



Scientific Committee on Consumer Products
SCCP

OPINION ON

2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE

COLIPA N° A99

The SCCP adopted this opinion during its 10th plenary of 19 December 2006

OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE**About the Scientific Committees**

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

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

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SCCP

Questions concerning the safety of consumer products (non-food products intended for the consumer).

In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE**ACKNOWLEDGEMENTS**

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OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYL PYRIDINE**1. BACKGROUND**

Submission I and II for 2,6-Dihydroxy-3,4-dimethylpyridine were submitted by COLIPA¹ in September 1992 and in May 1994 respectively.

The Scientific Committee on Consumer Products and Non Food Products intended for Consumers (SCCNFP) expressed its opinion (SCCNFP/0229/99) at the 11th plenary meeting of 17 February 2000 with the conclusion, that:

"Classification: 1 in permanent hair dye formulations at a maximum concentration of 2.0 %. However, as permanent hair dyes are mixed with hydrogen peroxide before application, the maximum in-use concentration should not exceed 1.0 %."

The substance is currently regulated by the Cosmetics Directive (76/768/EEC), Annex III, Part 2 under entry 6 on the Preliminary list of substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

According to the current submission III, submitted by COLIPA in July 2005, 2,6-Dihydroxy-3,4-dimethylpyridine is used as a precursor for hair colours. It reacts with primary intermediates to form the final dye. The reaction is accelerated by addition of an oxidising agent (e.g. hydrogen peroxide). The final concentration on the scalp is proposed to be 1.0%.

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

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider 2,6-Dihydroxy-3,4-dimethylpyridine safe for use as an ingredient in both oxidative and non-oxidative hair dye formulations with a concentration on the scalp of maximum 1.0% taking into account the scientific data provided?*
2. *Does the SCCP have any other concerns with regard to the use of 2,6-Dihydroxy-3,4-dimethylpyridine in both oxidative hair dyes and non-oxidative hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

2,6-Dihydroxy-3,4-dimethylpyridine

3.1.1.2. Chemical names

¹ COLIPA - European Cosmetics Toiletry and Perfumery Association

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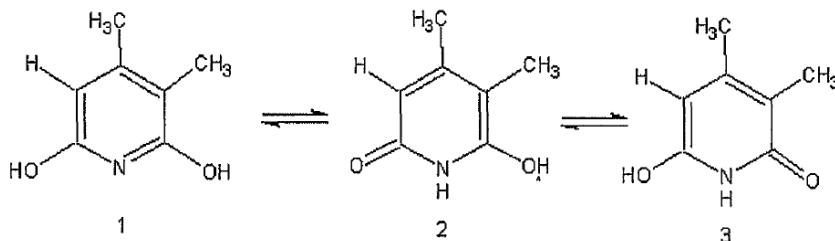
2(1H)-pyridone, 6-hydroxy-3,4-dimethyl- (CAS Index, 9CI)
 3,4-dimethylpyridine-2,6-diol
 6-hydroxy-3,4-dimethyl-2-pyridone
 6-hydroxy-2,3-dimethyl-2-pyridone

3.1.1.3. Trade names and abbreviations

Ro 598
 COLIPA n° A99

3.1.1.4. CAS / EINECS number

CAS: 84540-47-6
 EINECS: 283-141-2

3.1.1.5. Structural formula

NMR revealed the identity of 2,6-dihydroxy-3,4-dimethylpyridine in two tautomeric forms 2 and 3 in approx. equal proportions.

3.1.1.6. Empirical formula

Formula: C₇H₉NO₂

3.1.2. Physical form

Light yellow to beige amorphous powder

3.1.3. Molecular weight

Molecular weight: 139.15

3.1.4. Purity, composition and substance codes

Batch 01200081229 = 0120081229 = SAT 030659 = SAT 040292

Identification/characterisation: NMR, IR, UV (maxima at 237.5 nm and 325 nm)

NMR purity: 101%

HPLC purity (peak area): 98.4% (241 nm), 99.7% (314 nm)

Declaration concerning A99 used for acute toxicity:

"The batch of COLIPA A099 used in the acute oral toxicity test is not fully analytically described. However, information is available from the laboratories that have synthesized this batch concerning the identity and purity of the material produced at that time. From

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this information it can be concluded that the former not fully described batch is representative and its specification is quite similar to the fully characterised batch 01200081229".

