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Revision of 27 March 2012

Scientific Committee on Consumer Safety

SCCS

# OPINION ON
# 1-Hydroxyethyl-4,5-diamino pyrazole sulfate

# COLIPA n° A154

The SCCS adopted this opinion at its 13th plenary meeting

of 13-14 December 2011

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.
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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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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged. Revised opinions carry the date of revision.

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## 1. BACKGROUND

Submission I for 1-hydroxyethyl-4,5-diamino pyrazole sulphate was submitted in March 2003 by COLIPA[1] according to COLIPA.

Submission II with additional data for this substance has been submitted in July 2005 by COLIPA. According to this submission 1-hydroxyethyl-4,5-diamino pyrazole sulfate is used as an oxidative hair colouring agents (precursor).

In June 2006 the SCCP adopted an opinion (SCCP/0990/06) on 1-hydroxyethyl-4,5-diaminopyrazole sulphate with the conclusion:

*"The SCCP is of the opinion that the information submitted is inadequate to assess the safe use of the substance.*
*Before any further consideration, the following information is required:*

* *an in vitro percutaneous absorption study under oxidative conditions and conforming to the current Notes of Guidance for Safety Evaluation*
* *an appropriate mammalian cell gene mutation test in vitro*
* *Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with the Notes of Guidance".*

In the response to the above opinion is included the new *in vitro* percutaneous absorption study, the gene mutation study, the stability tests and the analytical data.

The intended maximum on-head concentration is 3.0%. The oxidative colouring agent and the developer are mixed in ratios between 1:1 to 1:3 (g+g peroxide). It is common practice to apply 100 g of the product over a period of about 30 minutes followed by rinse off with water and shampoo. The application may be repeated at monthly intervals.

## 2. TERMS OF REFERENCE

1. *Does the SCCS consider 1-hydroxyethyl-4,5-diamino pyrazole sulfate safe for use as an oxidative hair dyes with a concentration on-head of maximum 3.0% taken into account the scientific data provided?*

2. *And/or does the SCCS have any further scientific concern with regard to the use of 1-hydroxyethyl-4,5-diamino pyrazole sulfate in oxidative hair dye formulations?*

[1] COLIPA – The European Cosmetics Association

# 3. OPINION

## 3.1. Chemical and Physical Specifications

### 3.1.1. Chemical identity

#### 3.1.1.1. Primary name and/or INCI name

1-Hydroxyethyl-4,5-diamino pyrazole sulfate

#### 3.1.1.2. Chemical names

4,5-Diamino-1-(2-hydroxyethyl)-1H-pyrazole sulfate (1:1)

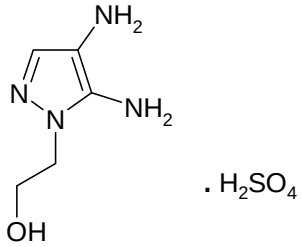
#### 3.1.1.3. Trade names and abbreviations

WR18247
DA 010894
Pyrazol DHE
COLIPA n° A154

#### 3.1.1.4. CAS / EC number

CAS: 155601-30-2
EC: 429-300-3

#### 3.1.1.5. Structural formula




. $H_2SO_4$

#### 3.1.1.6. Empirical formula

Formula: $C_5H_{10}N_4O.H_2O_4S$

### 3.1.2. Physical form

White to light pink powder

### 3.1.3. Molecular weight

Molecular weight: 240.24

### 3.1.4. Purity, composition and substance codes

Purity and impurities in various batches of 1-Hydroxyethyl-4,5-diamino pyrazole sulfate

| Description | Batch number | | | | |
|---|---|---|---|---|---|
| | **GST7-18099** | **GST4-20079** | **GST6-26089** | **ET046** | **09048045** |
| Chemical characterisation | NMR, HPLC-UV, UV | | | | HPLC, elemental analysis |
| Content, % (w/w) HPLC analysis, Ref. material: R00052659 | 99.8 | 99.8 | 96.8 | 98.5 | 99.4 |
| Water content, % (w/w) | n.d. | | | | |
| Loss on drying, % (w/w) | n.d. | | | | |
| Sulfate ash, % (w/w) | n.d. | | | | |

n.d.: not done because of lack of the necessary amount of substance for analysis, however reported values are:

Loss on drying: <1%
Ash content: <2%

### 3.1.5. Impurities / accompanying contaminants

4-((5-Amino-1(2-hydroxyethyl)-1H-pyrazol-4-yl)-imino)-
4,5-dihydro-1-(2-hydroxyethyl)-5-imino-1H-pyrazole-sulfate (2:1): max. 0.145% (w/w)

1-Methyl-4,5-diamino pyrazole sulphate: max. 0.7% (w/w)

Methanol, ethanol, isopropanol, n-propanol, acetone, ethylacetate, cyclohexane, methyl ethyl ketone and monochlorobenzene not detected (detection limit 100 ppm for each solvent)

### 3.1.6. Solubility

| | | |
|---|---|---|
| Water: | 666 g/l (w/w, 20°C)<br>pH of 1% solution: 1.82-1.94<br>pH of 5% solution: 1.61-1.66 | EC Method A.6 |
| Water/acetone (1:1): | >10% (w/w), pH 1.1 | |
| DMSO: | >10% (w/w) | |

### 3.1.7. Partition coefficient (Log $P_{ow}$)

Log $P_{ow}$: -1.75 (pH 7.0, 30°C) EC Method A.8

### 3.1.8. Additional physical and chemical specifications

Organoleptic properties
Melting point: 174.7°C
Boiling point: Not detectable (decomposition starting at 200°C)
Flash point: /
Vapour pressure: 1.65 x $10^{-8}$ hPa (20°C, extrapolated)
Density: 1.87 (20°C)
Viscosity: /
pKa: /
Refractive index: /
UV/Vis: λmax 226 nm

### 3.1.9. Stability

The stability of three different lots of 1-hydroxyethyl-4,5-diamino pyrazole sulphate was tested during storage for a period of 3 years at 25°C and 50% relative humidity as well as during storage for a period of one year at 40°C and 75% relative humidity. 1-hydroxyethyl-4,5-diamino pyrazole sulphate was shown to be stable under these storage conditions (variation <6%).

The stability of 3% 1-hydroxyethyl-4,5-diamino pyrazole sulphate in three batches of a typical hair colouring cream, stored in tubes with aluminium screw caps, was tested during storage for a period of 3 years at 25°C and 50% relative humidity as well as during storage for a period of one year at 40°C and 75% relative humidity. 1-Hydroxyethyl-4,5-diamino pyrazole sulphate was shown to be stable in the cream under these storage conditions (variation <6%).

**General Comments on Physico-chemical characterisation**

- Stability of 1-Hydroxyethyl-4,5-diamino pyrazole sulphate in solutions used for oral toxicity testing is not reported

## 3.2. Function and uses

1-Hydroxyethyl-4,5-diamino pyrazole sulphate is used in oxidative hair dye formulations at a final concentration of 3%, after mixing with peroxide.

## 3.3. Toxicological Evaluation

### 3.3.1. Acute toxicity

#### 3.3.1.1. Acute oral toxicity

***Taken from SCCP/0990/06***

| | |
|---|---|
| Guideline: | OECD 401 (1987) |
| Species/strain: | Rats, Him: OFA, Sprague Dawley, SPF |
| Group size: | 5 male + 5 female |
| Test substance: | DA 010894 |
| Batch: | GST 4-20079 |
| Purity: | 99.1% (HPLC, 254 nm) |
| Dose: | 2000 mg/kg bw |
| Observation: | 14 days |
| GLP: | in compliance |

The test article was administered in a total volume of 10 ml/kg bw deionized water by gastric intubation to five male and five female rats at a dose of 2000 mg/kg bw (limit test). The animals were checked daily for mortality and clinical signs. Body weights were recorded at start and on days 7 and 14. Animals were observed for 14 days. All animals were submitted to a gross necropsy at the end of the observation period.

