EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

1-HYDROXYETHYL-4,5-DIAMINO PYRAZOLE SULFATE

COLIPA N° A154

Adopted by the SCCP during the 8th plenary meeting
of 20 June 2006

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1. BACKGROUND

Submission I for 1-Hydroxyethyl-4,5-diamino pyrazole sulphate was submitted in March 2003 by COLIPA[1,2].

Submission II with additional data for this substance was 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). The intended maximum on-head concentration is 3.0%. The oxidative colouring agent and the developer are mixed in ratios between 1:1 and 1:3. 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.

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider 1-Hydroxyethyl-4,5-diamino pyrazole sulfate safe for use as an oxidative hair dye with a concentration on-head of maximum 3.0 % taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use of 1-Hydroxyethyl-4,5-diamino pyrazole sulfate in oxidative hair dye formulations?*

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

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

1-Hydroxyethyl-4,5-diamino pyrazole sulphate

3.1.1.2. Chemical names

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

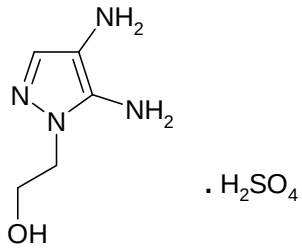
3.1.1.3. Trade names and abbreviations

WR18247
DA 010894
Pyrazol DHE
COLIPA n° A154

3.1.1.4. CAS / EINECS number

CAS: 155601-30-2
EINECS: /

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-4,5diamino 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)
pH of 1% solution: 1.82-1.94
pH of 5% solution: 1.61-1.66
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)

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:	/

3.1.9.	Stability

1-Hydroxyethyl-4,5-diamino pyrazole sulphate on storage in dryness is considered to be stable more than 5 years – *No data provided*

General Comments on Physico-chemical characterisation

- Stability of 1-Hydroxyethyl-4,5-diamino pyrazole sulphate in test solutions and in marketed products is not reported.
- EINECS/ELINCS number of 1-Hydroxyethyl-4,5-diamino pyrazole sulphate is not provided.

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

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

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

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

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

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

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 dermal induction: 40% test substance in white petrolatum, occluded 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

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) 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 48 h.

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

3.3.4. Dermal / percutaneous absorption

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:
I: static system
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 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.

3.3.5. Repeated dose toxicity

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

No data submitted

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

Guideline:	OECD 408 (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, 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. Body weight gain was slightly, but statistically significant decreased in females from the 800 mg/kg 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*

Bacterial gene mutation assay

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA1538
Replicates:	Two independent tests with and without S9 mix (in triplicate)
Test substance:	DA 010894
Vehicle:	water
Batch:	4-20079
Purity:	> 99% (HPLC)
Concentrations:	1-5000 µg/plate with and without S9 mix (6 concentrations)
GLP:	In compliance

The test substance was assessed for the induction of revertant mutations in various strains of *Salmonella typhimurium* with and without S9 mix containing Aroclor 1254 -induced rat liver postmitochondrial fraction. The test was performed according to the OECD guidelines and GLP.

Results
Some growth inhibiting effects (reduction in revertant counts or sparse bacterial background lawn) were observed with TA1337 and TA98 without S9 mix and TA100 with S9 mix at 3000 µg/plate. No increase in revertant colony numbers was observed in any tester strain following treatment with the test substance. 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

In vitro mammalian chromosome aberration test

Guideline:	OECD 473
Species/strain:	Human peripheral lymphocytes
Replicates:	Two independent experiments
Test substance:	A005767, Pyrazole DHE
Vehicle:	Ham's F-10 medium
Batch:	DA010894
Purity:	98.5%
Concentrations:	1st experiment: 50, 150, 500 µg/ml without S9 mix (24 h from the start); 2nd experiment: 500, 1500, 5000 µg/ml without (4 h) and with (3.5 h) S9 mix
GLP:	in compliance

Human lymphocytes were used to examine the induction of chromosomal aberrations by the test substance. 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 interval was 24 h and 4 h without metabolic activation in the first and second experiments, 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 2nd experiment with and without S9-mix. Mitomycin C and cyclophosphamide were used as positive controls.