3.1.5. Impurities / accompanying contaminants

Several impurities revealed by HPLC, peak areas 0.038 – 0.439%, are not identified

Solvent content: <0.1% (w/w)

Sulfated ash: <0.1% (w/w)

3.1.6. Solubility

Water: <1 g/l, room temperature

Ethanol: <1 g/l, room temperature

DMSO: 3 -30 g/l

Comment

Solubility has not been determined by EU Official Method, EEC A6. Exact solubility in water is not reported.

3.1.7. Partition coefficient ($\log P_{ow}$)

$\log P_{ow}$: 2.44 (calculated, Syracuse Vers. 1.66)

3.1.8. Additional physical and chemical specifications

Melting point: 185-191°C

Boiling point: /

Flash point: /

Vapour pressure: /

Density: /

Viscosity: /

pKa: /

Refractive index: /

3.1.9. Homogeneity and Stability

2,6-Dihydroxy-3,4-dimethylpyridine suspensions, used for oral toxicity studies, in water containing 1% carboxymethylcellulose and 0.5% Tween 80 were stable (maximum variation 14%)

General comments to physico-chemical characterisation

- No documentation is provided for the similarity of physico-chemical properties of batches 0120081229 = SAT 030659 = SAT 040292 of 2,6-dihydroxy-3,4-dimethylpyridine with those of batch 01200081229, which is fully characterised.
- Solubility data of 2,6-dihydroxy-3,4-dimethylpyridine is inadequate
- $\log P_{ow}$: calculated values can not be accepted as estimates of the true physical constants without justification, indicating that the reported values are realistic
- Stability of 2,6-dihydroxy-3,4-dimethylpyridine in marketed products is not reported
- Impurities in 2,6-dihydroxy-3,4-dimethylpyridine, revealed by HPLC have not been identified and quantified

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2,6-Dihydroxy-3,4-dimethylpyridine is used in oxidative hair dye formulations at a maximum final concentration of 1%, after mixing with peroxide developer.

3.3. Toxicological Evaluation**3.3.1. Acute toxicity**

Guideline:	/
Species/strain:	Wistar rats
Group size:	40 male
Test substance:	2,6-Dihydroxy-3,4-dimethylpyridine
Batch:	/
Purity:	/
Dose:	794, 1000, 2500, 5000 mg/kg bw
Route:	oral
Exposure:	single administration and a 14 days observation period
GLP:	/

On a day of exposure the animals (40 males) had a weight from 159 to 180 g.

DDP was suspended in olive oil and aliquots of 20 ml/kg bw were administered once. The doses were 794, 100, 2500, 5000 mg/kg body weight.

During a two weeks of observation, mortalities (794; 0/10, 1000; 1/10, 2500; 1/10 and 5000, 4/10) and clinical observations were recorded on a daily basis.

The main observations were prone position, crouching and apathy. At the highest dose diarrhoea was recorded. The death occurred 2 to 24 hours after administration. No histological examinations were reported

LD50 was determined to be between 2500 and 5000 mg/kg bw.

Ref.: 3

Comment

The study was not conducted according to GLP/OECD guideline. The major shortcuts were limited information of the test substance and that only one gender was used. However, the SCCP regards this study as sufficiently valid to avoid a repetition of the acute oral toxicity test.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE**3.3.2 Irritation and corrosivity****3.3.2.1. Skin irritation**

Guideline: OECD 404
 Species: New Zealand albino rabbit
 Group: 3 males
 Substance: A99
 Batch: 0120081229
 Purity: 99.5%
 Dose: 0.5 g
 Vehicle: water
 GLP: in compliance

The rabbit were exposed to 0.5 g A099 (moistened with 0.7 ml water), applied onto shaved skin for 4 h using a semi-occlusive dressing. Observations were made 1h, 24 h, 48 h and 72 h after exposure. Adjacent areas of the untreated skin of each animal served as control.

Results

No oedema or erythema was caused by 4 h exposure to A 099.

Green-purple staining of the treated skin by the test substance was observed throughout the observation period, which did not hamper the scoring of the skin reactions. No remnants of the test substance were present in the skin.

Conclusion

A 099 is not an irritant to rabbit skin.