Results
No mortality or any clinical signs of systemic toxicity were recorded in the test animals. Orange coloured urine was observed in all animals. Body weight gain was within a normal range. In one male, large mesenteric lymph nodes and a grey-white covering on the spleen capsule were noted.

Conclusion
The acute oral $LD_{50}$ in rats was greater than 2000 mg/kg bw.

Ref.: 15

| 3.3.1.2. Acute dermal toxicity |
|---|

No data submitted

| 3.3.1.3. Acute inhalation toxicity |
|---|

***Taken from SCCP/0990/06***

| | |
|---|---|
| Guideline: | OECD 403 (1981) |
| Species/strain: | Rats, Wistar (Crl:[WI]WU BR) |
| Group size: | 5 male + 5 female |
| Test substance: | 18247 Pyrazol |
| Batch: | ET046 |
| Purity: | 99.8% (HPLC, 225 nm) |
| Dose: | 5.24 ± 0.31g/m³ |
| Observation: | 14 days |
| GLP: | in compliance |

One group of 5 male and 5 female rats was exposed during a single period of four h to a test atmosphere containing the test substance at the limit concentration of 5.24 ± 0.31g/m³. The test material was milled before use to obtain an MMAD in the range of 1-4 µm. The mass median aerodynamic (MMAD) size of the particles in the test atmosphere was 3.3 µm and the distribution of particle sizes had a geometric standard deviation of 1.8.

Results
Treatment-related effects included a slight to moderate decreased breathing frequency during exposure, grey discoloured areas on the lungs in half of the animals at necropsy, and discolouration of the fur of the animals, which was visible from just after exposure until necropsy 14 days later. No mortality occurred.

Conclusion
In rats, the 4 h $LC_{50}$ value of an aerosol of the test substance was larger than 5.24 g/m³ for both sexes.

Ref.: 16

| 3.3.2. Irritation and corrosivity |
|---|

| 3.3.2.1. Skin irritation |
|---|

***Taken from SCCP/0990/06***

| | |
|---|---|
| Guideline: | OECD 404 (1987) |
| Species/strain: | New Zealand albino rabbit |
| Group size: | 3 females |
| Test substance: | DA010894 |
| Batch: | GST 4-20079 |

Purity: 99.8%
Dose: 0.5 g (soaked with 0.5 ml deionised water)
GLP: in compliance

A cellulose patch with 0.5 g test material was placed over an area of 6 $cm^2$ on the shaved skin of three female rabbits and covered with a semi-occlusive dressing for 4 h. Access to the application area was prevented by a plastic collar. After the 4-h application time, the area was wiped with a cellulose tissue. The animals were checked daily for mortality and systemic symptoms. Skin reactions were evaluated 1, 24, 48, and 72 h after removing the patches according to a Draize scoring system.

Results
Erythema was observed in all three animals 1 and 24 h after removing the patches, in one animal also at the 48 h observation time point. Oedema was noted in all three animals 24 h after removal of the patches.

Conclusion
The test substance is irritant to rabbit skin under the conditions of the experiment.

Ref.: 18

### 3.3.2.2. Mucous membrane irritation

***Taken from SCCP/0990/06***

Undiluted Test Compound

Guideline: OECD 405 (1987)
Species/strain: New Zealand albino rabbit
Group size: 3 females
Test substance: DA 010894
Batch: GST 4-20079
Purity: 99.8%
Dose: 0.1 ml (95-100 mg)
GLP: In compliance

The approximate equivalent of 0.1 ml of the test article (95-100 mg) was placed into the conjunctival sac of the right eye of each animal. Lids were then held together for about one second. The untreated left eye served as control. The animals were checked daily for mortality and signs of systemic toxicity. Ocular reactions were evaluated 1, 24, 48, and 72 h after instillation of the test article. Further examinations were performed 6, 8, 10, 13, 15, 17 and 21 days after instillation.

Results
Conjunctival redness up to grade 3 and oedema up to grade 3 were noted in all animals 1, 24, 48 and 72 h after instillation, in one animal persisting until day 21, the last day of observation. Cornea opacity up to grade 2 was observed in all animals 1, 24, 48 and 72 h after instillation, in one animal persisting until day 21. Iris reactions were noted in all three animals 1, 24, 48 and 72 h after instillation, in one animal persisting until day 8.

Conclusion
Under the conditions of the experiment, the test substance is very irritant to rabbit eyes; the undiluted test compound may cause serious damage to eyes.

Ref.: 19

***Taken from SCCP/0990/06***

Diluted Test Compound, 5%

| | |
|---|---|
| Guideline: | Directive 92/69/EEC, method B.5. (1992) |
| Species/strain: | New Zealand albino rabbit |
| Group size: | 3 female |
| Test substance: | DA 010894 |
| Batch: | GST 4-20079 |
| Purity: | 99.8% |
| Dose: | 0.1 ml of the 5% aqueous solution |
| GLP: | In compliance |

0.1 ml of a 5% solution of the test article in deionised water was placed into the conjunctival sac of the right eye of each animal. Lids were then hold together for about one second. The untreated left eye served as control. The animals were checked daily for mortality and signs of systemic toxicity. Ocular reactions were evaluated 1, 24, 48, and 72 h after instillation of the test article.

Results
Conjunctival redness up to grade 2 was noted in all animals 1 h after instillation, in one animal seen until the reading time point 48 h after instillation. Conjunctival oedema was noted in two of the three animals, one h after instillation only. No reactions of cornea or iris were observed at any time point in any of the animals.

Conclusion
Under the conditions of the experiment, 5% of the test substance is irritant to rabbit eyes.
Ref.: 20

### 3.3.3. Skin sensitisation

***Taken from SCCP/0990/06***

**Maximisation (Magnusson and Kligman) Test**

| | |
|---|---|
| Guideline: | Directive 92/69/EEC, method B.6. (1992) |
| Species/strain: | Hartley guinea pigs, Crl:(HA)BR |
| Group size: | 10 female in test group, 5 female in control group |
| Test substance: | DA 010894 |
| Batch: | 4-20079 |
| Purity: | > 99% |
| Concentrations: | intradermal induction: 1% test substance in physiological saline<br>dermal induction: 40% test substance in white petrolatum, occluded<br>challenge: 40% test substance in white petrolatum, occluded |
| GLP: | In compliance |

The test group consisted of 10 female Guinea pigs, the control group of five female Guinea pigs. In the first week of induction, the test group was treated with single intradermal injections of complete Freund's adjuvans/saline mixture 1:1 (v/v), 1% of the test substance in physiological saline and with 1% of the test substance in the adjuvant/saline mixture (w/v). The negative control group was treated with the adjuvant and the vehicle (physiological saline).

During the second week of induction, the test substance, suspended in white petrolatum (40% w/w; 0.6 g of preparation) and dermally applied under occlusive dressing for 48 h to the area (2 x 4 cm) of the intradermal injections. The negative control group was treated

with the vehicle alone. The day before epicutaneous application, 0.6g of 10% sodium lauryl sulphate in petrolatum had been applied).

After a two weeks treatment-free reaction period, sensitisation reactions were challenged in the test as well as in the negative control group by dermal administration of 40% test substance in white petrolatum (w/w) on one side and the vehicle alone on the contralateral flank, applied under occlusive dressing for 24.

Twenty-four and 48 h after removal of the patches the skin reactions were scored. All sites of treatment at challenge exposure and the contralateral flanks were examined histopathologically at the end of the experiment.

Results
No animal of the negative control group showed any reaction to the test compound, neither at visual nor at histopathological examination. A positive response was observed in all animals of the test group at 24 and 48 h. Histopathological examination revealed hyper- and parakeratosis, vesicle formation, lymphohistiocytic infiltration among other skin reactions.

