irritation Index was 189. Microscopic examination of the positive control (neat sodium hydroxide) corneas confirmed the severity of the corneal effects. The negative control (physiological saline) caused no corneal effects on cornea thickness, opacity or fluorescein retention and no histopathological effects.

Conclusion
1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) tested neat and at 1.5% (w/w) is identified as not irritating to eyes and is not classified according to the UN-GHS and EU-CLP classification schemes.
1-Hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) tested at 5% (w/w) is identified as mildly irritating to eyes and is classified as Category 2B (mildly irritant) in the UN-GHS and Category 2 (irritating to eyes) in the EU-CLP classification schemes with the latter identified as borderline between Category 2 and Not Classified. However, interpretation of this result must take into account that the presence of sodium hydroxide, as part of the vehicle system, is present at a level in the 1-hexyl-1H-pyrazole-4,5-diamine sulfate (2:1) tested at 5% (w/w) that is likely to have a small effect on the level of irritation observed and is therefore expected to be responsible for this borderline result between Category 2 and Not Classified.

Ref. 13

SCCS comment
No justification for the use of ascorbic acid in the test vehicle was given.
No information on a possible eye irritation potential of the vehicles used for the 1.5% (w/w) and 5% (w/w) solutions is given in the study report.

On the basis of the results obtained in the ICE study, it can be concluded that 1-hexyl 4,5-diamine pyrazole sulfate as neat substance and at 1.5% (w/w) in aqueous solution is not a strong eye irritant. Therefore, it is considered that the maximum on-head concentration of 1.0% (w/w) 1-hexyl 4,5-diamine pyrazole sulfate does not cause severe ocular irritation. This, however, does not exclude a mild eye irritant potential. Under the conditions of this study, an eye irritation potential of 1-hexyl 4,5-diamine pyrazole sulfate at 1.0% (w/w) cannot be excluded.

The SCCS further notes that based upon the ICE test results for 1-hexyl 4,5-diamine pyrazole sulfate as a 5% (w/w) aqueous solution no prediction can be made towards its eye irritation potential and thus does not allow its classification as mild irritant.

3.4.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD TG 429
Species/strain:	Mouse/CBA/J
Group size:	5 females per test concentration
Test substance:	4,5-Diamino-1-hexyl-1H-pyrazole dihydrochloride (WR804026)
Batch:	DTF654/074A-HAY
Purity:	99.7 % HPLC, 210 nm); 99.8% (HPLC 224 nm); 100.0 % (HPLC 254 nm)
Vehicle:	70% ethanol
Concentration:	1, 3, 10, 15%
Route:	Application to the dorsal surface of both ears on days 1, 2 and 3
Positive control:	35% (v/v) hexylcinnamaldehyde (HCA) in acetone/olive oil 4:1 (AOO)
GLP:	In compliance
Study period:	May-June 2009

The skin-sensitising potential of 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride was investigated in CBA/J mice by measuring the cell proliferation in the draining lymph nodes after topical application on the ear. A volume of 25 µl of 0 (vehicle only, i.e. 70% ethanol), 1, 3, 10 and 15% 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride was applied to the surface of the ear of five female mice per group for three consecutive days. Ethanol (70%) was selected as the vehicle because of the good solubility of 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride in this solvent.
As a positive control, hexylcinnamaldehyde (HCA) at a concentration of 35% in acetone:olive oil, 4:1 (AOO) was investigated in parallel under identical test conditions.
Animals were checked for morbidity/mortality daily on days 1 to 6. Observations for clinical signs were carried out immediately prior to and post-dose on days 1 to 3 and then once per day after dosing. Bodyweight was measured on days 1 and 6.
At day 6, the mice received an intravenous injection of 250 µl solution containing 20 µCi of [^{3}H]-methyl thymidine. Five hours later, the mice were euthanised, and the draining auricular lymph nodes were removed and collected in PBS. After preparing a single cell suspension for each mouse, cells were precipitated by 5% trichloro-acetic acid, and the radioactivity was determined (incorporation of [3H]-methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm).

Results
The solubility and stability of the test item in the solvent was considered sufficient. None of the animals died during the study, bodyweights were not altered and all animals appeared clinically normal throughout the study. Some orange staining on the ears and on the fur

occurred, which was considered to be related to the staining properties of 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride. There were no treatment-related effects on bodyweight or bodyweight gain.
The positive control, HCA, at a concentration of 35.0% in acetone: olive oil, induced a 10.6-fold increase in isotope incorporation in the draining auricular lymph nodes relative to the vehicle control. The mean stimulation index above 3 validated the experimental conditions.
4,5-Diamino-1-hexyl-1H-pyrazole dihydrochloride was positive in the LLNA, as there was a statistically significant increase in isotope incorporation in the draining auricular lymph nodes relative to the vehicle at 10% and 15%, which were greater than a 3-fold increase. The mean stimulation indices were 0.7, 1.0, 3.5 and 3.1 at the concentrations of 1%, 3%, 10% and 15%, respectively. The EC3 value was calculated to be 8.6%.

Conclusion
4,5-Diamino-1-hexyl-1H-pyrazole dihydrochloride is a moderate skin sensitiser under the defined experimental conditions, with a calculated EC3 value of 8.6%.

Calculation for the hemisulfate salt of 1-hexyl-1H-pyrazole-4,5-diamine:
The EC3 value of the hemisulphate salt was calculated from the dihydrochloride salt by using the conversion factor of 0.91 to account for the different molecular weight (see Reference 20).

EC3 value dihydrochloride: 8.6%
8.6% = 8.6 g/100 mL x 0.91 (conversion factor) = 7.8 g /100 mL = 7.8%
EC3 value hemisulfate: 7.8%

Therefore, the calculated EC3 value for 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate is 7.8%. Based on this calculation, 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate is considered a moderate skin sensitiser according to the relative skin sensitisation potency classification scheme published in the ECETOC technical report on contact sensitisation (see Reference 19).

Ref. 18

SCCS comment
The LLNA was performed with 1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride, *i.e.* the dihydrochlorde instead of the hemisulfate salt. 1-Hexyl-1H-pyrazole-4,5-diamine dihydrochloride was positive in the LLNA with an EC3 value of 8.6%. The sensitising potential and potency are attributable to the free base component. Therefore, the results of the LLNA carried out with the dihydrochloride salt can be used for the hazard assessment of 1-hexyl 4,5-diamine pyrazole sulfate. The EC3 value of the hemisulfate salt was calculated from the dihydrochloride salt by using the conversion factor of 0.91 to account for the different molecular weights. Therefore, the calculated EC3 value for 1-hexyl 4,5-diamine pyrazole sulfate is 7.8%. Based on the calculated EC3 value of 7.8%, 1-hexyl 4,5-diamine pyrazole sulfate is a moderate skin sensitiser.

3.4.4 Dermal / percutaneous absorption

Guideline:	OECD TG 428 (2004)
Test system:	Dermatomed frozen (390-400 µm) human skin
Membrane integrity:	Tritiated water method
Group size:	5 donors, 12 replicates
Test substance:	1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate
Batch:	RD-CRU 096-07/142-01
Purity:	99.99%

Test item:	n-Hexylpyrazole hemisulfate-[14C] ([14C]-C6P), radiochemical purity 99.6%
Concentration:	1.5%, 0.5% and 0.165% (w/w) in an oxidative hair dye formulation
Exposed area:	0.64 cm^2
Dose applied:	20 mg/cm²
Exposure time:	30 minutes
Sample volume:	250 µl
Sampling period:	72 hours
Receptor fluid:	Phosphate Buffered Saline (PBS) supplemented with sodium azide (0.01% w/v)
Solubility in receptor fluid:	0.3 mg/ml
Mass balance analysis:	Provided
Tape stripping:	Yes (20)
Method of Analysis:	Liquid Scintillation Counting
GLP:	In compliance
Study period:	April- May 2011

The percutaneous absorption of 1.5%, 0.5% and 0.165% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate in a typical oxidative hair dye formulation, in the presence of hydrogen peroxide and a equimolar concentration of a reaction partner (3-amino-2, 6-dimethylphenol), was investigated *in vitro*, by using human skin preparations. A total of 12 samples of human skin obtained from 5 different donors were used for each test formulation. Mean values were calculated from all valid skin samples (n=12, five donors).
The integrity of the skin was demonstrated prior to application by tritiated water method. Only skin samples within the acceptable range of <0.6% were used.
Split-thickness human skin membranes were mounted into flow-through diffusion cells.
Receptor fluid was pumped underneath the skin at a flow rate of 1.5 ml/h ± 0.15 ml/h. The skin surface temperature was maintained at 32°C ± 1°C throughout the experiment. Test item at 1.5%, 0.5% and 0.165% (w/w) was applied at an application rate of ca 20 mg/cm^2. Absorption of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was evaluated by collecting receptor fluid in 30 min fractions from 0 to 1 h post dose, then hourly fractions from 1 to 6 h post dose and then in 2-hourly fractions from 6 to 72 h post dose. At 30 min post dose, the skin was washed with water, sodium dodecyl sulphate (SDS) solution (2% w/v) and water again. The skin was then dried with tissue paper swabs. At 72 h post dose, the skin surface was washed and dried in the same manner as described for the 30 min wash. The underside of the skin was rinsed with receptor fluid. The skin was then removed from the flow-through cells and dried. The stratum corneum was removed by tape stripping and the skin divided into exposed and unexposed skin (i.e. the area of skin under the cell flange). The exposed epidermis was then heat separated from the dermis. Skin compartments were extracted separately. The radioactivity (1-hexyl-1H-pyrazole-4,5-diamine hemisulfate content) was quantified by liquid scintillation counting.

Results
The majority of the test substance was removed in the skin wash after the 30-minute exposure i.e. 94.24% (311.41 ± 10.39 µg/cm^2), 89.57% (107.39 ± 4.07 µg/cm^2) and 87.38% (27.89 ± 0.81 µg/cm^2) for application doses of 1.5%, 0.5% and 0.165%, respectively. Small amounts of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate were found in the stratum corneum (2.33 ± 0.75 µg/cm^2, 1.40 ± 0.77 µg/cm^2 and 0.55 ± 0.21 µg/cm^2, in the living part of the epidermis (0.99 ± 0.44 µg/cm^2 , 0.54 ± 0.18 µg/cm^2 and 0.16 ± 0.08 µg/cm^2), in the dermis (0.63 ± 0.24 µg/cm^2, 0.21 ± 0.10 µg/cm^2 and 0.07 ± 0.03 µg/cm^2), as well as in the receptor fluid fractions collected within 72 hours (3.71 ± 1.30 µg/cm^2, 1.02± 0.54 µg/cm^2 and 0.26 ± 0.16 µg/cm^2) for the applied doses of 1.5%, 0.5% and 0.165% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate, respectively.