Ref.: 4

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405
 Species: New Zealand albino rabbits
 Group: 3 males
 Substance: A099
 Batch: 0120081229
 Purity: 99.5%
 Dose: 62 mg (ca. 0.1 ml)
 GLP: in compliance

Each animal was treated by instillation of 61.5 mg (60.8 - 62.2 mg) of the test substance (a volume of approximately 0.1 ml) in the conjunctival sac of one of the eyes after gently pulling the lower lid away from the eyeball. The lids were then gently held together for about one second to prevent loss of the test substance. The other eye remained untreated and served as control.

Immediately after the 24 h observation, a solution of 2% fluorescein in water (pH 7.0) was instilled into both eyes of each animal to quantitatively determine corneal epithelial damage. Any bright green stained, indicating epithelial damage, was estimated as a percentage of the total corneal area.

Results

Instillation of A 099 into one eye of each of the three rabbits resulted in effects on the iris and conjunctivae. Iridial irritation grade 1was observed and had resolved within 24 h. The irritation of the conjunctivae consisted of redness, chemosis and discharge and had completely resolved in one animal within 72 h and in two animals within 7 days.

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No corneal opacity was observed, and treatment of the eyes with 2% fluorescein, 24 h after test substance instillation revealed no corneal epithelial damage.

There was no evidence of corneal corrosion.

Conclusion

The test substance was an irritant to the rabbit eye under the conditions of the test.

Ref.: 5

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species:	Mouse, CBA strain, inbred, SPF-quality
Group:	30 females, 6 groups 5 females
Substance:	A099
Batch:	Lot 0120081229
Purity:	99.5%
Dose:	2.5%, 10%, 25% and 50%
Vehicle:	Propylene glycol
Control:	Treatment by vehicle only
GLP:	Statement of compliance

Preliminary study

A preliminary irritation study was conducted to select the highest test substance concentration that could be used in the main study. The test system, procedures and techniques were identical to those used during days 1-3 of the main study.

Four young adult animals (5-11 weeks old) were selected. Each animal was treated with one concentration (5%, 10%, 25% and 50%) for two consecutive days. Approximately 4 hours after the last exposure, the skin was cleaned of residual test substance with water and the irritation was assessed. No oedema or erythema was observed at any dose level (Grade 0). No necropsy was performed and no body weights were determined after termination.

Based on the results and previous experience with similar test substances, the highest test substance concentration selected for the main study was 25%.

Main study

Initially, four groups of five animals were treated with the vehicle and 3 with test substance (concentration 2.5%, 10% and 25%). The dorsal surface of both ears was epidermally treated with test substance/vehicle (25 µl/ear) at approximately the same time each day, up to 3 days. On day 6, each animal was injected via the tail vein with 0.25 ml of sterile phosphate buffered saline (PBS) containing 20 µCi of ³H-methylthymidine. Five hours later the animals were killed by intraperitoneal injection with an overdose of phenobarbital and the draining (articular) lymph node of each animal was excised. The relative size of the nodes (as compared to control) was estimated by visual examination and the abnormalities of the nodes were recorded. Nodes were pooled for each animal in 3 ml PBS.

A single cell suspension of lymph node cells (LNC) was prepared in PBS by gentle separation through stainless steel gauze (diameter 125 µm). LNC were washed twice with an excess of PBS by centrifugation at 200g for 10 minutes at 4°C. The DNA was precipitated with 3 ml 5% trichloroacetic acid (TCA) at 4°C for approximately 18 hours. Precipitates were recovered by centrifugation at 200g for 10 minutes, re-suspended in 1 ml TCA and transferred to 10 ml of the scintillation fluid for the measurement of radioactivity.

Based on the results of the initial study, two additional groups of the animals were treated with the test substance concentration of 25% and 50% respectively using the same procedure as above.

Results

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The majority of nodes were equal in size, except for the large nodes found in the additionally treated group at 25% and some smaller nodes were found in the group treated with 50% test material. No other macroscopic abnormalities of the nodes were noted.

Table: Uptake of ^3H -methylthymidine by draining lymph nodes and Stimulation Index (SI)

Concentration of A99 % (w/w)	Uptake of ^3H - methylthymidine in Experiment 1		SI		Uptake of ^3H - methylthymidine in Experiment 2		SI	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
2.5	118	96	0.70	0.6				
10.0	435	319	0.25	2.0				
25.0	782	461	4.6*	2.8	187	148	1.1**	0.9
50.0					141	145	0.8	0.9
Control	172	162			173	160		

* individual values: 1.4, 2.7, 5.9, 11.2, 1.5

** individual values: 0.6, 0.9, 0.5, 2.0, 1.5

SI in all cases, except that based on mean value for 25% A099 in Experiment 1, was <3. The mean SI value of 4.6 in Experiment 1 was not confirmed in the Experiment 2. According to study authors SI that exceeded the SI=3 was influenced by two animals with an individual SI above 3. The study authors conclude that there is sufficient evidence that the test substance could not elicit a SI ≥ 3 . Based on these results should not be regarded as a skin sensitisier.