Conclusion
Based on the sensitization rate of 100%, the test compound is an extremely potent contact allergen.

Ref.: 21

***Taken from SCCP/0990/06***

**Buehler test**

| | |
|---|---|
| Guideline: | OECD 406 (1992) |
| Species/strain: | Hartley guinea pigs, Crl:(HA)BR |
| Group size: | 20 female in test group, 10 female in control group |
| Test substance: | DA010894 |
| Batch: | 4-20079 |
| Purity: | 99.8% |
| Concentrations: | dermal induction: 40% test substance in white petrolatum, occluded (3 induction exposures)<br>challenge: 40% test substance in white petrolatum, occluded |
| GLP: | in compliance |

The test group consisted of 20 female guinea pigs, the control group of 10 female guinea pigs. A positive control group, consisting of 5 female guinea pigs, was treated with 10% p-phenylenediamine in white petrolatum. A corresponding negative control group (5 female animals) was treated with white petrolatum alone.

A 40% formulation of the test compound in white petrolatum was prepared and applied to the clipped skin under occlusive dressing on days 0, 7 and 14 (induction phase) and on day 28 (challenge exposure) for 6 h each time. The positive control group was treated in the same way but with 10% p-phenylenediamine.

Twenty-four and 48 h after removal of the patches the skin reactions were scored. All animals were observed daily for signs of systemic toxicity. Body weights were recorded on days 0 and 30.

Results
No positive response was observed in any animal of the test group. Positive responses were noted in all 5 animals of the positive control group after 24 and/or 48h.

Conclusion
In this Buehler test, 4,5-diamino-1-(2-hydroxyethyl)-1H-pyrazol-sulfat (1:1) showed no sensitising potential when applied epicutaneously at a concentration of 40%.

Ref.: 22

***New study, submission II, 2007***

**Local Lymph Node Assay (LLNA)**

| | |
|---|---|
| Guideline: | OECD guideline 429 (2002) |
| Species: | Mouse, strain CBA/Ca01aHsd |
| Group size: | 5 females per test concentration |
| Test substance: | 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate |
| Batch: | GST 7-18099 |
| Purity: | 100 area% (HPLC, 254 nm) |
| Concentrations: | 0.5, 1.5, 5, 10% in acetone/water/olive oil and 0.5, 1.5, 5, 10% in DMSO |
| Route: | Dermal |
| Vehicle: | Acetone/water (1:1) mixed with olive oil (4:1) and dimethylsulfoxide (DMSO) |
| Dosing schedule: | Once daily on three consecutive days |
| GLP: | In compliance |

The skin sensitising potential of 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate was investigated in CBA mice by measuring the cell proliferation in the draining lymph nodes after topical application on the ear.
A standardised volume (25 µl) containing 0 (vehicles only), 0.5, 1.5, 5 and 10% of 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate in a mixture of acetone/water (1:1) mixed with olive oil (4:1) and 0.5, 1.5, 5 and 10% in dimethylsulfoxide (DMSO) were applied to the surface of the ear to each of five mice per group for three consecutive days. As an appropriate positive control for oxidative hair dye precursors, p-Phenylenediamine (PPD) at 1% in DMSO was used in parallel under identical test conditions.

On day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 20 µCi of [H3] methyl thymidine. Approximately five hours later, the mice were sacrificed by CO2-inhalation and the draining auricular lymph nodes were removed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [H3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate did not induce a biologically relevant immune response in local lymph nodes after dermal application up to the maximum test concentration of 10%.

| Compound | % | Stimulation index in DMSO | Stimulation index in Acetone/olive oil |
|---|---|---|---|
| p-Phenylenediamine | 1 | 14.2 | - |
| 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate | 0.5 | 1.4 | 0.9 |
| | 1.5 | 1.7 | 1.0 |
| | 5 | 1.5 | 0.8 |
| | 10 | 1.8 | 1.0 |

Conclusions
The applicant concluded that 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate does not pose a sensitising risk to consumers when used as intended.

Ref.: 7 (subm II)

Comment
The maximum test concentration was too low for hazard identification.

Overall conclusion for sensitisation
1-Hydroxyethyl-4,5-diamino pyrazole Sulfate is a potent skin sensitiser as demonstrated by the Guinea Pig Maximisation test.

### 3.3.4. Dermal / percutaneous absorption

***Taken from SCCP/0990/06***

**Main study, *in vitro***

| | |
|---|---|
| Guideline: | OECD draft guideline "Skin absorption: *in vitro* method" (2000) |
| Tissue: | Porcine back skin (thickness: 1000 µm) |
| Method: | Diffusion Teflon-chambers |
| Test substance: | WR18247 tested at a concentration of 3% in a commercial hair dye formulation. |
| Batch: | GST 7-18099 |
| Purity: | 100% (HPLC, 254 nm) |
| Concentration: | 100 mg/cm² of formulation, corresponding to 3 mg/cm² of dye |
| No. of chambers: | 6 (five for the formulation containing the dye stuff and one for the blank formulation) in each experiment:<br>I: static system<br>II: flow through system (5ml/h) |
| GLP: | in compliance |

Skin absorption of WR18247at the maximum concentration intended for hair colorants (3%), was investigated with pig skin (Schweizer Edelschwein, male, 120 kg) prepared from the back and the flanks. 3 mg of the dye was applied once to the skin in a commercial non-oxidative hair dye formulation (400 mg aqueous cream formulation containing 3% dye applied to 4 cm² skin). The integrity of the skin was monitored at the beginning of the experiment using tritiated water. Teflon-diffusion chambers were used. In experiment I, a static system was used. In experiment II, the receptor solution (physiological phosphate buffer containing NaCl and antibiotics) was pumped through the receptor chamber at a rate of 5 ml/h. Six chambers were investigated in each experiment.

In both experiments, 30 minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, then once with 4 ml washing solution (shampoo-formulation diluted to approximately 16.7%) and again twice with water. The washing solutions were combined and the amount of dye was determined by HPLC.
Fractions of the receptor fluid were collected after 16, 24, 40, 48, 64 and 72 h, concentrated directly after the pump and analysed immediately. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the dye content was quantified by means of HPLC.

Results
All samples/tissue extracts were analysed by HPLC. Data for solubility (153 mg/ml in receptor fluid) and stability are provided in the report. The limit of quantification was 174 ng/HPLC-injection for the HPLC method used.

The integrity of each skin preparation was demonstrated by examination of penetration characteristics with tritiated water resulting in 1.2 to 1.7% of the applied dose found in the receptor fluids in experiment 1 and 1.2 to 2.2% of the applied dose found in the receptor fluids in experiment 2, respectively. The values were within the limit of acceptance (≤ 1.5%) for 4 of the six skin samples in experiment 1 and 1 skin sample in experiment 2.Taken together, 5 skin samples with appropriate values in the integrity test are available for the final calculation of bioavailability of WR18247. The total recovery in these five skin samples was 87.7 ± 10.3%. The loss of test item is due to oxidation processes, the recovery is still within an acceptable range.
The majority of the applied dose of WR18247remained on the skin surface, representing 87.5 ± 10.2% of the applied dose.
At 72 h, 0.2 ± 0.3 µg/cm² was recovered in the epidermis and 0.2 ± 0.3 µg/cm² in the upper dermis. After 72 h, the content of WR18247in all fractions of the receptor fluid (six measuring points per skin) was below the limit of quantification of 174 ng per injection and corresponds to 3.6 µg/cm$^2$. Thus, a maximum amount of 3.6 ± 5.7 µg/cm$^2$ could have passed through the skin barrier during the 72 h permeation period.
For the systemic exposure of WR18247 a maximum amount of 3.8 ± 6.0 µg/cm$^2$ (n=5, one donor; receptor fluid + upper dermis) has to be regarded as biologically available under use conditions.