The mass balances for all three test preparations were complete, i.e. 98.31 ± 3.09 %, 94.27 ± 3.38 % and 93.23 ± 2.17 %. The results of the cutaneous absorption experiment are summarised in Table 1.

4.4.1. Table 1: Summary of the cutaneous absorption of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate

Amount of *1-HEXYL-1H-PYRAZOLE-4,5-DIAMINE HEMISULFATE* in:	Concentration of *1-HEXYL-1H-PYRAZOLE-4,5-DIAMINE HEMISULFATE* in typical hair dye formulation:								
	1.5 %			0.5 %			0.165 %		
	Recovery [µg/cm^2]								
Rinsing solution (after 30 minutes)	311.41	±	10.39	107.39	±	4.07	27.89	±	0.81
Dislodgeable dose* (after 30 minutes)	315.19	±	10.16	108.84	±	3.86	28.33	±	0.89
Stratum Corneum (72 hours)	2.33	±	0.05	1.40	±	0.77	0.55	±	0.23
Epidermis (72 hours)	0.99	±	0.44	0.54	±	0.18	0.16	±	0.08
Dermis (72 hours)	0.63	±	0.24	0.21	±	0.10	0.07	±	0.03
Receptor fluid (72 hours)	3.71	±	1.30	1.02	±	0.54	0.26	±	0.16
Total balance (recovery)**	324.83	±	10.21	113.04	±	4.06	29.76	±	0.69
	% Applied dose**								
Rinsing solution (after 30 minutes)	94.24	±	3.14	89.57	±	3.39	87.38	±	2.55
Dislodgeable dose* (after 30 minutes)	95.39	±	3.08	90.77	±	3.22	88.76	±	2.77
Stratum Corneum (72 hours)	0.71	±	0.23	1.17	±	0.64	1.73	±	0.65
Epidermis (72 hours)	0.30	±	0.13	0.45	±	0.15	0.49	±	0.25
Dermis (72 hours)	0.19	±	0.07	0.18	±	0.08	0.21	±	0.09
Receptor fluid (72 hours)	1.12	±	0.39	0.85	±	0.45	0.81	±	0.50
Total balance (recovery)***	98.31	±	3.09	94.27	±	3.38	93.23	±	2.17

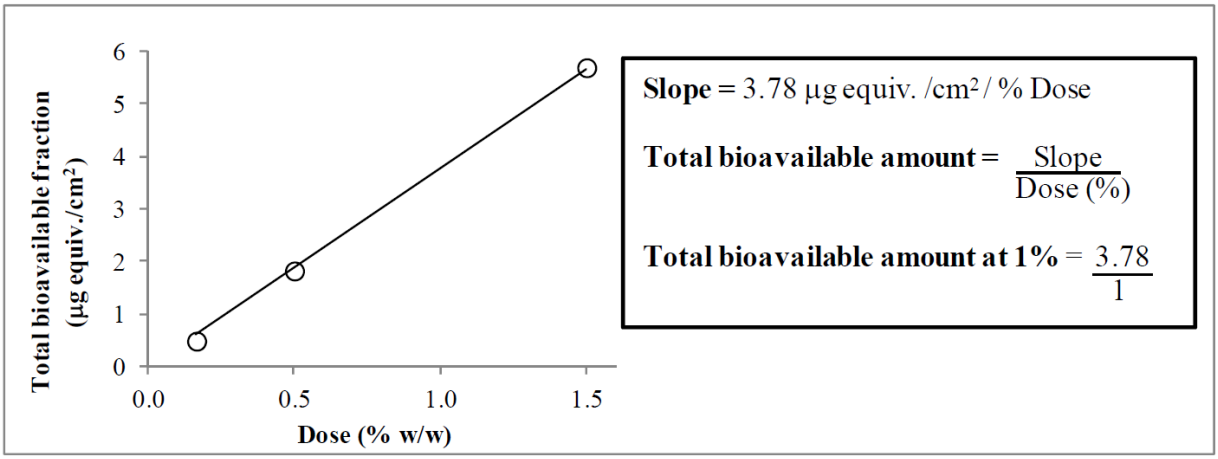
All values are mean ± SD, n=12

* The dislodgeable dose is the sum of the skin wash, tissue swab and pipette tips
** Corrected for individual applied dose
*** Total is corrected for losses on tips

For all three test concentrations, fractions of the receptor fluid collected within hours 0-3 contained the majority of the 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate detected in total receptor fluid. After this time, the amounts in the total receptor fluid declined over 72 hours below the limit of reliability (of 0.001 µg/cm² for 0.165% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate) or close to the limit of reliability (of 0.001/0.004 µg/cm² for 0.5% and 1.5% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate, respectively). This indicates that 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate remaining on or in the stratum corneum or in the epidermis after 72 hours does not exhibit a tendency to migrate to deeper layers and to become systemically available (no depot effect).
Consequently, due to the absence of a depot effect in the epidermis, the total bioavailable fraction (amounts in receptor fluid plus dermis) is considered to be 4.33 ± 1.38 µg/cm², 1.23 ± 0.56 µg/cm², and 0.33 ± 0.16 µg/cm², following topical application of 1.5%, 0.5% and 0.165% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate in a typical oxidative hair dye product, respectively.
In order to be compliant with current SCCS requirements for the calculation of the margin of safety, 1 SD was added to the mean total bioavailable fraction leading to values of 5.7, 1.83 and 0.49 µg equiv./cm^2, following topical application of 1.5%, 0.5% and 0.165% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate in a typical oxidative hair dye product, respectively.

Calculation of the percutaneous absorption from 1.0% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate:
The measured total bioavailable fraction values + 1 SD were found to be directly proportional to the dose applied (See Figure 1).
Consequently, this correlation was used to calculate the total bioavailable fraction + 1 SD following topical application of 1.0% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate in a typical oxidative hair dye product.
Using the equation provided in Figure 1, the total bioavailable fraction + 1 SD is 3.78 µg/cm² (i.e. the slope) following topical exposure to 1.0% 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate in a typical oxidative hair dye formulation. Consequently, the value of 3.78 µg/cm² was used for the calculation of the margin of safety.

4.4.1. Figure 1. Correlation between the dose applied and the total bioavailable fraction + 1SD.



Ref. 21

SCCS comment
The *in vitro* dermal absorption study was performed using three different hair dye formulations of 1-hexyl 4,5-diamine pyrazole sulfate at concentrations of 1.5, 0,5 and 0.165%. A typical hair dye formulation containing the anticipated in-use concentration of

1% was not tested. Nevertheless, the dermal absorption for 1% 1-hexyl 4,5-diamine pyrazole sulfate has been calculated via interpolation. In accordance with the Notes of Guidance, the material available in the living epidermis has not been considered as no movement of the chemicals from the skin reservoir to the receptor fluid occurred.
The calculated systemically available dose for 1% 1-hexyl 4,5-diamine pyrazole sulfate of 3.78 µg/cm² (mean + 1 SD) will be used for the MoS calculation.

3.4.5 Repeated dose toxicity

3.4.5.1 Repeated Dose (14 days) oral toxicity

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3.4.5.2 Sub-chronic (90 days) toxicity (oral)

Guideline:	OECD TG 408 (1998)
Species/strain:	Rat, strain Hsd:Sprague Dawley®™SD®™
Group size:	15/sex/group (control and high-dose groups), 10/sex/group (other groups)
Test substance:	1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate
Batch:	RD-CRU 096-07/142-01
Purity:	99.1%
	Vehicle: 10% (w/v) propylene glycol and 0.1% (w/w) ascorbic acid in reverse osmosis water
Dose levels:	0, 2.5, 5, 8 and 20 mg/kg bw/day
Dose volume:	5 ml/kg bw
Route:	Oral
Administration:	Gavage
GLP:	In compliance
Study period:	9 April 2012 – 18 July 2013

1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate was administered daily by oral gavage to Sprague Dawley rats of both sexes at dose levels of 0, 2.5, 5, 8 and 20 mg/kg bw/day for 90 days; the vehicle was 10% (w/v) propylene glycol and 0.1% (w/w) ascorbic acid in reverse osmosis water and the pH was adjusted using ammonium hydroxide solution based on formulation concentration. The groups comprised 10 animals per sex, which were euthanised after 90 days of treatment. An additional 5 rats per sex and group at 0 and 20 mg/kg bw were treated for 90 days and then allowed a 14-day treatment-free recovery period, after which they were euthanised.
Test solutions were prepared freshly daily. Samples from the formulations were analysed for actual concentrations of the test item on days 1, 2, 3, 8, 15, 22, 50, 79, and 85 of the dosing phase. Stability was determined and reported under Covance Study No. 8256312.
Animals were checked twice daily for mortality, abnormalities, and signs of pain or distress. Bodyweights were recorded once during the pre-dose phase, before dosing on day 1, weekly thereafter through the dosing phase, on the day prior to dosing phase necropsy, and on days 1, 8, and 14 of the recovery phase. Food consumption was measured weekly during weeks 1 to 13 of the dosing phase, for days 85 to 90 of the dosing phase, and from days 1 to 8 and 8 to 14 of the recovery phase. Ophthalmoscopic examinations were performed once during the pre-dose phase, on day 85/89 (males/females) of the dosing phase, and on day 9/8 (males/females) of the recovery phase. Functional observational battery, locomotor activity and grip strength were assessed once during the pre-dose phase and then on day 86 of the dosing phase. Samples were collected on the day of scheduled sacrifices from all surviving animals. Blood samples were collected for haematology, coagulation, and clinical

chemistry and urine samples were collected for urinalysis. After 90 days of dosing, 10 animals/sex/group were sacrificed, necropsied and examined post mortem. After 90 days of dosing followed by 14 days of recovery, all surviving animals were sacrificed, necropsied and examined post mortem. Protocol-specified organ weights were recorded at each scheduled sacrifice. Histological examinations were performed on protocol-specified organs and tissues from all control and treated animals in both the main study and the recovery groups.

Results

According to the study report authors, the stability test of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate demonstrated that the dose formulations were stable under the conditions employed in this study. No 1-hexyl-1H-pyrazole-4,5-diamine was detected in vehicle control article formulations. Mean measured concentrations of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate ranged from 92.2 to 101.1% of theoretical and were within the limits of acceptability and suitable for use on the study.

One high-dose female was found dead on day 1 of the recovery phase. No toxicologically relevant findings were observed microscopically. Due to the temporal relationship to clinical pathology blood collection, this death was considered accidental by the study report authors and not related to 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate administration. All other animals survived to their scheduled necropsy, and no clinical signs were noted, which were considered as related to the administration of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate.

No effects on mean food consumption, mean bodyweight, mean bodyweight change, ophthalmoscopic observations, locomotor activity, expanded clinical observations or elicited behaviours were noted, which were considered as related to the administration of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate.