Ref.: 6

Comment

In one of the experiments, 25% A099 was found to be sensitisier (SI 4.6) based on mean values, but this was not confirmed in another experiment. No explanation for this contradictory finding has been found in the dossier submitted. The SCCP considers A99 as a possible skin sensitisier.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428
Tissue:	Pig dermatomed skin membranes
Group size:	For each experiment skin (thickness 400 μm) was used from 3 pigs, 2 membranes coming from each. Number of donor pigs is not identified.
Test substance:	A099 (2,6-dihydroxy-3,4-dimethyl pyridine) and ^{14}C -A099 (the ^{14}C -labelled position in the molecule is not identified)
Batch:	0120008129 (unlabelled) and 012000829 (^{14}C -labelling)
Purity:	99.5% (unlabelled) and 99% labelled, defined as HPLC peak area
Vehicle:	A cream formulation (TM0032-1a) containing 2% A099, and 1% A099 in PEG 300. Cream formulation was spiked with the ^{14}C -A099 to a final concentration of A099 to 2.133% (w/w). Final concentration of A099 (inclusive ^{14}C -A099) in the PEG 300 solution was 0.967% (w/w).
Doses:	20 mg/cm 2 or 20 $\mu\text{l}/\text{ml}$, exposed area 2.54 cm 2
Receptor fluid:	Phosphate buffered saline (composition and pH not identified)
GLP:	in compliance

The penetration of A099 was measured *in vitro* through dermatomed pig skin from a standard cream formulation (mixed with developers with and without hydrogen peroxide)

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and from a solution in polyethylene glycol 300 (PEG 300). The PEG 300 solution and formulated material were applied to the dermatomed skin at a nominal rate of 20 mg /cm² or 20 µl / cm² and left unoccluded.

The applications were washed off after a 0.5 h contact period and the penetration of A099 through the membrane was assessed throughout the entire 48h exposure period. At the end of the exposure period, the distribution of A099 in the test system was assessed, which included a tape stripping technique to determine its distribution in the skin. Samples collected during this study were analysed by liquid scintillation counting.

Results

The amount of A099 penetrated the skin after 48 h was as given in the following table.

Test compartment	µg/cm² (Mean ± SD)		
	Cream formulation + developer with H₂O₂	Cream formulation + developer without H₂O₂	A 099 in PEG 300
<i>Stratum corneum</i>	0.562±0.066	0.423±0.246	0.164±0.068
Epidermis + dermis	0.331 ± 0.129	0.426±0.185	0.107±0.131
Receptor fluid	0.558±0.320	0.405±0.201	0.052±0.042
Systemically available*	0.889±0.340 Range 0.526 - 1.430	0.832±0.198 Range 0.624 – 1.070	0.159±0.163 Range 0.035 - 0.476
Recovery	96.5±3.1	98.3±2.6	93.0±3.6

* sum of amounts in epidermis + dermis + receptor fluid

Conclusion

The maximum amount of A099 absorbed into the skin (Epidermis + dermis + receptor fluid) in the presence of developer containing H₂O₂, i.e. 1.43 µg/cm² will be used for the calculation of margin of safety.

Ref.: 15

Comment

The solubility of the test substance in the receptor fluid was not reported.

3.3.5. Repeated dose toxicity

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

Guideline: OECD 407
 Species/strain: Wistar
 Group size: 40 animals (5 per sex and per dosage)
 Test substance: 2,6-Dihydroxy-3,4-dimethylpyridine (DDP)
 Batch: 0120081229
 Purity: >98 %
 Dose: 0, 100, 300 and 1000 mg/kg day
 Route: Oral, in 1.0 % aqueous carboxymethylcellulose and 0.5% Tween 80
 Exposure: 28 days
 GLP: in compliance

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A 28-Day study was used for a range finding for Wistar rats exposed to DDP by gavage at doses of 100, 300 and 1000 mg/kg day. A negative control groups was used. Altogether 40 rats were used in this study (5 animals per sex). Clinical signs, food consumption and body weight were recorded. At the end of the experiment, all animals were killed, necropsied and examined post mortem. Only the kidneys were studied using histopathology.