Conclusion
Under the described test conditions, a skin penetration rate of 3.8 ± 6.0 µg/cm$^2$ of WR18247 is obtained from the amounts in the receptor fluid and for the lower skin compartment (upper dermis).

Ref.: 25

Comment
The test substance has not been studied in the presence of hydrogen peroxide.
In the study, two experiments were combined, in which a static system and a dynamic system (flow through) were used. As the results of the skin integrity test were not within the acceptance criteria for several skins, 5 skins were selected that were within the range of acceptance. The use of two different chamber systems in combination with the selection of acceptable skin samples does not meet the guideline. The study is considered inadequate.

Because of these concerns, the studies are not used for calculating margin of safety.

**New study, submission II, 2007**

| | |
|---|---|
| Guideline: | OECD Guideline No. 428 |
| Tissue: | Porcine back or flank skin (frozen/thawed; thickness: ≤ 1000 µm) from 3 donors (2 males, 1 female) |
| Membrane integrity: | Penetration characteristics with tritiated water |
| Method: | In-house Diffusion Teflon-chambers |
| No. of chambers: | Two independent experiments were performed with 6 diffusion cells per experiment. For calculations, the mean value of all valid skin samples (n=10) were used |
| Test substance: | 3% 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate in a typical hair dye formulation in the presence of a reaction partner |
| Batch: | GST 7-18099 |
| Purity: | 99.8 weight% (HPLC, 254 nm) |
| Area Dose: | 100 mg/cm² (3.0 mg/cm², tested as part of an oxidative hair dye formulation) |
| Time period: | 30 min (16, 24, 40, 48, 64 and 72 hours) |
| Receptor fluid: | 0.14 M NaCl, 2 mM $K_2HPO_4$, 0.4 mM $KH_2PO_4$, 100 IU penicillin/ml, 76 IU streptomycin/ml and 3% of Ethanol, pH = 7.3 |
| Solubility in receptor: | 0.139 mg/ml in receptor fluid (at pH 7.3) |

Stability: 85% recovery after 3 days (1 mg/ml, in the presence of 0.3% ascorbic acid and 0.4% sodium sulfite)
Analyses: liquid scintillation counting
Date: 2006-2007
GLP: in compliance

The cutaneous absorption of 3% 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate (WR18247) in a typical hair dye formulation with a reaction partner was investigated *in vitro*, using pig skin preparations, which were continuously rinsed from underneath with physiological receptor fluid at a temperature of 32 ± 2 °C. Two independent experiments were performed with 6 diffusion cells per experiment. For calculations, the mean value of all valid skin samples (n=10) in contact with 3% 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate (WR18247) in formulation was used.
The integrity of each skin preparation was determined by examination of penetration characteristics with tritiated water. Two skin samples with skin integrity values above 2% of the applied dose were not within the limit of acceptance and were not taken into consideration for the calculation of the mean.

After checking the skin integrity, 400 mg of the formulation (= 100 mg/cm$^2$), containing 3% 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate, was applied to the skin samples (= 3.0 mg of test item/cm$^2$) for 30 minutes and subsequently washed off with water and shampoo. The determination of the amount of 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate in the washings and in the receptor fluid was performed by measuring the radioactivity by means of a scintillation counter. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the radioactivity was quantified by means of scintillation counter.

Results

Table 1: Summary of the cutaneous absorption of 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate

| Amount of WR 18247 in: | µg/cm$^2$ (mean ± S.D, n=10) | | | %* (mean ± S.D, n=10) | | |
|---|---|---|---|---|---|---|
| Receptor fluid (72 hours) | 0.509 | ± | 0.062 | 0.017 | ± | 0.002 |
| Lower skin (72 hours) | 0.654 | ± | 0.390 | 0.023 | ± | 0.014 |
| Upper skin (72 hours) | 3.492 | ± | 0.906 | 0.122 | ± | 0.031 |
| Rinsing solution (after 60 min.) | 2689.76 | ± | 138.76 | 93.47 | ± | 2.84 |
| Total balance (recovery)** | 2817.46 | ± | 78.42 | 97.99 | ± | 2.88 |

* Corrected for individual applied dose; ** Total is corrected for losses on tips

Under the assumption that a depot effect is absent, a maximum amount of 1.163 ± 0.395 µg/cm$^2$ (0.040% of the applied dose) of 1-Hydroxyethyl 4,5-Diamino Pyrazole Sulfate is considered as biologically available by the applicant.

Ref.: 5 (subm II)

Comment
- The amount applied (100 mg/cm²) is too high compared to the normal value of 20 mg/cm².
- The SSCS considers that the amount measured in the "upper skin" was not biologically available as this is a 72-h estimation.

For the MoS calculation, the dermal absorption of 1-hydroxyethyl 4,5-diamino pyrazole sulfate may be considered as mean value + 2SD = 1.163 + 2 x 0.395 = 1.953 µg/cm$^2$

### 3.3.5. Repeated dose toxicity

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

No data submitted

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

***Taken from SCCP/0990/06***

| | |
|---|---|
| Guideline: | OECD 408 (1981) |
| Species/strain: | Rats, Sprague Dawley, crl: CD(SD)BR – strain |
| Group size: | 15 male + 15 female, one group with vehicle (control) |
| Test substance: | DA 010894 |
| Batch: | GST 6-26089 |
| Purity: | 99.2% (HPLC, 254 nm) |
| Dose levels: | 80, 250, 800mg/kg bw/day, 7 days/week by gavage |
| Exposure period: | 13 weeks |
| GLP: | In compliance |

Three groups of 15 male and 15 female Sprague Dawley rats each were dosed daily intragastrically at levels of 80, 250 and 800 mg/kg bw of the test compound for a period of 13 weeks. In addition, an equally sized control group received the same dose volume of the vehicle (10 ml/kg bw, distilled water) throughout the dosing period.
The effects of the test compound were assessed using daily clinical observations and mortality checks, weekly body weight determinations, food consumption (cagewise) at weekly intervals, ophthalmoscopic examinations (pre-test and week 12) and blood biochemical as well as haematological investigations. As additional in-life observations, the functional observational battery (weeks 4, 8 and 12) and assessment of motor activity (week 12/13) were included. At terminal sacrifice, all animals were subjected to gross necropsy, organ weights were determined of brain, adrenals, epididymides, heart, kidneys, liver, spleen, testes and thymus. A large number of organs and tissues from all animals in all study groups were preserved and the majority of these specimens from the control and high-dose groups were examined histopathologically.

Results
No compound-related effects were observed at the low dose of 80 mg/kg bw. Body weight gain was slightly, but statistically significant decreased in females from the 800 mg/kg bw dose group. In high dose males, slight changes in red blood cell parameters (increase in mean corpuscular haemoglobin and in red blood cell volume) and an increase in relative spleen weight was observed.

Conclusion
In a 13-week study using daily intragastric dosing of the test animals at the highest tested dose of 800 mg/kg bw, marginal changes in some blood biochemical, haematological and spleen weight were observed. Based on these effects, the NOAEL in this study was 250 mg/kg bw/day.