Treatment-related effects on haematology included lower red blood cell count for males given 20 mg/kg bw/day (95% of the mean count in the control group) and females given 8 and 20 mg/kg bw/day (96 and 88% of the mean count in the control group, respectively), lower haemoglobin for animals given 20 mg/kg bw/day (96 and 92% of the values in the control group for males and females, respectively), haematocrit for females given 20 mg/kg bw/day (94% of the value in the control group), higher mean corpuscular volume for females given 20 mg/kg bw/day (107% of the value in the control group), higher mean corpuscular haemoglobin for females given 20 mg/kg bw/day (104% of the value in the control group), lower mean corpuscular haemoglobin concentration for animals given 20 mg/kg bw/day (98% of the values in the control group for both males and females), higher reticulocyte count for males given 20 mg/kg bw/day (136% of the value in the control group) and females given 8 and 20 mg/kg bw/day (126 and 230% of the mean count in the control group, respectively), higher absolute neutrophil counts for females given 20 mg/kg bw/day (151% of the value in the control group), and shorter prothrombin time in males given 20 mg/kg bw/day (96% of the value in the control group). Effects on mean corpuscular volume and mean corpuscular haemoglobin did not exhibit reversibility. According to the study report authors, these findings supported a minimal to mild reduction in red blood cell mass with an appropriate regenerative reticulocytosis that correlated with the increased splenic extramedullary haematopoiesis observed microscopically; the higher absolute neutrophil count may have represented an inflammatory response.

Effects on clinical chemistry test results were limited to higher bilirubin for females given 20 mg/kg bw/day (200% of the value in the control group), which, according to the study report authors could be associated with haemolysis, which would correlate with the haematology findings and pigment-laden macrophages observed in the liver.

Mean absolute and relative spleen weights were greater than control values in females given 20 mg/kg bw/day (125 and 129% of the control values, respectively). These weight differences were considered test article-related by the study report authors and correlated with an increased incidence of increased extramedullary haematopoiesis noted microscopically in this group. No statistically significant or test article-related organ weight changes were noted in animals at the recovery phase necropsy.

All macroscopic findings were considered spontaneous, incidental, and/or unrelated to the test article by the study report authors.

Test article-related microscopic findings were noted in the liver and spleen of males and females at the dosing phase necropsy. Test article-related findings in the liver included minimal infiltrates of pigment-laden macrophages/Kupffer cells in females given 20 mg/kg bw/day, and minimally increased Prussian blue staining in males and females given 20 mg/kg bw/day. In the spleen, test article-related findings included minimal infiltrates of pigment-laden macrophages in males given 20 mg/kg bw/day, minimally increased extramedullary haematopoiesis in males and females given 20 mg/kg bw/day, and minimally increased Prussian blue staining in males given 20 mg/kg bw/day and females given 8 or 20 mg/kg bw/day. Prussian blue stain detects ferric ions in loosely bound protein complexes, as in hemosiderin. Because increased Prussian blue staining occurred concurrently with the presence of pigment-laden macrophages and/or Kupffer cells in the liver and spleen, the pigment likely represented hemosiderin, according to the study report authors, and suggested red blood cell damage and/or haemolysis as a possible aetiology. This was further supported by the minimal to mild reduction in red blood cell mass in males given 20 mg/kg/day and females given 8 and 20 mg/kg bw/day. Because evidence for effects on red blood cells persisted in the recovery phase females, the test article-related morphological changes were considered toxicologically relevant by the study report authors. No differences were observed in the 2.5 and the 5.0 mg/kg bw/day dose groups.

Conclusion
In conclusion, daily administration of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate by oral gavage to Sprague Dawley rats at a dose level of 20 mg/kg bw/day to males, and 8 and 20 mg/kg bw/day to females for 90 days resulted in minimal to mild decreases in red blood cell mass. Although reversible, this dose and sex dependent effect on haematopoiesis was considered of toxicological relevance. Accordingly, the no observed adverse effect level (NOAEL) is 8.0 mg/kg bw/day for males and 5.0 mg/kg bw/day for females, which is also the no observed effect level (NOEL).

Ref. 22

SCCS comment
No justification for the use of ascorbic acid in the test vehicle was given.
The study report (Covance Study No. 8256312) demonstrating the stability of 1-hexyl 4,5-diamine pyrazole sulfate has not been submitted.
The SCCS agrees with the NOAEL of 5.0 mg/kg bw/day; this NOAEL is used for the MOS calculation.

3.4.5.3 Chronic (> 12 months) toxicity

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3.4.6 Mutagenicity / Genotoxicity

3.4.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537
Replicates:	triplicates in a single experiment
Test substance:	4,5-Diamino-1-hexyl-1H-pyrazole dihydrochloride
Batch:	DTF654/074A-HAY
Purity:	100 area% at 254 nm

Solvent:	deionised water
Concentrations:	0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate with and without S9-mix
Treatment:	direct plate incorporation with at least 48 h incubation, with and without S9-mix (experiment I) Pre-incubation test with 60 minutes pre incubation and 48 h incubation with and without S9-mix (experiment II)
GLP:	in compliance
Study period:	31 August 2009 – 8 September 2009

4,5-Diamino-1-hexyl-1H-pyrazole dihydrochloride was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment for toxicity and mutation induction with all strains both with and without S9-mix. Toxicity was evaluated for 8 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 clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as the main experiment. Experiment I was performed with the direct plate incorporation method; in experiment II the pre-incubation method with 60 minutes pre-incubation was used. Negative and positive controls were in accordance with the OECD guideline.

Results
No precipitation occurred up to the highest concentration investigated. Toxic effects evident as a reduction in the number of revertants or reduced background growth were not observed up to the highest concentrations with S9-mix in all strains. In the presence of metabolic activation, a reduction in the number of revertants and a reduced background growth was observed in all strains at the higher concentrations above 2500 µg/plate. Toxic effects also at lower concentrations were found for TA100 and TA102.
In both experiments, a biologically relevant increase in the number of revertant colonies was not observed with any strain used, at any concentration both in the presence and absence of metabolic activation.

Conclusion
Under the experimental conditions used 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride was not genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref. 23

***In vitro* Mammalian Cell Gene Mutation Test (*hprt*-locus)**

Guideline:	OECD 476 (1997)	
Cells:	V79 cells	
Replicates:	duplicate cultures in two independent experiments	
Test substance:	4,5-Diamino-1-hexyl-1H-pyrazole, dihydrochloride	
Batch:	RD-CRU 096-07/47-01	
Purity:	99.71 area% (at 254 nm, HPLC)	
Solvent:	deionised water	
Concentrations:	experiment I:	5, 10, 20, 30 and 40 µg/ml without S9-mix 40, 80, 160, 320 and 640 µg/ml with S9-mix
	experiment II	2.5, 5, 10, 15 and 20 µg/ml without S9-mix 320, 640, 720, 800 and 880 µg/ml with S9-mix
Treatment:	experiment I:	4 h treatment both with and without S9-mix;

		Expression period 7 days and a selection period of 8 days.
	experiment II:	4 h treatment both with and without S9-mix; expression period 7 days and a selection period of 8 days. 24 h treatment both without S9-mix; expression period 7 days and a selection period of 8 days.
GLP:	in compliance	
Study period:	3 May 2011 – 4 July 2011	

4,5-Diamino-1-hexyl-1H-pyrazole dihydrochloride was assayed for gene mutations at the *hprt* locus of V79 cells in both the absence and presence of S9 metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test on toxicity. Toxicity of 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride is indicated by a reduction of the cloning efficiency. Eight concentrations were tested with and without metabolic activation and exposure for 4 h with concentrations up to 2560 µg/ml or without metabolic activation and exposure for 24 h with concentrations up to 40 µg/ml.
In the main tests, cells were treated for 4 h or 24 h (experiment II, without S9-mix only) followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured in the main experiments as percentage cloning efficiency of the treated cultures relative to the cloning efficiency of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
At higher concentrations, the pH was neutralised with 2N sodium hydroxide. No relevant shift of the osmolarity of the medium was seen at the two higher concentrations used.
In the pre-test, relevant cytotoxic effects were observed at 20 µg/ml and above in the absence of metabolic activation and at 1280 µg/ml and above with metabolic activation. After 4 h treatment, turbidity of 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride occurred at 160 µg/ml and 320 µg/ml without S9-mix and at 1280 µg/ml and 2560 µg/ml with S9-mix. At 640 µg/ml and above precipitation occurred. After 24 h of treatment, neither turbidity nor precipitation was observed.
The data on cloning efficiency after the selection period did not indicate that the required 10-20% survival was reached at any concentration.
In both experiments, no biologically relevant and concentration dependent increase in the mutant frequency was observed, either in the presence or in the absence of metabolic activation. Moreover, a linear regression analysis did not indicate a significant concentration-dependent trend of the mutant frequency by a probability value of <0.05 in any of the experimental groups.

Conclusion
Under the experimental conditions used, 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride did not induce gene mutations in this gene mutation test in mammalian cells and, consequently, 4,5-diamino-1-hexyl-1H-pyrazole dihydrochloride is not mutagenic in V79 cells.

Ref. 24

SCCS comment
The data on cloning efficiency after the selection period did not indicate that the required 10-20% survival was reached at any concentration.

***In vitro* Micronucleus Test in human lymphocytes**

Guideline:	In compliance with the OECD draft guideline OECD 487 (2009)
Test system:	human peripheral blood lymphocytes
Replicates:	duplicate cultures in a single experiments with 2 exposure times
Test item:	4,5-Diamino-1-hexyl-1H-pyrazole, dihydrochloride
Batch:	DTF654/074A-HAY
Purity:	100%
Solvent:	water
Concentrations:	25, 50, 100 and 150 µg/ml without S9-mix and 4 h exposure 25, 50, 100 and 175 µg/ml with S9-mix and 4 h exposure 12.5, 25, 50 and 80 µg/ml without S9-mix and 24 h exposure
Treatment	24 h PHA, 4 h treatment and 20 h recovery without or with S9-mix 24 h PHA, 24 h treatment without S9-mix
GLP:	in compliance
Study period:	3 August 2010 – 3 September 2010

4,5-Diamino-1-hexyl-1H-pyrazole, dihydrochloride has been investigated for the induction of micronuclei in cultured human lymphocytes in the absence and presence of metabolic activation. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
Blood from two healthy, non-smoking female volunteers was used in this study. The donors had no recent history of radiotherapy, viral infection or drug administration. After isolation of the donor cells, the mitogen phytohaemagglutinin (PHA) was included in the culture medium in order to stimulate the lymphocytes to divide, and blood cultures were incubated at 37° C for 48 h (pre-experiment) or 24 h (main experiment). The preliminary toxicity test and the main experiments were performed after this mitogenic treatment.
The concentration selection for the micronucleus test was based on the proliferation and cytotoxicity data from a preliminary toxicity test with 9 concentrations up to 2550 µg/ml measuring cytokinesis blocked proliferation index (CBPI) or replication index (RI) relevant to the solvent control. The top concentration for micronucleus analysis was to be the one at which approximately 60% reduction in RI occurred.
Both in the range finder and in the main test, cells were treated either for 4 h in the absence or presence of S9-mix or 24 h in the absence of S9-mix, and sampled 24 h after start of treatment. Cytochalasin B (final concentration 6 µg/ml) was added after the 4 h treatment or during the complete 24 h treatment. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Measurements on post-treatment media in the absence or presence of S9-mix indicated that 4,5-diamino-1-hexyl-1H-pyrazole, dihydrochloride had no marked effect on osmolarity or pH as compared to concurrent vehicle controls.
In the preliminary toxicity test, visible precipitation and haemolysis were observed at concentrations of 765 µg/ml and above after 4 h exposure both with and without S9-mix and at 255 µg/ml and above after 24 h exposure without S9-mix.
Substantial cytotoxicity was seen at concentration levels of 255 µg/ml and above after 4 h exposure both with and without S9-mix and at 76.5 µg/ml and above after 24 h exposure without S9-mix.
In the main micronucleus test, visible precipitate was only observed after 24 h treatment without S9-mix at concentrations of 80 µg/ml and above. At the highest concentrations evaluated both after 4 and 24 h treatment and both in the absence or presence of S9-mix cytotoxicity was between 53% and 59 % relative to the solvent control.
A biologically relevant and concentration dependent increase in the number of cells with micronuclei was not observed in cells treated for 4 h both in the absence or presence of S9-mix or treated for 24 h in the absence of S9-mix.