DDP was well tolerated and did not produce early mortality, clinical signs or effects on food intake and body weight gain.

After 28 days of DDP exposure gross lesions were not reported. However, some organ weights were affected; liver (increased at 300 and 1000 mg/kg day), thymus (decreased at 1000 mg/kg day) kidneys (increased at 300 and 1000 mg/kg day and spleen (1000 mg/kg day). The histopathological evaluation was done only for kidneys and osmonephrotic effects consisting of tubular vacuolization and accompanying epithelial hypertrophy were reported at doses 300 and 1000 mg/kg day. Also hyaline inclusions and tubular basophilia were increased in 1000 mg/kg day group.

The NOAEL was defined at 100 mg/kg bw per day.

Ref.: 11

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

Guideline:	OECD 408
Species/strain:	Wistar
Group size:	20 animals per dosage
Test substance:	2,6-Dihydroxy-3,4-dimethylpyridine (DDP)
Batch:	0120081229
Purity:	>98 %
Dose:	0, 100, 200 and 400 mg/kg day
Route:	Oral
Exposure:	91 days in 1.0 % aqueous carboxymethylcellulose and 0.5% Tween 80
GLP:	in compliance

The subchronic toxicity of DDP was investigated in a 91 days by gavage toxicity study in Wistar rats where DDP was given at 0, 100, 200 or 400 mg/kg/day. There were no treatment related unscheduled deaths in the study.

Mortality, signs of intoxication, body weight, functional observations and the food consumption data was collected. Animals in the recovery groups were examined after four weeks of exposure.

Food intake was not affected and the body weight development was similar in controls and the exposed groups. Water intake was increased in high dose males and in mid (200 mg/kg day) and high (400 mg/kg day) dose groups of females.

Hypersalivation was observed in high dose animals, but no further evidence of neurotoxic potential of DDP was reported.

The clinical chemistry indicated at the treatment end a slight anaemia by changes in red blood cell parameters. The levels of red blood cells, haemoglobin concentration, haematocrit values were lower than in the control group and the mean corpuscular volume, mean corpuscular haemoglobin and red blood cell volume were increased in mid and high exposure groups. The signs of anaemia stayed to the end of the recovery period in the highest exposure group.

At the treatment end, increased absolute/relative mean liver and kidney weights were recorded for mid and high exposure groups. In high exposure group absolute/relative weights of spleen was elevated in high exposure group. These observations were not detected after the 4-week recovery period.

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The following histopathological findings were reported in the highest exposure group; an increased extramedullary hemopoiesis in spleen, increased erythropoiesis in bone marrow, increased mean grade and incidence of vacuolization in the adrenal cortices and follicular hypertrophy in the thyroids.

The NOAEL was defined at 100 mg/kg bw per day, based on liver and kidney weight.

Ref.: 12

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity/Genotoxicity *in vitro*

Guideline: OECD 471 (1997)

Species/strain: *Salmonella typhimurium*, TA 98, TA 100, TA 102, TA 1535, and TA 1537

Replicates: Triplicates per concentration in two independent experiments both in the presence and absence of Aroclor 1254 induced rat liver S9

Assay conditions: Plate incorporation method and pre-incubation method

Test substance: A099

Batch: 01200081229

Purity: 99.5% (area %, HPLC)

Concentrations: Experiment I (strain TA 98, TA 100): 3; 10; 33; 100; 333; and 1000 µg/plate; Experiment I (all other strains): 1, 3; 10; 33; 100; 333; and 1000 µg/plate; Experiment II: 0.3; 1; 3; 10; 33; 100; and 333 µg/plate

Solvent: DMSO

GLP: in compliance

The study was performed to investigate the potential of A099 to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535, and TA 1537. The assay was performed in two independent experiments both with and without liver microsomal activation. The second experiment had to be repeated with strain TA 98, due to a very low spontaneous revertant number in the negative and solvent control. Each concentration, including the controls, was tested in triplicate.