Ref.: 17

#### 3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

| 3.3.6. | Mutagenicity / Genotoxicity |
|---|---|

| 3.3.6.1. | Mutagenicity / Genotoxicity *in vitro* |
|---|---|

***Taken from SCCP/0990/06, re-evaluated***

**Bacterial gene mutation assay**

| | |
|---|---|
| Guideline: | OECD 471 |
| Species/strain: | *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA1538 |
| Replicates: | Triplicate in two independent experiments |
| Test substance: | DA 010894 |
| Vehicle: | water |
| Batch: | 4-20079 |
| Purity: | > 99% (HPLC) |
| Concentrations: | experiment 1: 1, 10, 100, 1000 and 5000 μg/plate with and without S9-mix<br>experiment 2: 30, 100, 300, 1000 and 3000 μg/plate with and without S9-mix |
| Treatment: | direct plate incorporation with 48 incubation |
| GLP: | in compliance |
| Study period: | 15 September 1994 - 6 December 1994 |

DA 010894 was assessed for the induction of gene mutations in strains of *S. typhimurium.* Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Both experiments were performed according to the direct plate-incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Some growth inhibiting effects (reduction in revertant counts or sparse bacterial background lawn) were observed with TA1537 and TA98 without S9-mix and TA100 with S9-mix at 3000 μg/plate. A biological relevant increase in revertant colony numbers was not observed in any tester strain following treatment with DA 010894. Positive controls showed a distinct increase in revertant colonies.

Conclusions
Under the experimental conditions reported, the test substance was not mutagenic to *Salmonella typhimurium*.

Ref.: 27, Submission I, updated 2005

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

| | | |
|---|---|---|
| Guideline: | OECD 476 (1997) | |
| Species/strain: | V79 cells | |
| Replicates: | duplicate cultures in 2 independent experiments | |
| Test substance: | 1-hydroxyethyl-4,5-diaminopyrazole sulphate (WR 18247) | |
| Batch: | MJ253 (R0038744) | |
| Purity: | 99.87 area % by HPLC | |
| Vehicle: | deionised water | |
| Concentrations: | experiment I: | 40, 50, 60, 70 and 80μg/ml, without S9-mix<br>600, 900, 1200, 1800 and 2400 μg/ml, with S9-mix |
| | experiment II: | 20, 25, 30 and 35 μg/ml without S9-mix |
| Treatment | experiment I: | 4 h both treatment without and with S9-mix; expression period 7 days and a selection period of 8 days. |

| | | |
|---|---|---|
| | experiment II: | 24 h treatment without S9-mix; expression period 7 days and a selection period of 8 days. |
| GLP: | in compliance | |
| Study period: | 7 November 2006 – 27 March 2007 | |

1-hydroxyethyl-4,5-diaminopyrazole sulphate was assayed for mutations at the *hprt* locus of V79 cells both in the absence and presence of 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 measuring the cloning forming ability of approximately 500 cells after treatment with the test item. Toxicity of 1-hydroxyethyl-4,5-diaminopyrazole sulphate is indicated by a reduction of the cloning efficiency. Exposure to the maximum concentration should result in a reduced cloning efficiency to approximately 10-20%. In the main test, cells were treated for 4 h (experiment I) or 24 h (experiment II) followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Negative and positive controls were in accordance with the OECD guideline.

Results
No precipitation was observed up to the maximum concentration tested. In the pre-test without S9-mix following 4 h treatment strong toxic effects occurred at 82.5 µg/ml and following 24 h treatment at 20.6 µg/ml. At the next higher concentrations the cell growth was completely inhibited. With S9-mix, no relevant toxicity occurred up to the maximum soluble concentration of 660 µg/ml.
In both experiments in the absence of S9-mix the appropriate level of toxicity (10-20% survival after the highest concentration) was reached. In the presence of S9-mix no relevant toxicity occurred up to the maximum concentration of 2400 µg/ml (approximately 10mM the required top concentration for *in vitro* genotoxicity tests).
Biological relevant or statistically significant increases in mutant frequency were not found following treatment with 1-hydroxyethyl-4,5-diaminopyrazole sulphate at any dose level tested, either in the absence nor presence of S9-mix in both experiments with one exception. In the second culture of experiment I at 1200 µg/ml and above (with S9-mix) an increase in mutant frequency was observed exceeding the threshold of 3 times the corresponding solvent control. As the absolute value of the mutant frequency was low and remained well within the historical control range and as the increase was not reproduced in the other culture, it was considered not biologically relevant.

Conclusion
Under the experimental conditions used, 1-hydroxyethyl-4,5-diaminopyrazole sulphate was considered not mutagenic in this *hprt* gene mutation assay in V79 cells.

Ref. 6, Submission II 2007

***Taken from SCCP/0990/06, re-evaluated***

***In vitro* mammalian chromosome aberration test**

| | | |
|---|---|---|
| Guideline: | OECD 473 | |
| Species/strain: | Human peripheral lymphocytes | |
| Replicates: | duplicates in two independent experiments | |
| Test substance: | A005767 (DA010894) | |
| Vehicle: | Ham's F-10 medium | |
| Batch: | DA010894 | |
| Purity: | 98.5% | |
| Concentrations: | experiment 1: | 50, 150, 500 µg/ml without and with S9-mix |
| | experiment 2: | 500, 1500, 5000 µg/ml without and with S9-mix |
| Treatment: | experiment 1: | 24 h without S9-mix and 3.5 h with S9-mix; harvest time 24 h after start of treatment. |

| | | |
|---|---|---|
| | experiment 2: | 4 h without S9-mix and 3.5 h with S9-mix; harvest time 24 h after start of treatment. |
| GLP: | in compliance | |
| Study period: | 14 April 1999 – 9 July 1999 | |

Human lymphocytes were used to examine the induction of chromosomal aberrations by A005767. The chromosome aberration assay was performed in the presence and absence of S9-mix containing S9 fraction from Aroclor 1254-stimulated rat liver. The test procedure followed the OECD guideline and was conducted in compliance with the principles of GLP. The cells were harvested 24 h after the start of treatment. The treatment intervals were 24 h and 4 h without metabolic activation in the first and second experiment, respectively. With metabolic activation, a 3.5-h treatment was used. 100 metaphases per culture were scored for structural chromosome aberrations. The test substance, dissolved in medium, was tested at concentrations of 50, 150, and 500 µg/ml in the absence of S9-mix in the first experiment, and 500, 1500, and 5000 µg/ml in the second experiment with and without S9-mix. Mitomycin C and cyclophosphamide were used as positive controls.

Results
The highest concentration tested induced 44% and 52% cytotoxicity in experiment 1 and 81% and 59% cytotoxicity in experiment 2 without and with S9-mix respectively. The test substance induced a significant and dose-related increase in the number of cells with chromosomal aberrations in the absence of metabolic activation, with a stronger effect after the 24 h treatment with 500 µg/ml than after the 4 h treatment with 5000 µg/ml. With metabolic activation, only one test point gave a positive response. The positive controls induced statistically significant increases in cells with structural chromosome aberrations.

Conclusion
Under the experimental conditions reported, the test substance induced structural chromosome aberrations in human lymphocytes in the absence of metabolic activation and was thus found to be clastogenic *in vitro*.

Ref.: 28, Submission I, updated 2005

### 3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

***Taken from SCCP/0990/06, re-evaluated***

**Mouse bone marrow micronucleus test *in vivo***

| | |
|---|---|
| Guideline: | OECD 474 |
| Species: | Mouse, NMRI |
| Group size: | 5 males + 5 females |
| Test substance: | DA 010894 (4,5-diamino-1-(2'-hydroxyethyl)pyrazolsulfat) |
| Vehicle: | gum Arabic, 4% |
| Batch: | 4-20079 |
| Purity: | > 99% |
| Dose levels: | 500, 1000, 2000 mg/kg bw |
| Route: | oral gavage |
| Sacrifice time: | 24 and 48 (highest dose group only) h after the treatment |
| GLP: | in compliance |
| Study period: | 9 October 1995 – 24 November 1995 |

This study was performed to investigate the potential of DA 010894 to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the mouse. The test item was formulated in gum Arabic (4%) which was also used as vehicle control. The compound was administered by a single oral treatment, and bone marrow cells were collected 24 h and 48 h later. Ten animals (5 males, 5 females) per test group were included and at least 1000 polychromatic erythrocytes (PCEs) per animal were scored for micronuclei. 9,10-dimethyl-

1,2-benzanthracene was used as a positive control agent (48 h after the treatment). Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic (PCEs) and normochromatic erythrocytes (NCEs). The following dose levels of the test item were investigated: 24 h preparation interval: 500, 1000, 2000 mg/kg bw, 48 h preparation interval: 2000 mg/kg bw.