Conclusion
Under the experimental conditions used 4,5-Diamino-1-hexyl-1H-pyrazole, dihydrochloride did not induce an increase in cells with micronuclei and, consequently, is not genotoxic (clastogenic and/or aneugenic) in cultured human peripheral lymphocytes *in vitro*.

Ref.25

SCCS overall comment on mutagenicity / genotoxicity
The *in vitro* mutagenicity tests were performed with 1-hexyl-1H-pyrazole-4,5-diamine dihydrocloride, *i.e*. the dihydrochloride instead of the hemisulfate salt. 1-Hexyl-1H-pyrazole-4,5-diamine dihydrocloride was found non-genotoxic in these three *in vitro* genotoxicity assays covering all genotoxicity endpoints, thereby demonstrating its absence of a genotoxicity potential. The toxic potential of both salts of 1-hexyl-1H-pyrazole-4,5-diamine is attributable to the free base component. Therefore, the results of the *in-vitro* mutagenicity studies carried out with 1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride were taken into consideration for the risk assessment of 1-hexyl 4,5-diamine pyrazole sulfate. Accordingly, 1-hexyl 4,5-diamine pyrazole sulfate is, therefore, considered not to pose a risk to humans with regard to genotoxicity.

3.4.6.2 Mutagenicity / Genotoxicity *in vivo*

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3.4.7 Carcinogenicity

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3.4.8 Reproductive toxicity

3.4.8.1 Two generation reproduction toxicity

/

3.4.8.2 Other data on fertility and reproduction toxicity

/

3.4.8.3 Developmental Toxicity

Guideline:	OECD TG 414
Species/strain:	Rat, strain Sprague-Dawley rats Crl:CD(SD)
Group size:	25/females/group
Test substance:	1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate
Batch:	RD-CRU 096-07/142-01
Purity:	99.1%
Vehicle:	10% (w/v) propylene glycol and 0.1% (w/w) ascorbic acid in reverse osmosis water, pH 7.0
Dose levels:	0, 2.5, 5, 8 and 20 mg/kg bw/day
Dose volume:	5 ml/kg bw
Route:	Oral

Administration: Gavage
GLP: In compliance
Study period: 9 April 2012 – 18 July 2013

1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate was administered daily by oral gavage to pregnant female Sprague Dawley rats at dose levels of 0, 2.5, 5, 8 and 20 mg/kg bw/day from gestation day 6 to day 20; the vehicle was 10% (w/v) propylene glycol and 0.1% (w/w) ascorbic acid in reverse osmosis water, pH 7.0. The groups comprised 25 females.
The dosing formulations were stirred continuously during dose administration. Test solutions were prepared freshly daily. Samples from the formulations were analysed for actual concentrations at the first, mid-interval and the last day of dosing (all 5 groups) and for stability at the mid-interval day of dosing (from the 2.5, 8 and 20 mg/kg bw/day dose groups).
The rats were assessed for viability at least twice daily during the study. The animals were observed for general appearance twice during the acclimation period, on gestation day 0, daily before each dose was administered, and once daily during the post-dose period. Bodyweights were recorded twice during the acclimation period, on gestation day 0, and daily during the dose and post-dose periods. Food consumption data were recorded on gestation days 0, 6, 9, 12, 15, 18, and 21. Prior to scheduled euthanasia blood samples were collected for haematology. On gestation day 21, all mated females were euthanised followed by Caesarean section and a complete autopsy and a macroscopic examination of the organs were carried out. The gravid uterus, liver, and spleen were weighed for all rats at scheduled euthanasia. The ovaries and uterus were examined for number and distribution of corpora lutea, implantation sites, placentae (size, colour or shape), live and dead foetuses, and early and late resorptions. Uteri of apparently non-pregnant rats were examined to confirm the absence of implantation sites. Uteri (with cervix) and ovaries of apparently non-pregnant animals were preserved for histopathological examination. Representative samples of the tissues were collected from the rats that were euthanised before scheduled termination and all rats that survived to scheduled euthanasia and preserved for histopathological examination. Each viable foetus was euthanised and then weighed, sexed and examined for external malformations. Late resorptions were examined for external abnormalities and sex to the extent possible. Half of the foetuses were prepared for examination of soft tissues abnormalities and the remaining foetuses were examined for skeletal abnormalities.

Results
Concentration results for samples obtained from all dose formulations on the first, intermediate, and last days of preparation met acceptance criteria (± 10% of nominal concentrations), with the exception of samples obtained from the 2.5 mg/kg bw/day dose group from the intermediate preparation (-12.8% of nominal concentration). Analysis of the backup sample confirmed the original result (-13.8%). According to the study report authors, this excursion from acceptance criteria did not affect the outcome or interpretation of study because: 1) the excursion was considered minimal (2.8% to 3.8% difference); and 2) the results for this concentration from the first and last days of preparation met acceptance criteria. Formulation stability for the 2.5 and 8 mg/kg bw/day dose groups was established for 21 days and for the 20 mg/kg bw/day dose group for 27 days. Sample concentration results after storage were within or equal to ±10% of the initial (time 0) mean sample concentration results.
There were no unscheduled deaths or adverse clinical signs in any group. Spontaneous delivery (gestation days 20 and 21) were observed in the control group (one) and in the 2.5 and 8.0 mg/kg bw/day dose groups (one each). These spontaneous deliveries occurred in a non-dose-dependent manner and were not attributed to administration of the test substance.
A transient reduction in mean bodyweight gain occurred in the 20 mg/kg bw/day dose group on gestation days 6 to 9 (89% of the control group value). Despite this transient reduction, mean body weight gains in the 20 mg/kg/day bw dose group during the entire

dose period (gestation days 6 to 21) and the overall gestation period (gestation days 0 to 21) were 96% and 98% of the control group value, respectively. However, a slight reduction (89% of the control group value, not statistically significant) was apparent in the 20 mg/kg bw/day dose group during the entire dose period following correction for gravid uterine weights. Food consumption values were generally comparable among the five dose groups and did not differ significantly.
A statistically significant increase ($p \leq 0.05$) in mean reticulocyte count occurred in the 20 mg/kg/day dose group (153% of the control group value). Mean platelet volume was increased in all dose groups (statistically significantly ($p \leq 0.05$) from 5 mg/kg bw/day) in each of the groups (106, 107, 109 and 110% of the control group value, respectively); all of the values were within the range of historical control data for female Sprague-Dawley rats (5 to 12 weeks of age) provided by the Test Site, with the exception of the 20 mg/kg bw/day dose group.
No treatment-related macroscopic changes were noted at necropsy of the adult females in any group. Non-statistically significant increases (107% and 109% of the control group value) in absolute and relative spleen weights occurred in the 20 mg/kg bw/day dose group; no other substance related organ weight changes were observed. Microscopic examination of the bone marrow (femur), liver, and spleen from all female rats on study revealed no microscopic changes that could be attributed to administration of the test substance.
Pregnancy occurred in 23 to 25 rats in each dose group. Due to premature deliveries in the control group and in the 2.5 and 8.0 mg/kg bw/day dose groups, ovarian/uterine examinations on gestation day 21 were based on 24, 24, 25, 22, and 25 pregnant rats in the control group and the 2.5, 5.0, 8.0, and 20 mg/kg bw/day dose groups, respectively.
The litter averages for *corpora lutea*, implantations, percentage of preimplantation loss, litter sizes, live foetuses, early and late resorptions, percentage of post-implantation loss, and percentage of resorbed conceptuses per litter were comparable among the five dose groups and did not significantly differ. No dam had a litter consisting of only resorbed conceptuses, and there were no dead foetuses. All placentae appeared normal.
Mean foetal bodyweights (female and total) in the 20 mg/kg bw/day dose group were statistically significantly lower ($p \leq 0.05$, both 96% of the control group value). The mean percentage of live male foetuses per litter was statistically significantly lower ($p \leq 0.01$) in the 5.0 mg/kg bw/day dose group.
No foetal gross external, soft tissue or skeletal alterations (malformations or variations) were related to the administration of C6 of the test substance at dose levels up to 20 mg/kg bw/day. The average number of ossification sites per litter was also comparable among the five dose groups and did not significantly differ.

Conclusion
Based on these results, the NOAEL for maternal and developmental toxicity was 8.0 mg/kg bw/day. Neither malformations nor variations occurred at doses as high as 20 mg/kg bw/day, the highest dose tested.

Ref.26

SCCS comment
No justification for the use of ascorbic acid in the test vehicle was given.