Results

Reduced background growth was observed in experiment I at 100 µg/plate and above in strains TA 98, TA 100 with and without S9 mix, TA 1535, TA 1537 without S9 mix, and TA 102 with S9 mix) at 333 µg/plate and above reduced background growth was observed in strains TA 1535 and TA 1537 with S9 mix. In experiment II reduced background growth was observed at 333 µg/plate with and without S9 mix in all strains. Strong toxic effects, evident as a reduction in the number of revertants, were observed in all strains used. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with A099 at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

Conclusion

It can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or

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frameshifts in the genome of the strains used. Therefore, A 099 was considered to be non-mutagenic in the *Salmonella typhimurium* reverse mutation assay.

Ref.: 7

Mammalian Cell Gene Mutation Test in Mouse Lymphoma Cells (*tk locus*)

Guideline:	OECD 476
Species/strain:	Mouse lymphoma cell line L5178Y
Replicates:	Duplicate cultures in two independent experiments (single cultures for positive controls)
Metabolic act.:	Phenobarbital/β-naphthoflavone-induced rat liver S9
Test substance:	A099
Batch:	01200081229
Purity:	≥ 98%
Concentrations:	1.3, 2.5, 5, 10, 15, 20 µg/ml without S9 mix, 4 h; 2.5, 5, 10, 20, 40, 60, 80 with S9 mix, 4 h; 0.2, 0.3, 0.5, 1.3, 2.5, 5, and 10 µg/ml without S9 mix, 24h (lowest % is missing)
Treatment:	4 h in the absence and presence of S9
Solvent:	DMSO
GLP:	In compliance

The study was performed to investigate the potential of A 099 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y. The assay was performed in three independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The experimental part with metabolic activation was terminated prematurely due to insufficient cell growth. This part of the first experiment was repeated in experiment IA using identical concentrations. The data of the repeat experiment IA were included in the first experiment. The second experiment was solely performed in the absence of metabolic activation with a treatment period of 24 hours. The dose range of the main experiments was adjusted to toxicity data generated in a range finding pre-experiment.

Results

No substantial and reproducible dose dependent increase in mutant colony numbers was reportedly observed in the main experiments. No relevant shift of the ratio of small versus large colonies was observed up to the maximal concentration of the test item. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were valid.

In the first experiment, culture II, with S9 mix, 40 µg/ml A 099 resulted in a 3.0-fold increase in mutant colonies and a 3.5-fold increase in small mutant colonies, in comparison to solvent controls. This effect was at the same level as observed for 3.0 µg/ml of cyclophosphamide, the positive control agent. However, as cell survival at 40 µg/ml A 099 with S9 mix was below the set 10% threshold. These data were not considered. A slight (1.9-fold) increase in mutant colonies and small mutant colonies was observed at 20 µg/ml A 099 with S9 mix also in culture I of experiment I, but this effect was not considered significant.

In the second experiment (24 h), culture II, the number of mutant colonies/ 10^6 cells exceeded the range of negative and solvent controls at 0.3 µg/ml and at 1.3 µg/ml (both cultures); 2.5 µg/ml A 099 was toxic. However, the increase in the mutation frequency (1.9-fold) did not quite reach the set threshold of twice the mutation frequency of the corresponding solvent control. Therefore, these increases were not considered as biologically relevant.

OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE

Conclusion

It was stated that, under the experimental conditions reported, the test item did not induce mutations in the mouse lymphoma thymidine kinase locus assay using cell line L5178Y in the absence and presence of metabolic activation. Therefore, A 099 was considered to be non-genotoxic (mutagenic/clastogenic) in the mouse lymphoma assay.

Ref.: 8

OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE***In Vitro* Micronucleus Test in cultured Human Lymphocytes**

Guideline:	OECD draft guideline 487
Species/strain:	Chinese hamster V79 cells
Metabolic act.:	Phenobarbital/β-naphthoflavone-induced rat liver S9
Test substance:	A 099
Batch:	01200081229
Purity:	98.5%
Concentrations:	1.3, 2.5, 5.0, and 10.0 µg/ml without S9 mix and 3.1, 6.3, 12.5, 25.0 µg/ml with S9 mix
Treatment:	4 hours (20 recovery)
Solvent:	Minimal essential medium
GLP:	In compliance

The test item A 099, dissolved in MEM (minimal essential medium), was assessed for its potential to induce micronuclei in Chinese hamster V79 cells *in vitro* in the absence and presence of metabolic activation by S9 mix. Exposure time was 4 h followed by a recovery time of 20 h. In each experimental group, two parallel cultures were set up. At least 1000 cells Per culture were scored for micronuclei. Dose selection was performed considering toxicity data from a pre-test. The following test item concentrations were applied in the main experiment: 1.3, 2.5, 5.0, and 10.0 µg/ml without S9 mix and 3.1, 6.3, 12.5, 25.0 µg/ml with S9 mix.