Results
After treatment with test agent, all animals showed reduced motility, with the most pronounced effect at the highest dose. In the male mice, the highest dose of the test substance the ratio of PCEs to NCEs was decreased. DA 010894 induced no increase in the number of PCEs with micronuclei. However, the positive control treatment clearly increased micronucleated PCEs.

Conclusion
Under the experimental conditions reported, DA 010894 did not induce an increase in the number of polychromatic erythrocytes with micronuclei of treated mice and, therefore, the test substance is not genotoxic (clastogenic and/or aneugenic) in polychromatic erythrocytes of mice

Ref.: 30, Submission I, updated 2005

***Taken from SCCP/0990/06, re-evaluated***

**Rat bone marrow chromosomal aberration test *in vivo***

| | | |
|---|---|---|
| Guideline: | OECD 475 | |
| Species: | Rat, Wistar | |
| Group size: | 5 males and 5 females | |
| Test substance: | A005767 WR 18247 | |
| Vehicle: | water | |
| Batch: | GST 7-18099 | |
| Purity: | 98.5% (NMR) | |
| Dose levels: | males: | 100, 200, 400 mg/kg bw/day |
| | females: | 150, 300, 600 mg/kg bw/day |
| Route: | intraperitoneally, twice 24 h apart | |
| Sacrifice time: | 24 h after the last treatment | |
| GLP: | In compliance | |
| Study period: | 25 April 2000 – 25 August 2000 | |

This study was performed to investigate the potential of A005767 WR 18247 to induce chromosomal aberrations in rat bone marrow. The test item was formulated in deionised water which was also used as vehicle control. The compound was administered by two intraperitoneal injections, 24 h apart. Bone marrow cells were collected 24 h later. Ten animals (5 males, 5 females) per test group were included, and at least 100 metaphases per animal were scored for chromosomal aberrations. Cyclophosphamide was used as a positive control agent. The ratio between polychromatic and normochromatic erythrocytes (NCEs) was determined in the same sample and reported as the number of PCEs per 2000 erythrocytes. The maximum dose levels were determined by preliminary experiments for toxicity to be near the maximum tolerated dose. The following dose levels of the test item were investigated: 100, 200, and 400 mg/kg bw/day in males and 150, 300, 600 mg/kg bw/day in females.

Results
In the preliminary experiments at the maximum tolerated doses toxic symptoms such as reduction of spontaneous activity, eyelid closure (females only), and apathy were observed. In all pre-experiments and at all dose levels used the urine colour of the treated animals turned orange.

In the main experiment, one female rat died after the highest dose of the test substance. No relevant reduction of mitotic indices was seen after the treatment, indicating no bone marrow toxicity. However, systemic availability of the compound was indicated by the urine colour change and the strong toxic reactions. A005767 WR 18247 did not induce a biological relevant increase in the number of cells with chromosomal aberrations. However, the positive control treatment did increase the number of cells with chromosomal aberrations.

Conclusion
Under the experimental conditions reported, the test item did not induce an increase in the number of cells with chromosomal aberrations in rat bone marrow and therefore, A005767 WR 18247 was not clastogenic in the chromosome aberration assay *in vivo*.

Ref.: 29, Submission I, updated 2005

### 3.3.7. Carcinogenicity

No data submitted

### 3.3.8. Reproductive toxicity

#### 3.3.8.1. One generation reproduction toxicity

***Taken from SCCP/0990/06***

| | |
|---|---|
| Guideline: | OECD 415 (1983) |
| Species/strain: | Rat, Hsd: Sprague-Dawley SD |
| Group size: | 24 per sex and dose |
| Test substance: | Pyrazole DHE |
| Batch: | 09048045 |
| Purity: | 99.4% (HPLC, 254 nm) |
| Dose levels: | 150, 300, 900 mg/kg bw/day |
| Route: | Oral, gavage |
| Vehicle: | Distilled water |
| Exposure period: | Males: 9 weeks prior to mating, females two weeks before mating, during gestation and up to day 20 of lactation |
| GLP: | in compliance |

Three groups of 24 male and female rats each were dosed daily intragastrically at levels of 150, 300 and 900 mg/kg bw during a premating phase (at least 9 weeks for males and at least 2 weeks for females), during mating and the subsequent gestation and lactation periods (females only; until postnatal day 21). In addition, an equally sized control group received the same dose volume of the vehicle throughout the described dosing period. After a mating period of maximally 42 days, all pregnant females were allowed to litter and raise their offspring until postnatal day 21.

The effects of the test compound were assessed using daily clinical observations and mortality checks, weekly body weight determinations in males throughout the study period and in females during the premating and mating periods. Pregnant/lactating females were weighed on gestation days 0, 6, 10, 15, 20 and on postnatal days 0, 4, 7, 14 and 21. Food consumption was recorded at weekly (premating) or 3 to 4-days intervals (females during gestation and lactation).
On the day of delivery, litter size was recorded and live pups were sexed, weighed and examined for external anomalies. On day 4 post partum, litters were culled to 8 pups. Postnatal development of the offspring was monitored by survival checks, body weight gain (per litter), recording of developmental landmarks (pinna unfolding, hair growth, incisor eruption, eye opening and testes descend) and by certain functional tests (startle response, air righting reflex, pupil reflex). At terminal sacrifice, all study animals were submitted to

gross necropsy (including pups of the $F_0$-generation). Organ weights were recorded for testis, epididymides and spleen. In addition, histopathological examination was performed on major reproductive system organs and spleen.

Results
No treatment-related adverse effects were noted in males and females in body weight, food consumption or clinical signs. Red staining of the skin/fur was noted for high dose animals. In addition, violet staining was noted in the cage tray of all animals treated with the test item. These signs were considered probably to be due to the excretion of the test item in urine. Reproductive parameters, litter data, sex ratios, gestation and pre-weaning development of pups were unaffected by treatment. Red staining on the dorsum was noted in pups of the mid- and high dose groups. This sign is considered related to the colour of the test item. At necropsy, a decrease in testes weight and increase in spleen weight, were noted in high dose $F_0$ animals when compared to controls. No treatment related changes were seen at the macroscopic and histopathologic examinations.

Conclusion
No adverse effects were observed on gonadal function, mating, fertility, implantation, prenatal development and postnatal development of the offspring. The highest tested dose of 900 mg/kg bw was the foetal NOAEL. Due to the decrease of testes weight and an increase in spleen weight in the high dose group (900 mg/kg bw/day) the NOAEL for parental males and females is 300 mg/kg bw/ day.

Ref.: 24

### 3.3.8.2. Teratogenicity

***Taken from SCCP/0990/06***

| | |
|---|---|
| Guideline: | OECD 414 (1983) |
| Species/strain: | Rat, Sprague-Dawley, Crl: CD (SD) BR strain |
| Group size: | 24 females per dose |
| Test substance: | DA 010894 |
| Batch: | GST 6-26089 |
| Purity: | 99.2% (HPLC, 254 nm) |
| Dose levels: | 100, 300, 1000 mg/kg bw/day |
| Exposure period: | Days 6 to 17 of pregnancy, inclusive |
| GLP: | In compliance |

Three groups of 24 pregnant female rats each were dosed daily intragastrically at levels of 100, 300 or 1000 mg/kg bw of the test compound. In addition, an equally sized control group received the same dose volume of the vehicle (10 ml/kg bw, distilled water) throughout the dosing period on days 6 through 17 of gestation (p.c.). The effects of the test compound were assessed using daily clinical observations in pregnant dams, body weight determination (on days 0, 2, 4, 6 through 17 and on day 20 p.c.), food consumption and macroscopic inspection of major organs and tissues on day 20 p.c. (gross necropsy observations). The uteri and ovaries were removed for counting of corpora lutea, implantations, viable foetuses as well as early and late resorptions. Intact pregnant uteri and placentae were weighed. Viable foetuses were weighed, sexed and examined for gross external defects. About 50% of the foetuses were processed for skeletal examination. The remainder was examined for visceral defects.