3.4.9 Toxicokinetics

3.4.9.1 Toxicokinetics in laboratory animals

Guideline:	OECD TG 417 and TG 427
Species/strain:	Rat, Sprague Dawley, (Hsd:Spraque Dawley SD)
Group size:	6 Females in the mass balance groups (groups 1, 2, 3, 4) 4 Females in the iv and oral toxicokinetic groups (groups 5 and 6) 8 Females in the dermal toxicokinetic groups (groups 7 and 8)
Test substance:	1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate
Batch:	RD-CRU-096-07/142-01
Purity:	99.1 %
Test substance:	^{14}C-1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate, labelled on the carbon in the 5th position
Batch:	CFQ41662
Radiopurity:	98.4 %; specific activity: 25 mCi/mmol
Vehicle:	Intravenous administration (groups 1 and 5): 0.1% ascorbic acid in aqueous sodium hydroxide (pH 6.81) Oral administration (groups 2 and 6): 0.1% ascorbic acid in aqueous sodium hydroxide (pH 6.06) Dermal administration – 0.5 hour (groups 3 and 7): 0.1% ascorbic acid in aqueous sodium hydroxide Dermal administration – 24 hours (groups 4 and 8): 10% (w/v) propylene glycol and 0.1% (w/v) ascorbic acid in aqueous sodium hydroxide (pH 6.90)
Dose levels:	Intravenous administration: 11.9 mg/kg bw (containing approximately 19.7 µCi/mg of radioactivity) Oral administration: 12 mg/kg bw (containing approximately 19.5 µCi/mg of radioactivity) Dermal administration: 11.0 mg/kg bw (groups 3 and 7) and 11.5 mg/kg bw (groups 4 and 8) – equal to 0.186 mg/cm² and 0.208 mg/cm², respectively (containing approximately 83.0 / 83.1 µCi/mg of radioactivity, respectively)
Route:	Intravenous, oral (gavage), dermal
Administration:	Single administration
GLP:	yes
Study period:	February - June 2013

The absorption, distribution, metabolism, and excretion of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate following a single intravenous (iv, Groups 1 and 5), oral (gavage, Groups 2 and 6), or dermal (Groups 3, 4, 7, and 8) administration was investigated in female rats. ^{14}C-1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate was administered at a target dose level of 12 mg/kg (iv and oral doses: 250 µCi/kg, and dermal doses: 1000 µCi/kg). During dermal application, the skin was exposed for 0.5 (Groups 3 and 7) or 24 (Groups 4 and 8) hours.
The animals were observed twice daily for mortality and signs of pain and distress. Cage side observations for general health and appearance were done once daily.

Mass balance groups (groups 1, 2, 3, 4):
Groups 1 and 2: Blood was collected at 0.25, 0.5, 1, 2, 8, 24, 48, 72, 96, 120, 144 and 168 hours post-dose from 2 animals/group/time point (protocol deviation).
Groups 3 and 4: Blood was collected at 0.5 (prior to skin wash, if applicable), 1, 2, 4, 8, 24 (prior to skin wash, if applicable), 48, 72, 96, 120, 144 and 168 hours post-dose from 2 animals/group/time point.

After the last blood collection, all animals were sacrificed and blood was collected for use in metabolite profiling.

Toxicokinetic groups (groups 5, 6, 7, 8):
Urine was collected at 0-8 and 8-24 hours post-dose, and at 24-hour internals through 168 post-dose. Faeces were collected at 24-hour internals through 168 post-dose. After each 24-hour excreta collection through 144 hours post-dose, cages were rinsed with water and the samples were collected. After the last excreta collections, cages were washed and wiped and cage wash samples and gauze pads were collected. Animals were sacrificed and the residual carcass was weighed and retained for radioanalysis.

Dermal groups (groups 3, 4, 7 and 8):
At 0.5 or 24 hours post-dose, the applicators/spreaders, non-occlusive cover, enclosures, skin wash/rinses, gauze, dosed skin, and non-dosed skin (abdominal region) were collected and retained for radioanalysis.

Concentrations of radioactivity in whole blood, plasma, urine, faeces (after combustion), cage rinse, cage wash, and dermal apparatus/skin washes/rinse samples were determined by liquid scintillation counting (LSC). The toxicokinetic parameters for total radioactivity in blood and plasma were calculated. Selected samples of plasma, urine, faeces, and cage rinse were profiled for the metabolites of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate.

Metabolite profiling and identification:
Following all routes of exposure, selected plasma, urine, faeces, and cage rinse samples were pooled and profiled for metabolites of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate. Plasma pools (representing the majority of radioactivity exposure in plasma) for all dose groups were generated using a time-weighted pooling scheme to obtain a single area under the curve (AUC)-representative sample from 0.25 hour (0.5 hour for dermal groups) to 144 hours (168 hours for dermal groups) post-dose. Selected urine, faeces and cage rinse samples collected post-dose were pooled across animals and collection intervals, as applicable. Aliquots of pooled plasma and faeces samples were extracted and analysed by HPLC. For the determination of exposure to major metabolites in plasma, the total radioactivity in the HPLC run (sum of % total for all individual peaks detected) was considered to be the total radioactivity exposure in plasma from 0.25 hour (0.5 hour for dermal groups) to 144 or 168 hours post-dose, and the percent of radioactivity that each peak represented in the HPLC run was considered as the percent exposure of that metabolite from 0.25 hour (0.5 hour for dermal groups) to 144 or 168 hours post-dose, as applicable. Structures of the metabolites were predicted by liquid chromatography mass spectrometry (LC-MS and/or LC-MS/MS).

Results
Stability under conditions of administration was demonstrated by analysing pre-dose and post-dose aliquots by HPLC.

All animals appeared healthy and no overt signs of toxicity were observed throughout the study. At approximately 0.5 hours post-dose, all animals in the dermal groups (groups 3, 4, 7 and 8) had an orange-coloured dose site. At approximately 24 hours post-dose, all animals (in groups 4 and 8) had a red-coloured dose site.

Mass balance groups (groups 1, 2, 3, 4):
The toxicokinetic parameters in blood and plasma for total radioactivity are presented in the table below:

Matrix	Groups (Dose Route)	C_0 (ng eq/g)	C_{max} (ng eq/g)	T_{max} (hours)	$t_{½}$ (hours)	AUC_{0-t} (ng eq·hours/g)	$AUC_{0-\infty}$ (ng eq·hours/g)	$AUC_{0-\infty}$ (Dose Normalized) [(ng eq·hour/g)/ mg/kg)]	% of iv $AUC_{0-\infty}$
Blood	1 (iv)	18900	N.A.	N.A.	48.6	227000	237000	19900	N.A.
Blood	2 (oral)	N.A.	17000	0.500	62.4	207000	221000	18400	93.2
Blood	3 (0.5-hr dermal)	N.A.	193	4.00	47.5	4810	5150	468	2.17
Blood	4 (24-hr dermal)	N.A.	364	8.00	60.3	17100	18900	1660	7.97
Plasma	1 (iv)	16600	N.A.	N.A.	40.0	105000	106000	8940	N.A.
Plasma	2 (oral)	N.A.	12500	0.500	51.7	105000	107000	8920	101
Plasma	3 (0.5-hr dermal)	N.A.	164	4.00	33.4	2450	2510	228	2.37
Plasma	4 (24-hr dermal)	N.A.	281	8.00	71.3	9310	10600	932	10.0

eq Equivalents ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine.
iv Intravenous.
N.A. Not applicable.

Following oral administration of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate, the test item was rapidly absorbed with blood and plasma reaching maximum concentration at the same time (T_{max}: 0.5 hours, first time point) for total radioactivity in blood and plasma, indicating a rapid absorption. Following dermal application, the T_{max} values for total radioactivity in blood and plasma were longer (T_{max} values between 4 to 8 hours) when compared to oral administration, indicating a slower absorption.

Following a single iv, oral, or dermal administration, the exposure ($AUC_{0-\infty}$) in blood was 2.23-, 2.06-, 2.05-, or 1.78-fold greater than in plasma in groups 1, 2, 3, and 4, respectively. The average blood to plasma concentration ratios for total radioactivity following iv, oral, or dermal administration in groups 1 through 4 were greater than one (ranging from 1.11 to 7.24), indicating preferential distribution of radioactivity to the cellular component of blood. Radioactivity in blood and plasma declined with similar elimination half-life values for the respective dose routes; likewise, the elimination half-life values were generally comparable between dose routes.

Following iv and oral dosing, exposures ($AUC_{0-\infty}$) of total radioactivity in blood and plasma were comparable between dose routes, whereas, the maximum exposure ($AUC_{0-\infty}$) of total radioactivity in blood and plasma following dermal application was lower (2.17 and 2.37% for group 3, and 7.97 and 10.0% for group 4), when compared to exposure following iv administration.

Toxicokinetic groups (groups 5, 6, 7, 8):
The amounts of radioactive dose (mean ± standard deviation, SD) recovered in urine, faeces, cage rinse, and skin wash (groups 7 and 8) following a single iv, oral, or dermal administration are presented in the table below:

Group/Dose Route	% Total Recovery*	% Dose in Faeces	% Dose in Urine	% Dose in Cage rinse	% Dose in Skin Wash
5/iv	94.3 ± 1.69	11.6 ± 1.38	69.2 ± 6.59	9.94 ± 4.12	N.A.
6/Oral	95.1 ± 0.998	14.9 ± 1.93	68.9 ± 5.75	8.41 ± 4.12	N.A.
7/Dermal (0.5 hr)	71.6 ± 3.28	3.99 ± 1.75	3.44 ± 3.09	0.288 ± 0.139	61.3 ± 5.70**
8/Dermal (24 hr)	73.1 ± 2.31	6.18 ± 2.26	7.82 ± 1.92	1.23 ± 0.665	47.8 ± 7.10**

N.A. Not applicable.
* Total recovery also includes the radioactivity levels in the carcasses, the cage wash, the cage wipe, the non-dosed and dosed skin, the faeces wipe and the tape strips, which were not indicated in this table.
** The low recovery in the dermal groups is considered to be caused by limited extraction of radioactivity from the skin and the cage wash; and considered to account for more than 20% of the applied dose. (as demonstrated in an additional test: refer to test description and results in Appendix 8.)

The percentage of the administered dose (mean ± SD) potentially absorbed following dermal administration is presented in the table below:

	Percent of Radioactive Dose				
Group/Dose Route	Dislodged dose[a]	Absorbed dose[b]	Skin Residue dose[c]	Total recovery	(%) Potentially Absorbed Dose[d]
5/iv	N.A.	N.A.	N.A.	94.3 ± 1.69	100
6/Oral	N.A.	N.A.	N.A.	95.1 ± 0.998	82.4
7/Dermal (0.5 hr)	62.8 ± 5.84	8.53 ± 5.01	0.259 ± 0.176	71.6 ± 3.28[e]	8.79 ± 4.95
8/Dermal (24 hr)	56.2 ± 4.47	16.4 ± 4.46	0.440 ± 0.102	73.1 ± 2.31[e]	16.8 ± 4.52

The excretion profiles of radioactivity were similar following iv or oral administration, with the majority of radioactivity recovered in urine. The extent of absorption following oral administration is represented by the sum of fraction excreted in urine (F_u) and the fraction of radioactivity remaining in the carcasses at the end of the study (F_c). The extent of

absorption following iv administration is represented by the sum of fractions excreted in urine (F_u) and faeces (F_f). Therefore the extent of oral absorption (F_a) of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate in this study can be calculated as the sum of fraction excreted in urine (F_u) following oral administration, the remaining radioactivity in the carcasses (F_c) following oral administration and the amount excreted in faeces (F_f) following intravenous administration ($F_a = F_u + F_c + F_f$). Based on the mean radioactivity recoveries in urine (F_u = 68.9%) following oral administration; the remaining radioactivity in the carcasses at the end of the study (F_c = 1.39%); and the mean radioactivity recoveries in faces (F_f = 11.6%) following iv administration, a mean of total of at least 81.89% (F_a = 68.9% + 1.39% + 11.6%) of the administered radioactivity was absorbed.