Results

In the absence and the presence of S9 mix, clear cytotoxicity, as measured by relative cell numbers and XTT activities (below 40 % of control level) were observed after treatment with the test item. In the presence as well as in the absence of metabolic activation, statistically significant and biologically relevant increases in the percentage of micronucleated cells were observed after treatment with the test item. Without S9 mix, the effect was dose-dependent and the level of micronucleated cells was increased by 24-fold (-S9 mix, 10 µg/ml) in comparison with vehicle control. Already the lowest concentration (1.3-12.5 µg/ml) tested produced a 3.7-fold increase. With S9 mix, the highest response (7.6-fold) was obtained at 12.5 µg/ml, and also the highest dose assessed (25 µg/ml) gave a statistically significant increase in micronucleated cells. Cyclophosphamide (+S9 mix) and colcemid (-S9 mix) were used as positive controls and showed a clear response as well.

Conclusion

It could be stated that under the experimental conditions reported, the test item A 099 induced micronuclei in Chinese hamster V79 cells *in vitro* in the absence and the presence of metabolic activation. Therefore, A 099 was considered to be genotoxic in this *in vitro* test system when tested up to cytotoxic test item concentrations.

The positive effect was very clear and was obtained with a relatively low concentration of the test item.

Ref.: 9

3.3.6.2 Mutagenicity/Genotoxicity <i>in vivo</i>
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Mouse bone marrow micronucleus test

Guideline:	OECD 474 (1997)
Species/strain:	NMRI mice
Group size:	Six/sex/dose level
Test substance:	A 099
Batch:	01200081229
Purity:	99.5% (Area %, HPLC)

OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYL PYRIDINE

Dose level: 75, 150, and 300 mg/kg bw (24 h) and 300 mg/kg bw (48 h); administered as a single dose
 Route: Intraperitoneal
 Vehicle: 20% aqueous ethanol
 Sacrifice times: 24 and 48 h after dosing.
 GLP: In compliance

This study was performed to investigate the potential of A 099 to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the mouse. The test item was dissolved in aqueous ethanol (20%), which was also used as vehicle control. The volume administered intraperitoneally (ip) to 12 mice was 10 ml/kg bw. 24 and 48 h after a single administration of the test item, bone marrow cells were collected for micronuclei analysis. One male and one female of the 300 mg/kg bw group died. Ten animals (5 males, 5 females) per test group were evaluated for the occurrence of micronuclei (at least 2000 polychromatic erythrocytes (PCEs) per animal scored). Cytotoxicity of the treatment was assessed by the ratio of polychromatic erythrocytes and total (reported as the number of PCEs per 2000 erythrocytes). The following dose levels of the test item were investigated: 75, 150, and 300 mg/kg bw for the 24 h preparation interval, and 300 mg/kg bw for the 48 h preparation interval.

Results

According to pre-experiments, 300 mg A 099 per kg bw was estimated to be the highest applicable dose without significant effects on survival rates, but with clear signs of toxicity. In the main experiment, one male and one female animal (out of 64 and 47, respectively) died after treatment with this dose. After treatment with the test item, the number of PCEs was not relevantly decreased as compared to the mean value of PCEs of the vehicle control, thus indicating that A 099 did not have cytotoxic effects on the bone marrow. In comparison with the corresponding vehicle controls, there was no biologically relevant or statistically significant enhancement in the frequency of micronucleated PCEs at any preparation interval after the administration of the test item and with any dose level used. Cyclophosphamide (40 mg/kg bw; administered ip.) was used as positive control and showed a substantial increase in micronuclei.

Conclusion

It could be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the mouse. Therefore, A 099 was considered to be negative in the micronucleus assay.

Ref.: 10

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity
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No data submitted

3.3.8.2. Teratogenicity

Guideline: OECD 414
 Species/strain: Wistar
 Group size: 88 animals
 Test substance: 2,6-Dihydroxy-3,4-dimethylpyridine (DDP)
 Batch: 0120081229

OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYL PYRIDINE

Purity: >98 %
 Dose: 0, 80, 160 and 480 mg/kg/day
 Route: Orally in 1.0 % aqueous carboxymethylcellulose and 0.5% Tween 80
 Exposure: female rats from day 6 to day 20 of pregnancy
 GLP: in compliance

DDP at dose levels of 0, 80, 160, 480 mg/kg bw were daily administered by gavage. Distilled water containing 1.0 % carboxymethylcellulose and 0.5% Tween 80 was used as an appropriate solvent and vehicle for DDP. Dosages were based on the data of the dose range finding study in which doses of 0, 100, 500, and 1000 mg/kg bw were used (ref. 13). Mortality and body weight gain were observed on a daily basis.