Results
No compound-related effects were observed at the low and intermediate dose levels (100 and 300 mg/kg bw). There was no indication of maternal toxicity at any of the tested dosages. Survival of the foetuses during prenatal development was not affected at any dose level. At the high dose of 1000 mg/kg bw, marginal adverse effects of the test compound were evident as indicated by a slightly delayed ossification, abnormal ossification patterns

and an increased incidence of foetuses with supernumerary ribs. The distribution of sporadically observed malformations over all study groups including controls did not indicate a specific teratogenic activity of the test compound.

Conclusion
The NOAEL for maternal toxicity was 1000 mg/kg bw/day. The highest dose of 1000 mg/kg bw exerted slight embryotoxic effects manifested as delayed or disturbed ossification and increased occurrence of supernumerary ribs. The intermediate dosage of 300 mg/kg bw was found as the NOAEL for foetotoxicity.

Ref.: 23

### 3.3.9. Toxicokinetics

***Taken from SCCP/0990/06***

| | |
|---|---|
| Guideline: | OECD 417 (1984); OECD 427 (draft, 2000) |
| Species/strain: | Rat, strain Wistar Kyoto, WKY/NR Crl BR (inbred) |
| Group size: | 4 Females in the mass balance groups (four groups) per dose<br>6 Females in the toxicokinetics groups (four groups) per dose |
| Test substance: | WR 18247 |
| Vehicle: | Milli-Q (oral and dermal dosing) and 0.9% saline (intravenous dosing); solutions contained 0.3% sodium sulphite and were adjusted to pH 7-8 with ammonia 25% |
| Batch: | 01BLY099 [ring-$^{14}$C(U)]-*1-Hydroxyethyl-4,5-diamino pyrazole sulfate*<br>R0070335 (Robinson 7326HLO73) non-radiolabelled *1-Hydroxyethyl-4,5-diamino pyrazole sulfate* |
| Purity: | Radiochemical purity: >98% by HPLC, specific activity 15 mCi/mmol<br>Non-labelled: 99.6% (HPLC, 254 nm) |
| Dose levels: | Intravenous administration: 10 mg/kg bw<br>Oral administration: 10, 250 mg/kg bw<br>Dermal administration: 20 mg/kg bw (equal to 0.3 mg/cm² skin and 30 mg/ml or 3%) |
| Route: | Intravenous, oral (gavage), dermal |
| GLP: | In compliance |

A single dose of $^{14}$C-*1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate* was administered orally by gavage at 10 mg/kg bw and 250 mg/kg bw to fasted rats, or by intravenous (i.v.) administration at 10 mg/kg bw, or by dermal application during 30 minutes at a dose of 0.3 mg/cm$^2$ (equal to 20 mg/kg bw/day and 30 mg/ml) on the back of the animals.
Urine and faeces were collected over the following time intervals after dosing: 0-8 h, 8-24 h, 24-48 h, 48-72 h, 72-96 h for animals in the oral and i.v. administration groups. They were euthanised 96 h after dose administration, and several tissues and organs were collected. In the dermally dosed groups, urine and faeces samples were collected at a 24 h interval and animals were sacrificed at 120 h. Total radioactivity in urine, faeces, tissues, and organs was determined. For metabolism evaluation, urine and faeces samples were pooled per group, and the metabolite profile of the pooled samples was obtained by HPLC and LC-MS/MS.
In the toxicokinetic groups, blood was sampled alternately from several rats per time point at 15 and 30 min and 1, 2, 4, 8, 24, and 48 h after dosing for the oral and i.v. groups, and at 30 min and 1, 2, 4, 8, 24, 48 and 72 h after dosing for the dermal group. Total radioactivity and parent compound equivalent concentrations were determined.

Results
Homogeneity and stability of test substance formulations in the vehicle were demonstrated by HPLC. Accuracy of concentrations was sufficient to fulfil the study objectives.
$^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was readily absorbed and rapidly excreted after oral administration in Wistar Kyoto rats. Mean cumulative recovery of radioactivity in

the urine after 96 h was 73.3 ± 8.3% (low dose) and 75.7 ± 2.5% (high dose) of the applied dose. Mean cumulative recovery of radioactivity in faeces was 28.3 ± 2.3% (low dose) and 22.9 ± 1.0% (high dose) of the applied dose. Mean residual radioactivity in the carcass, tissues and blood was 0.9% (low dose) and 0.6% (high dose) of the applied dose. Less than 5% of the total radioactivity was recovered in the cage wash. The mean mass balance was 107.1 ± 8.8% (low dose) and 101.3 ± 2.4% (high dose).
The mean percent recovery of radioactivity after intravenous administration after 96 h was 86.9 ± 8.0% in urine and 6.0 ± 2.6% in faeces. Mean residual radioactivity in the carcass, tissues, and blood was 0.9% of the applied dose. Less than 3% of the total radioactivity was recovered in the cage wash. The mean mass balance was 96.7 ± 4.6%.
After dermal application, the mean cumulative recovery of radioactivity was 0.8 ± 0.5% of the applied dose for the urine and 0.8 ± 0.5% of the applied dose for the faeces. Mean residual radioactivity in the carcass, tissues and blood was 2.4%, and the majority of this was recovered from treated skin (1.7 ± 0.8%). Less than 0.1% of the total radioactivity was recovered in the cage wash. The mean mass balance was 91.1 ± 3.3%.
In the urine, five different metabolites could be distinguished after oral $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate administration. In the high oral dose group, the parent compound could be detected. The majority of the radioactivity present in urine, about 90%, could be assigned to three major metabolites. In the faeces, five different metabolites could also be distinguished. The majority of the radioactivity present in faeces, about 90%, could be assigned to two major metabolites. The urine and faeces samples appear to have one major metabolite in common. Detection of unabsorbed $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate in faeces remained questionable due to inconsistent analytical results.
Radioactivity in urine after dermal application was too low for an accurate detection of metabolites. However, LC-MS/MS analysis suggested a quantitatively similar profile of metabolites as the one seen after oral administration. After i.v. dosing inconsistent analytical results were obtained.
Characterisation of metabolites was difficult, since no standards were available. However, three metabolites were characterised (by PDA and MS detector). It appears that $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate is metabolised through oxidative reactions and N-acetyl conjugation. Two metabolites appeared to be present as dimers probably due to chemical conversion after sampling but in vivo formation cannot be excluded.
The most important route of excretion of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate and its metabolites was via urine after oral and i.v. administration. In both oral dose groups, 63-78% of the administered dose was recovered in the urine, showing that no metabolic saturation occurred at the high dose level.
Urinary excretion after dermal administration was low, (0.8%), reflecting the poor dermal absorption. The terminal rate of excretion was much slower than in the other groups.
Excretion in faeces was a far less important route of excretion, representing 3-30% of the dose after oral and i.v. administration, and 0.8% after dermal application. The amount of radioactivity excreted in the faeces was higher after oral dosing compared to intravenous dosing, indicating that the majority of the radioactivity in the faeces after oral dosing may represent $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate.
After oral, iv and dermal dosing radioactivity in organs was very low. The highest residues were found in the carcass (0.4-0.8%).
After dermal dosing radioactivity recovered from treated skin was 1.7%.