Radioactivity was eliminated rapidly, with a mean of 84.6% of the administered radioactive dose recovered by 48 hours post-dose following either a single oral or iv administration**.** However, the remaining radioactivity was slowly eliminated, with measurable levels in excreta through 168 hours post-dose. The mean recovery of 11.6% of the administered dose in faeces following iv administration indicated that hepato-biliary excretion, other non-biliary route(s) of excretion (such as direct secretion into the gut) of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate-related radioactivity from the systemic circulation into the gastrointestinal tract, or metabolism (by gut microflora) within the gastrointestinal tract (GI) was involved to some extent in the elimination of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate-related radioactivity in rats.

Following dermal application, a mean of 8.79 and 16.8% of the applied radioactive dose was absorbed following a 0.5 and 24 hour exposure, respectively. The radioactivity recoveries in urine (3.44 ± 3.09 and 7.82 ± 1.92% of the administered radioactive dose following 0.5- or 24-hour exposure, respectively) and faeces (3.99 ± 1.75 and 6.18 ± 2.26% of the administered radioactive dose following 0.5- or 24-hour exposure, respectively) were of the same order of magnitude for respective groups, indicating that both renal and hepato-biliary excretion were almost equally involved in the elimination of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate-related radioactivity in rats. Radioactivity was eliminated relatively slowly, with a mean of 61.6 and 49.8% of the absorbed radioactive dose recovered by 48 hours post application following 0.5- or 24-hour exposure, respectively. The remaining radioactivity was slowly eliminated, with measurable levels in excreta through 168 hours post-dose. The recovery in the dermal groups was 71.6% (dermal 0.5 hour exposure) and 73.1% (dermal 24 hours exposure). Further investigations were performed to determine the cause of lower recoveries in the dermal groups. Based on these results, the lower recoveries were considered to be caused by insufficient skin and cage wash, i.e. the use of water as cage rinse solvent was inefficient to extract the radioactivity from the cage surfaces and the extraction solvent and/or the procedures used in analysing skin wash samples were inefficient. In conclusion, lower recoveries observed following dermal administration are, according to the study report authors, likely to be caused by insufficient skin and cage wash considered to account for more than 20% of the applied dose. According to the applicant, this is considered to be of no relevance for systemic availability following dermal exposure.

Metabolite profiling and identification:
A summary of metabolites detected and/or predicted in plasma, urine, faeces, and cage rinse is presented in the table below:

Metabolite Designation	Proposed Identification	Chemical Name
M1	Di-oxidation	6-(4,5-diamino-1H-pyrazol-1-yl)-5-hydroxyhexan-2-one
M2	Tri-oxidation	(6-(4,5-diamino-1H-pyrazol-1-yl)-4,5-dihydroxyhexan-2-one or 6-(4,5-diamino-1H-pyrazol-1-yl)-3,5-dihydroxyhexan-2-one)
M3	N-acetylation coupled	(N-(4-amino-1-hexyl-1H-pyrazol-5-yl)acetamide

Metabolite Designation	Proposed Identification	Chemical Name
	with mono-oxidation	monoxide or N-(5-amino-1-hexyl-1H-pyrazol-4-yl)acetamide monoxide)
M4	N-acetylation coupled with mono-oxidation	(N-(4-amino-1-hexyl-1H-pyrazol-5-yl)acetamide monoxide or N-(5-amino-1-hexyl-1H-pyrazol-4-yl)acetamide monoxide)
M5	N-acetylation coupled with di-oxidation	(N-(4-amino-1-hexyl-1H-pyrazol-5-yl)acetamide dioxide or N-(5-amino-1-hexyl-1H-pyrazol-4-yl)acetamide dioxide)
M6	N-acetylation coupled with mono-oxidation	Could not be provided as definitive structure not proposed
M7	N-acetylation coupled with mono-oxidation	(N-(4-amino-1-hexyl-1H-pyrazol-5-yl)acetamide monoxide or N-(5-amino-1-hexyl-1H-pyrazol-4-yl)acetamide monoxide)
M8[a]	Unknown	Could not be provided as peak is unknown
M9 [a]	Unknown	Could not be provided as peak is unknown
M10 [a]	Unknown	Could not be provided as peak is unknown
M11	Oxidative deamination coupled with glucuronidation	4-amino-1-hexyl-1H-pyrazol-5-ol glucuronide or 5-amino-1-hexyl-1H-pyrazol-4-ol glucuronide
M12 [a]	Unknown	Could not be provided as peak is unknown
M13	N-acetylation coupled with glucuronidation	(N-(4-amino-1-hexyl-1H-pyrazol-5-yl)acetamide glucuronide or N-(5-amino-1-hexyl-1H-pyrazol-4-yl)acetamide glucuronide)
M14	N-acetylation	(N-(4-amino-1-hexyl-1H-pyrazol-5-yl)acetamide or N-(5-amino-1-hexyl-1H-pyrazol-4-yl)acetamide)
M15 [a]	Unknown	Could not be provided as peak is unknown
M16 [a]	Unknown	Could not be provided as peak is unknown
M17 [a]	Unknown	Could not be provided as peak is unknown
M18 [a]	Unknown	Could not be provided as peak is unknown
M19 [a]	Unknown	Could not be provided as peak is unknown
M20 [a]	Unknown	Could not be provided as peak is unknown
M21 [a]	Unknown	Could not be provided as peak is unknown
M22 [b]	Oxidative deamination	4-amino-1-hexyl-1H-pyrazol-5-ol or 5-amino-1-hexyl-1H-pyrazol-4-ol

a) Definitive identification of the metabolites was not possible due to low concentrations or mass spectrometric sensitivities
b) Metabolite not found by profiling, but was predicted by LC-MS

Plasma:
Radio HPLC profiles obtained from analysis of plasma extracts from all dose groups were generally qualitatively similar and showed up to seven radioactive peaks.
Unchanged ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was not detected in plasma from any dose groups, suggesting that ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was extensively metabolised and that the radioactivity exposure observed in plasma was related to its metabolites.

Following iv, oral or dermal administration, the predominant metabolite detected (representing greater than 60% of the radioactivity exposure in plasma) was metabolite M14, representing 62.09% (0.5-hour dermal application group) to 75.74% (iv group) of the total radioactivity exposure in plasma from 0.25 hour (0.5 hour for dermal groups) to 144 or 168 hours post-dose, as applicable.

Following iv or oral administration, the metabolites that represented greater than 3.5% of the radioactivity exposure in plasma were M3 and M7, and represented 4.10 (iv) to 5.08% (oral) of the total radioactivity exposure in plasma from 0.25 to 144 hours post-dose.
Following dermal administration, the metabolites that represented greater than 3.5% of the radioactivity exposure in plasma were M3 and M7 in 0.5-hour dermal application group, and metabolites M7 and M11 in 24-hour dermal application group, and represented 3.02 (0.5-hour dermal) to 4.10% (24-hour dermal) of the total radioactivity exposure in plasma from 0.5 to 168 hours post-dose.

Urine:
Radio HPLC profiles obtained from analysis of urine samples from all dose groups were generally qualitatively similar and showed up to 16 radioactive peaks.
Following iv, oral, 0.5-hour dermal and 24-hour dermal administration, samples pooled included totals of 66.9, 66.4, 3.04, and 6.91% of the administered radioactive dose excreted in urine through 96 and 168 hours post-dose, as applicable, respectively. Totals of 63.7, 63.0, 2.85, and 6.45% of the total administered radioactive dose were quantitated as 14C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate metabolites. Unchanged ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was not detected in urine, suggesting that the absorbed ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was extensively metabolised and that the metabolites were excreted in urine.

Following iv or oral administration, the metabolites that represented greater than 3.0% of the administered radioactive dose were metabolites M2, M3, M4, M5, M7, M13, M14 and M18.
Following 0.5-hour dermal application, the metabolites that represented greater than 0.2% of the administered radioactive dose were metabolites M3, M4, M5, M7, M13 and M14; whereas, following 24-hour dermal application, in addition the metabolites M2, M10 and M18 were detected.

Faeces:
Radio HPLC profiles obtained from analysis of faecal extracts from dermal groups differed when compared to those of faecal extracts obtained from the iv and oral groups. Together, all the faecal extracts showed up to 20 radioactive peaks. Following iv, oral, or dermal administration, the presence of metabolites in faecal extracts may suggest hepato-biliary excretion of ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine-related radioactivity, other non-biliary route(s) of excretion (such as direct secretion into the gut) of ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine-related radioactivity from systemic circulation into the gastrointestinal (GI) tract, or metabolism (by gut microflora) within the GI tract.