Results
The highest concentration tested induced a 44% (1st experiment) and 81% (2nd experiment) cytotoxicity without S9 mix and a 52 (1st experiment) and 59% (2nd experiment) cytotoxicity with S9 mix. The test substance induced a significant and dose-related increase in the frequency of 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
It could be stated that in the study described and 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

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test *in vivo*

Guideline:	OECD 474
Species/strain:	Mouse, NMRI
Group size:	5 males + 5 females
Test substance:	4,5-diamino-1-(2'-hydroxyethyl)pyrazolsulfat
Vehicle:	gum Arabic, 4%
Batch:	4-20079
Purity:	> 99%
Dose levels:	500, 1000, 2000 mg/kg bw (once orally)
Sacrifice time:	24 and 48 (highest dose group only) h after the treatment
GLP:	in compliance

This study was performed to investigate the potential of the test substance 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). 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 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 led a decrease in the ratio of PCEs to NCEs. The test compound induced no increase in the incidence of micronucleated PCEs. However, the positive control treatment clearly increased micronucleated PCEs.

Conclusion
Under the experimental conditions reported, the test item did not significantly induce micronuclei in mouse bone marrow polychromatic erythrocytes. Therefore, the test substance was considered to be negative in the micronucleus assay. Negative and positive controls were in accordance with the OECD guideline.

Ref.: 30

Rat bone marrow chromosomal aberration test *in vivo*

Guideline:	OECD 475
Species/strain:	Rat, Wistar
Group size:	5 males + 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; females: 150, 300, 600 mg/kg bw; twice intraperitoneally
Sacrifice time:	24 h after the last treatment
GLP:	In compliance

This study was performed to investigate the potential of the test substance 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 in males and 150, 300, 600 mg/kg bw in females.

Results
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 urine colour change and the strong toxic reactions. The test compound induced no statistically significant increase in the incidence of chromosomal aberrations. However, the positive control treatment clearly increased chromosomal aberrations.

Conclusion
Under the experimental conditions reported, the test item did not significantly induce chromosomal aberrations in rat bone marrow. Therefore, the test substance was not clastogenic in the chromosome aberration assay *in vivo*. Negative and positive controls were in accordance with the OECD guideline.

Ref.: 29

General conclusion on mutagenicity
A mammalian gene mutation test *in vitro* is required.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. One generation reproduction toxicity

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/day) the NOAEL for parental males and females is 300 mg/kg/bw day.

Ref.: 24

3.3.8.2. Teratogenicity

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, 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). 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

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 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* R0070335 (Robinson 7326HLO73) non-radiolabelled *1-Hydroxyethyl-4,5-diamino pyrazole sulfate*
Purity:	Radiochemical purity: >98 % by HPLC, specific activity 15 mCi/mmol Non-labelled: 99.6 % (HPLC, 254 nm)
Dose levels:	Intravenous administration: 10 mg/kg bw Oral administration: 10, 250 mg/kg bw 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 $\pm$ 8.3% (low dose) and 75.7 $\pm$ 2.5% (high dose) of the applied dose. Mean cumulative recovery of radioactivity in faeces was 28.3 $\pm$ 2.3% (low dose) and 22.9 $\pm$ 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 $mg_{eq}hr/kg$ 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 $mg_{eq}h/kg$ 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

Not applicable

3.3.14. Discussion

Physico-chemical specifications
1-Hydroxyethyl-4,5-diamino pyrazole sulphate is used in oxidative hair dye formulations at a final concentration of 3%, after mixing with peroxide. The EINECS or ELINCS number is not provided. The stability of 1-Hydroxyethyl-4,5-diamino pyrazole sulphate in test solutions and in marketed products is not reported.

General toxicity
The acute oral LD_{50} in rats was greater than 2000 mg/kg bw. 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.
In a 13-week study, the NOAEL was set at 250 mg/kg bw/day.
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.

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 %.

Dermal absorption
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).
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. Moreover, the test substance has not been studied in the presence of hydrogen peroxide. Because of these concerns, the studies are not used for calculating margin of safety.
In a toxicokinetics study in rats, the dermal absorption was 4% of the applied dose, or 0.01 μg/cm^2.

Mutagenicity
Under the experimental conditions reported, the test substance was not mutagenic to *Salmonella typhimurium*. It induced structural chromosome aberrations in human lymphocytes in the absence of metabolic activation and was thus found to be clastogenic *in vitro*. It did not significantly induce micronuclei in mouse bone marrow polychromatic erythrocytes. Therefore, it was considered to be negative in the micronucleus assay. Negative and positive controls were in accordance with the OECD guideline.
The test item did not significantly induce chromosomal aberrations in rat bone marrow. Therefore, it was not clastogenic in the chromosome aberration assay in vivo. Negative and positive controls were in accordance with the OECD guideline.

Carcinogenicity
No data submitted

4. CONCLUSION

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

Before any further consideration, the following information is required:

* 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

5. MINORITY OPINION

Not applicable

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