Toxicokinetic results indicate that a good dose proportionality was achieved with $C_{max}$ values of 5.50 mg/kg bw (low dose) and 170.81 mg/kg bw (high dose). The rate of oral absorption of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was fast, with maximum plasma concentrations reached one hour after administration in both groups indicating no saturation of absorption at the high dose level. $AUC_{0\rightarrow\infty}$ values were 24.10 and 600.46 $mg_{eq}$hr/kg bw for the low and high dose groups, respectively. The dose-normalised AUC values were quite similar, i.e. 2.23 and 2.38, respectively.
The rate of absorption after dermal administration was faster than after oral administration, since the maximum plasma concentrations were reached 30 min after dosing. However, plasma concentration values remained low, leading to relatively low AUC values ($AUC_{0\rightarrow\infty}$ =

0.36 $mg_{eq}h/kg$ bw and 0.02 for the respective dose-normalized value, both calculated as approximations since at some time points the plasma concentrations were below the limit of quantification).
In plasma samples of the high oral dose group taken within 2h after dosing, 14C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate could be detected. The concentrations rapidly decreased with time. Therefore, 1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate appears to be quickly metabolised. No 14C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was detected in any of the samples from the other groups. One metabolite peak was present in the plasma samples following oral and i.v. administration. After dermal application nothing was detected at any time point due to low radioactivity levels.

Conclusion
Absorption, distribution, metabolism and excretion have been investigated in the female Wistar Kyoto rat, a strain with a low acetylator phenotype. After oral administration, $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was extensively absorbed, readily distributed into all organs, extensively metabolised and excreted via the urine and faeces. The oral absorption of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was high, between 78-83%.
Dermal absorption of an aqueous solution containing 3% of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was low: 2.4% of the applied dose or 0.006 $mg/cm^2$. Since it cannot be excluded that the amount retained in the application site skin may eventually become systemically available, the skin residue dose was considered potentially absorbed and potentially systemically available. Thus, as a worst case assumption, dermal bioavailability was calculated as 4% of the applied dose, or 0.01 $mg/cm^2$ of an aqueous solution containing 3% of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate. When dermally absorbed, excretion took place mainly via the faeces and the rate of elimination was slower than after oral administration.
Analytical results indicate extensive metabolism after both oral and dermal administration. Efforts to characterise the urinary metabolites indicate the presence of oxidative and N-acetylated metabolites.

Ref.: 26

### 3.3.10. Photo-induced toxicity

No data submitted

### 3.3.11. Human data

No data submitted

### 3.3.12. Special investigations

No data submitted

### 3.3.13. Safety evaluation (including calculation of the MoS)

**CALCULATION OF THE MARGIN OF SAFETY**

**1-Hydroxyethyl-4,5-diamino pyrazole sulfate**

**Oxidative conditions**

| | | | |
|---|---|---|---|
| **Absorption through the skin** | **A** | **=** | **1.953 µg/cm²** |
| **Skin Area surface** | **SAS** | **=** | **580 cm²** |
| **Dermal absorption per treatment** | **SAS x A x 0.001** | **=** | **1.132 mg** |
| **Typical body weight of human** | | **=** | **60 kg** |
| **Systemic exposure dose (SED)** | **SAS x A x 0.001/60** | **=** | **0.02 mg/kg bw/d** |
| **No Observed Adverse Effect Level (13-week oral study, rat, gavage)** | **NOAEL** | **=** | **250 mg/kg bw/d** |
| **MOS** | **NOAEL/SED** | **=** | **12500** |

### 3.3.14. Discussion

*Physico-chemical specifications*
1-Hydroxyethyl-4,5-diamino pyrazole sulfate is used in oxidative hair dye formulations at a final concentration of 3%, after mixing with peroxide. The stability of 1-Hydroxyethyl-4,5-diamino pyrazole sulphate in solutions used for oral toxicity testing is not reported

*General toxicity*
The acute oral LD50 in rats was greater than 2000 mg/kg bw. In rats, the 4 h LC50 value of an aerosol of the test substance was larger than 5.24 g/m³ for both sexes.
In a reproductive toxicity study, the NOAEL for parental males and females is 300 mg/kg bw/ day. The foetal toxicity was 900 mg/kg bw.
In a teratogenicity study, the NOAEL for maternal toxicity was 1000 mg/kg bw/day. 300 mg/kg bw was found as the NOAEL for foetotoxicity.
In a 13-week study using daily intragastric dosing of the test animals at the highest tested dose of 800 mg/kg bw, marginal changes in some blood biochemical, haematological and spleen weight were observed. Based on these effects, the NOAEL in this study was 250 mg/kg bw/day.

*Toxicokinetics*
Absorption, distribution, metabolism and excretion have been investigated in the female Wistar Kyoto rat, a strain with a low acetylator phenotype. After oral administration, $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was extensively absorbed, readily distributed into all organs, extensively metabolised and excreted via the urine and faeces. The oral absorption of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was high, between 78-83%.
Dermal absorption of an aqueous solution containing 3% of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate was low: 2.4% of the applied dose or 0.006 mg/cm$^2$. Since it cannot be excluded that the amount retained in the application site skin may eventually become systemically available, the skin residue dose was considered potentially absorbed and potentially systemically available. Thus, as a worst case assumption, dermal bioavailability was calculated as 4% of the applied dose, or 0.01 mg/cm$^2$ of an aqueous solution containing 3% of $^{14}$C-1-Hydroxyethyl-4,5-Diamino Pyrazole Sulfate. When dermally absorbed, excretion took place mainly via the faeces and the rate of elimination was slower than after oral administration. Analytical results indicate extensive metabolism after both

oral and dermal administration. Efforts to characterise the urinary metabolites indicate the presence of oxidative and N-acetylated metabolites.

*Irritation / sensitisation*
This test substance is irritant to rabbit skin under the conditions of the experiment.
A 5% of the test substance is irritant to rabbit eyes. The undiluted test compound may cause serious damage to eyes.
The test compound was found an extremely potent contact allergen in a Magnusson and Kligman test. In a Buehler test, the substance showed no sensitising potential when applied epicutaneously at a concentration of 40%.
1-Hydroxyethyl 4,5-diamino pyrazole sulfate did not induce a biologically relevant immune response in local lymph nodes after dermal application up to the maximum test concentration of 10%. However, the maximum test concentration was too low for hazard identification.

*Dermal absorption*
The amount applied (100 mg/cm²) is too high compared to the normal value of 20 mg/cm². Then, under oxidative conditions, the mean value + 2SD = 1.163 + 2 x 0.395 = 1.953 µg/cm$^2$ has been used to calculate the MoS.

In a toxicokinetics study in rats, the dermal absorption was 4% of the applied dose, or 0.01 µg/cm².

Mutagenicity
Overall, the genotoxicity of 1-hydroxyethyl-4,5-diamino pyrazole sulfate is investigated in genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Both in bacteria and in mammalian cells treatment with 1-hydroxyethyl-4,5-diamino pyrazole sulfate did not result in an increase of the mutant frequency. However, 1-hydroxyethyl-4,5-diamino pyrazole sulfate induced structural chromosome aberrations in human lymphocytes in the absence of metabolic activation and was thus found to be clastogenic *in vitro*.
The positive *in vitro* findings with 1-hydroxyethyl-4,5-diamino pyrazole sulfate could not be confirmed in *in vivo* assays. a micronucleus test in mice and a chromosome aberration test in rats were both negative. Consequently, 1-hydroxyethyl-4,5-diamino pyrazole sulphate can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

*Carcinogenicity*
No data submitted

## 4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that 1-hydroxyethyl-4,5-diamino pyrazole sulfate in oxidative hair dye formulations with a concentration on-head of maximum 3.0% does not pose a risk to the health of the consumer, apart from its sensitising potential.

## 5. MINORITY OPINION

Not applicable

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