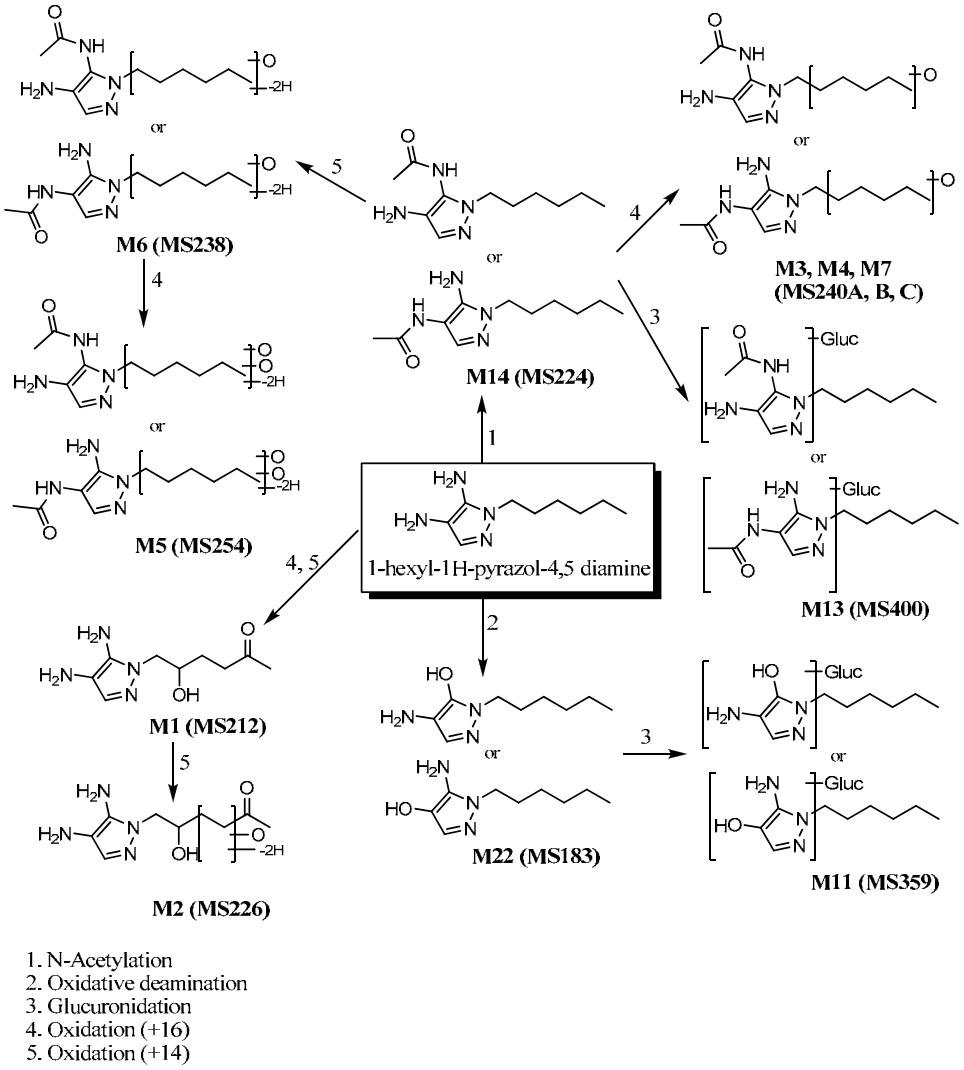
Following iv, oral, 0.5 hour dermal, and 24 hour dermal administration, samples pooled included totals of 9.88, 11.7, 2.40, and 3.38% of the total administered radioactive dose were excreted in faeces, respectively. Totals of 6.79, 7.80, 1.27, and 1.83% of the total administered radioactive dose were quantitated as ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate metabolites.
Unchanged ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine was not detected in faecal extracts, suggesting that ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine was extensively metabolised and the metabolites were excreted in faeces. Since unchanged 14C-1-hexyl-1H-pyrazol-4,5 diamine was not detected in faecal extracts following oral administration, it is likely that ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine was completely absorbed or that any unabsorbed ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine was degraded (chemical or by gut microflora) in the GI tract to other components.

Following iv or oral administration, the metabolites that represented greater than 1.0% of the administered radioactive dose were metabolites M5 and M14. All other metabolites detected represented less than 1.0% of the administered radioactive dose in faeces.
Following 0.5-hour dermal application, the metabolites that represented greater than 0.1% of the administered radioactive dose in faeces were metabolites M10, M14, M15, M18, and

M19. It should be noted that metabolites M12 and M15 were detected following 0.5- hour dermal application only and were not detected following iv or oral administration.
Following 24-hour dermal application, the metabolites that represented greater than 0.1% of the administered radioactive dose in faeces were metabolites M10, M14, M15, M18, M19 and M20. It should be noted that metabolites M12, M15, M16, and M17 were detected following 24-hour dermal application only and were not detected following iv or oral administration.

Cage rinse:
Radio HPLC profiles obtained from analysis of cage rinse samples from dose groups profiled were generally qualitatively similar and showed up to 13 radioactive peaks, most of which were similar to those observed in urine.
Following iv, oral, and 24-hour dermal administration, pooled samples included totals of 9.54, 7.90, and 0.836% of the total administered radioactive dose, respectively. Totals of 8.97, 7.58, and 0.753% of the total administered radioactive dose were quantitated as ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine metabolites. Unchanged parent compound was not detected in cage rinse, suggesting that the absorbed ^{14}C-1-hexyl-1H-pyrazol-4,5 diamine was extensively metabolised and the metabolites were excreted and recovered in cage rinse.

Biotransformation:
The proposed metabolic pathway and the predicted metabolites of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate in female rats are shown in the figure below:



Conclusion

In conclusion, ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate administered orally was extensively absorbed (at least 82%), readily distributed, extensively metabolised, and excreted mainly in the urine. Dermal absorption of ^{14}C-1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was relatively slower and lower (8.79%) after a 30-min exposure period and moderate (16.8%) after a 24-hour exposure period. When absorbed, radioactivity was excreted almost similarly in both urine and faeces.

^{14}C-1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate was extensively metabolised via both Phase I and Phase II biotransformation pathways after all dosing routes. The major biotransformation pathway was *N*-acetylation mainly in combination with oxidation and with glucuronidation.

Ref. 34

SCCS comments

No justification for the use of ascorbic acid (0.1%) in the test vehicles for iv, oral and dermal administration was given. No justification for the addition of 10% (w/v) propylene glycol in the test vehicle for the 24-hour dermal administration was given.

The SCCS noted the relatively low total recovery of radioactivity in the dermal groups of 71.6% (dermal 0.5 hour exposure) and 73.1% (dermal 24 hours exposure) which,

according to the study report authors, was likely caused by insufficient skin and cage wash. According to the applicant, this is considered to be of no relevance for systemic availability following dermal exposure. The calculated systemically available dose for 1% 1-hexyl 4,5-diamine pyrazole sulfate of 3.78 µg/cm² (mean + 1 SD) from the dermal *in vitro* study will be used for the MoS calculation.

^{14}C-1-Hexyl 4,5-diamine pyrazole sulfate administered orally was extensively absorbed (> 82%). An oral absorption of 82% is used for the MOS calculation.

3.4.9.2 Toxicokinetics in humans

/

3.4.9.3 Toxicokinetics *in vitro*

Bioavailability of 1-hexyl 4,5-diamine pyrazole sulfate across the intestinal barrier

Guideline:	/
Cells:	Human intestinal epithelial Caco-2 cell line
Test substance:	[ring ^{14}C] 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate
Batch no:	CFQ41662
Radiochemical purity:	98.4 %
Concentration:	10 µg free base/ml in Dulbecco's Phosphate Buffered Saline
No. of experiments:	1
GLP:	Not in compliance
Study period:	/ (Report date: 26 August 2013)

The purpose of this study was to assess the permeability of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate through Caco-2 monolayers. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37 °C in 24-well plates with orbital shaking for 4 hours. Radiolabelled 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was prepared at a concentration of 10 µg/ml in Dulbecco's Phosphate Buffered Saline (DPBS). The cells in Transwells were allowed to acclimatise in an incubator for 30 minutes prior to the addition of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate or the reference chemicals, mannitol, propranolol and ranitidine.
The trans-epithelial electrical resistance (TEER) was measured prior to and after the 4-hour incubation with test and reference chemicals. Incubations were performed in triplicate. Sampling time points were 60, 120, 180 and 240 minutes post-dose. Analysis of the donor (apical) and receiver (basolateral) samples was by liquid scintillation counting, apart from ranitidine samples, which were analysed by HPLC-MS/MS. The cumulative amount of compound was measured over time, and the linear portion of the curve was used to calculate the slope. The apparent *in vitro* permeability coefficient (k_p) was calculated for each compound (units of cm/minute).
To validate the test system, ranitidine and propranolol were used as control chemicals for low and high permeability, respectively, and mannitol was used to evaluate the monolayer integrity and cell functionality.
According to the laboratory's classification system, a low permeability is considered for test items with a k_p of < 1.2 x 10^{-4} cm/minute, medium permeability with a k_p of 1.2 - 12 x 10^{-4} cm/minute, and high permeability with a k_p ≥ 12.0 x 10^{-4} cm/minute.

Results
The k_p of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was 12.0 x 10^{-4} cm/minute and indicates that this substance would likely be moderately to highly absorbed through the human intestinal wall. There was a slight decrease in the TEER values over the course of the

incubation which may show a slight affect from the test substance; however, at the end of the study the TEER values were still above what is typically considered an acceptable TEER value (300 Ohms*cm^2) and the decrease was not considered to have affected the permeability value measured in this study.
The kp for the low permeable reference compound, ranitidine, was 0.3 x 10^{-4} cm/minute and was within the acceptable permeability range for this compound (0.12 - 1.2 x 10^{-4} cm/minute).
The mannitol kp was 1.1 x 10^{-4} cm/minute and was an acceptable rate for this compound (<1.5 x 10^{-4} cm/minute), indicating that the Caco-2 monolayer was intact with functioning tight junctions.
The kp for the highly permeable reference compound, propranolol was 14.4 x 10^{-4} cm/minute and was within the acceptable permeability range for this compound (12.0 – 27.0 x 10^{-4} cm/minute).

Conclusion
The permeability constant (kp) obtained for 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate indicates that it is considered to be moderately to highly absorbed in the human intestine.

Ref. 28

SCCS comment
The study was not performed under GLP conditions. There is no official test guideline for this assay. Only one cell batch was used and 3 replicate incubations were done on the same cell batch; usually, at least 3 different cell batches are used. The generated data are considered to provide an estimation of gastrointestinal absorption of 1-hexyl 4,5-diamine pyrazole sulfate after oral administration. The substance is expected to be highly absorbed after oral administration.

***In vitro* metabolism of 1-hexyl 4,5-diamine pyrazole sulfate in cryopreserved human hepatocytes**

Guideline:	/
Cells:	Cryopreserved human hepatocytes
Test design:	Plated cells
Replicates:	Triplicates for each test concentration and time point
Test substance:	1-hexyl-1H-pyrazole-4,5-diamine hemisulfate
Batch:	RD-CRU-096-07/142-01 (non-radiolabelled)
Radiolabelled:	/
Purity:	99.1 area% (non-radiolabelled)
Radiolabelled:	99% (Radio-HPLC)
Concentrations:	1.276, 12.76 and 127.6 µg/mL (7, 70 and 700 µM)
Incubation times:	3 and 24 hours
GLP:	/
Study period:	/ (Report date: 2 July 2013)

The metabolism of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate was tested following a 3- and 24-hour incubation with human hepatocytes. 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate was tested at final concentrations of 1.276, 12.76 and 127.6 µg/mL (7, 70, and 700 µM, respectively). The 7 µM solution was prepared using only radiolabelled test substance (100% radiolabelled), while the 70 and 700 µM solutions were prepared using both radiolabelled and non-radiolabelled test substance (28.6 and 2.86% radiolabelled, respectively). The plated hepatocytes were seeded at a concentration of 350,000 cells/well (700,000 cells/ml). The parent compounds and their metabolites were analysed by HPLC-UV-RAD/QToF mass spectrometry.

7-Ethoxycoumarin (1000 µM) was incubated with cells for 3 and 24 hours as a positive control to show metabolic activity (production of 7-hydroxycoumarin) in the plated hepatocytes over the entire incubation period.

Results
The plated human hepatocytes were metabolically active over 24 hours in culture, as determined by the formation of 7-hydroxycoumarin at both 3 and 24 hours, which was comparable to historical data.
Two metabolites of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate were detected, namely N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine and N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine glucuronide. No parent compound was found at the 70 µM incubation after 24 hours.
N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine was observed in incubations with hepatocytes, at both 3 and 24 hours, and at all test concentrations. Increasing the dose level of the test article from 7 to 70 to 700 µM resulted in an increase in the amount of the metabolite produced; however, the increase was not proportional to the increase in test article dosed.
The glucuronide was only observed in the 70 µM incubation, but was below the limit of quantification.

Conclusion
Two metabolites, N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine and N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine glucuronide were detected in human hepatocytes. Of these, only N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine was produced in quantifiable amounts.

Ref. 31

SCCS comment
The study was not performed under GLP conditions. There is no official test guideline for this assay.

***In vitro* metabolism of 1-hexyl 4,5-diamine pyrazole sulfate in human keratinocytes (HaCaT cell line)**

Guideline:	/
Cells:	HaCaT cells (human keratinocyte cell line)
Test design:	Plated cells
Replicates:	Triplicates for each concentration and time point
Test substance:	1-hexyl-1H-pyrazole-4,5-diamine hemisulfate
Batch:	RD-CRU-096-07/142-01 (non-radiolabelled)
Radiolabelled:	/
Purity:	99.1 area% (non-radiolabelled)
Radiolabelled:	99% (Radio-HPLC)
Test concentration:	1.276, 12.76 and 36.45 µg/ml (7, 70 and 200 µM)
Incubation time:	3 and 24 hours
GLP:	/
Study period:	/ (Report date: 2 July 2013)

The study was carried out to assess the metabolism of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate following incubation with human keratinocytes (HaCaT cell line). Cells were seeded at 500,000 cells/well and allowed to grow for 24 hours prior to dosing with the test article. The incubations were performed in triplicate with 2.0 ml of the dosing solution for 3 or 24 hours. Dosing solutions were prepared at final test article concentrations of 1.276, 12.76 and 36.45 µg/ml (7, 70 and 200 µM, respectively).
Chemical stability was evaluated by incubating each dose solution for 24 hours in the incubator, without cells present.

The samples were analysed for metabolic loss and formation of potential metabolites using an HPLC-UV-RAD/QToF-mass spectrometry for quantification and structure elucidation.
p-Aminobenzoic acid (PABA) was used as a positive control for N-acetyltransferase-1 (NAT-1) enzyme by monitoring the formation of its metabolite, 4-acetamidobenzoic acid, for 3 and 24 hours.
Cell density was measured in all test and positive control wells. Additional wells were seeded for the purpose of cell density measurement at the time of dosing and after 3 and 24 hours in treatment media.

Results
The addition of 1-hexyl-1H-pyrazole-4,5-diamine hemisulfate or PABA did not affect the growth or viability of the cells during the course of the study.
One metabolite, N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine, was found in the incubate samples at all three dose levels at 3 and 24 hours. Increasing the dose level of the test article from 7 to 70 to 200 µM resulted in an increase in the amount of the metabolite produced at the 3 hour time point; however, the increase was not proportional to the increase in test article dosed. At the 24-hour time point, there was an increase in the amount of metabolite produced when the test article was increased from 7 to 70 µM; however, less metabolite was produced at the 200 µM test article dose level that at the 70 µM dose level. The increase was not proportional to the increase in test article dosed.
No parent compound was found at the 70 µM incubation after 24 hours.
The PABA positive control samples showed a production of the known metabolite, 4-acetamidobenzoic acid. The results indicated that the cells were metabolically active for the duration of the study.

Conclusion
1-hexyl-1H-pyrazole-4,5-diamine hemisulfate did not affect the growth of HaCaT cells up to a dose of up to 200 µM. One metabolite, N-acetyl-1-hexyl-1H-pyrazole-4,5-diamine was observed after incubating with HaCaT cells for 3 and 24 hours.

Ref. 32

SCCS comment
The study was not performed under GLP conditions. There is no official test guideline for this assay.

3.4.10 Photo-induced toxicity

/

3.4.11 Human data

/

3.4.12 Special investigations

/

3.4.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(under oxidative conditions)
(on head concentration 1.0%)

Absorption through the skin	**A**	**=**	**3.78 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.19 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/...**	**=**	**0.037 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**5.0 mg/kg bw/d**
Bioavailability 82%*		**=**	**4.1 mg/kg bw/d**
Margin of Safety	**adjusted NOAEL/SED**	**=**	**110**

* based on the toxicokinetic study (ref. 32a).

3.4.14 Discussion

Physico-chemical properties
1-Hexyl 4,5-diamine pyrazole sulfate is used as an oxidative hair colouring agent (precursor). The intended maximum on-head use concentration is 1.0% in oxidative hair dye formulations.
Stability of 1-hexyl 4,5-diamine pyrazole sulfate in typical hair dye formulations has not been reported.

General toxicity
No acute oral toxicity studies were performed with 1-hexyl 4,5-diamine pyrazole sulfate. However, no deaths were observed in the sub-chronic (13-week) oral toxicity study or in the developmental toxicity study in rats at dose levels up to 20 mg/kg bw/day (Ref. 22 and Ref. 26).
Daily administration of 1-hexyl 4,5-diamine pyrazole sulfate by oral gavage to Sprague Dawley rats at a dose level of 20 mg/kg bw/day to males, and 8 and 20 mg/kg bw/day to females for 90 days resulted in minimal to mild decreases in red blood cell mass; no other toxicological significant or relevant findings were noted. The NOAEL of 5.0 mg/kg bw/day is used for the MOS calculation.
In the developmental toxicity study in rats, daily administration of 1-hexyl 4,5-diamine pyrazole sulfate by oral gavage to pregnant female Sprague Dawley rats at a dose level of 20 mg/kg bw/day during gestation days 6 to 20 resulted in minimal to mild maternal and developmental toxicity. The NOAEL for maternal and developmental toxicity was 8.0 mg/kg bw/day. Neither malformations nor variations occurred at the highest dose level of 20 mg/kg bw/day.

Irritation / sensitisation
Based on a TER assay and an EpiSkin™ test, 1 % 1-hexyl 4,5-diamine pyrazole sulfate is not considered to cause skin corrosion or skin irritation, respectively.
Based on an ICE study, it can be concluded that 1% 1-hexyl 4,5-diamine pyrazole sulfate does not cause severe ocular irritation. This, however, does not exclude a mild eye irritant potential. Under the conditions of this study, an eye irritation potential of 1-hexyl 4,5-diamine pyrazole sulfate at 1% cannot be excluded.
Skin sensitisation of 1-hexyl 4,5-diamine pyrazole sulfate was tested with the dihydrochloride instead of the hemisulfate salt. 1-Hexyl-1H-pyrazole-4,5-diamine

dihydrochloride was positive in the LLNA with an EC3 value of 8.6%. The sensitising potential and potency are attributable to the free base component. Therefore, the results of the LLNA carried out with the dihydrochloride salt can be used for the hazard assessment of 1-hexyl 4,5-diamine pyrazole sulfate. The EC3 value of the sulfate salt was calculated from the dihydrochloride salt by using the conversion factor of 0.91 to account for the different molecular weights. Therefore, the calculated EC3 value for 1-hexyl 4,5-diamine pyrazole sulfate is 7.8%. Based on the calculated EC3 value of 7.8%, 1-hexyl 4,5-diamine pyrazole sulfate is a moderate skin sensitiser.

Dermal absorption

The *in vitro* dermal absorption study was performed using three different hair dye formulations of 1-hexyl 4,5-diamine pyrazole sulfate at concentrations of 1.5, 0,5 and 0.165%. A typical hair dye formulation containing the anticipated in use concentration of 1% was not tested. Nevertheless, the dermal absorption for 1% 1-hexyl 4,5-diamine pyrazole sulfate has been calculated via interpolation. The calculated systemically available dose for 1% 1-hexyl 4,5-diamine pyrazole sulfate of 3.78 µg/cm² (mean + 1 SD) is used for the MoS calculation.

Mutagenicity

Mutagenicity of 1-hexyl 4,5-diamine pyrazole sulfate was tested with the dihydrochloride instead of the hemisulfate salt.

Overall, the genotoxicity of 4,5-diamino-1-hexyl-1H-pyrazole, dihydrochloride is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. 4,5-Diamino-1-hexyl-1H-pyrazole, dihydrochloride did not induce gene mutations both in bacteria nor in mammalian cells. Treatment with 4,5-diamino-1-hexyl-1H-pyrazole, dihydrochloride did also not result in an increase in cells with micronuclei in an in vitro micronucleus test in human blood cells.

Consequently, on the basis of these tests, 4,5-diamino-1-hexyl-1H-pyrazole, dihydrochloride can be considered to have no genotoxic potential and additional tests are unnecessary.

The toxic potential of both salts of 1-hexyl-1H-pyrazole-4,5-diamine is attributable to the free base component. Therefore, the results of the *in-vitro* mutagenicity studies carried out with 1-hexyl-1H-pyrazole-4,5-diamine dihydrochloride were taken into consideration for the risk assessment of 1-hexyl 4,5-diamine pyrazole sulfate. Accordingly, 1-hexyl 4,5-diamine pyrazole sulfate is, therefore, considered not to pose a risk to humans with regard to genotoxicity.

Carcinogenicity

No data were submitted.

Toxicokinetics

^{14}C-1-Hexyl 4,5-diamine pyrazole sulfate administered orally to rats was extensively absorbed (at least 82%), readily distributed, extensively metabolised, and excreted mainly in the urine. Dermal absorption of ^{14}C-1-hexyl 4,5-diamine pyrazole sulfate was relatively slower and lower (8.79%) after a 30-min exposure period and moderate (16.8%) after a 24 hour exposure period. When absorbed, radioactivity was excreted almost similarly in both urine and faeces. ^{14}C-1-hexyl 4,5-diamine pyrazole sulfate was extensively metabolised via both Phase I and Phase II biotransformation pathways after all dosing routes. The major biotransformation pathway was *N*-acetylation mainly in combination with oxidation and with glucuronidation. *In vitro* studies with human hepatocytes and HaCaT cells (human keratinocyte cell line) suggest that the same metabolic pathway, N-acetylation, predominates in rats and humans.

An oral absorption of 82% is used for the MOS calculation.

4. CONCLUSION

1. In light of the data provided, does the SCCS consider 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate (A163) safe when used as an oxidative hair colouring agent (precursor) in oxidative hair dye formulations at a maximum on-head concentration of 1.0%?

The SCCS is of the opinion that 1-hexyl 4,5-diamine pyrazole sulfate is safe for use in oxidative hair dye formulations with an on-head concentration of maximum 1.0% taken into account the scientific data provided.

2. Does the SCCS have any further scientific concerns with regard to the use of 1-Hexyl-1H-pyrazole-4,5-diamine hemisulfate (A163) in cosmetic products?

1-hexyl 4,5-diamine pyrazole sulfate is a moderate skin sensitiser.

5. MINORITY OPINION

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