Dams were sacrificed on day 21 post-coitum and subjected to necropsy. The number of alive and dead foetuses, their distribution and site in the uterus, implantation and number of corpora lutea were determined. Also the weight of the foetuses, gravid uteri, uteri without foetuses, placenta and the sex of foetuses were recorded.

All dams were pregnant and no macroscopic findings were noted at necropsy. At 160 and 480 mg/kg bw day a dose dependent reduction in mean food consumption was recorded. On the same exposure levels the mean body weight was dose-dependently and statistically significantly reduced. The mean numbers of the corpora lutea and implantation sites were similar in all groups.

Exposure to DDP did not have an effect to the sex ratio and the mean foetal body weights were similar in all groups. No abnormalities related to treatment were noted in foetal external examinations. In addition, DDP related findings were not noted in visceral, skeletal and cartilage examinations.

Oral administration of DDP at the dose levels of 160 and 480 mg/kg bw caused reduced food consumption and reduced body weight gain in dams. DDP was not embryolethal, embryotoxic or teratogenic in any doses tested. Based on the maternal toxicity the NOAEL can be considered to be 80 mg/kg day.

The NOAEL for foetal toxicity was considered to be 480 mg/kg/bw, as no teratogenic potential of the test substance was observed in any dose group.

Ref.: 14

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

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No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(2,6-Dihydroxy-3,4-dimethylpyridine)
(Oxidative/permanent)

Maximum absorption through the skin A	=	1.43 µg/cm²
Skin Area surface SAS	=	700 cm²
Dermal absorption per treatment SAS x A x 0.001	=	1.001 mg
Typical body weight of human	=	60 kg
Systemic exposure dosage (SED) SAS x A x 0.001/60	=	0.017 mg/kg
No observed adverse effect level (mg/kg) NOAEL	=	80 mg/kg (maternal toxicity, oral, rat)

Margin of Safety	NOAEL / SED	=	4706
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3.3.14. Discussion

Physico-chemical properties

2,6-Dihydroxy-3,4-dimethylpyridine is used in oxidative hair dye formulations at a maximum final concentration of 1%, after mixing with hydrogen peroxide. The reported solubility data of 2,6-Dihydroxy-3,4-dimethylpyridine is inadequate. Experimental Log P_{ow}, and stability of 2,6-Dihydroxy-3,4-dimethylpyridine in marketed products are not reported. Impurities in 2,6-dihydroxy-3,4-dimethylpyridine, revealed by HPLC, have not been identified and quantified

General toxicity

LD50 was determined to be between 2500 and 5000 mg/kg bw. In 90 days oral toxicity study, NOAEL was set at 100 mg/kg/bw for the increased liver and kidney weights. NOAEL for maternal toxicity was 80 mg/kg/bw, and the NOAEL of foetal toxicity was considered to be 480 mg/kg bw.

Irritation / sensitisation

2,6-Dihydroxy-3,4-dimethylpyridine is not an irritant to rabbit skin. It is an irritant to rabbit eye.

2,6-Dihydroxy-3,4-dimethylpyridine is considered to be a possible skin sensitisier.

Dermal absorption

The maximum amount of 2,6-Dihydroxy-3,4-dimethylpyridine absorbed into the skin (Epidermis + dermis + receptor fluid) in the presence of developer containing H₂O₂, i.e. 1.43 µg/cm² should be used for the calculation of the margin of safety. Solubility and stability of 2,6-Dihydroxy-3,4-dimethylpyridine in PBS is not reported. According to GLP declaration, "the stability and achieved concentration of the test formulation in the vehicle used were not determined by analysis.

Mutagenicity

2,6-Dihydroxy-3,4-dimethylpyridine was not mutagenic in the *Salmonella typhimurium* reverse mutation assay. It did not induce mutations in the mouse lymphoma thymidine kinase locus assay using cell line L5178Y in the absence and presence of metabolic activation, and therefore it was considered to be non-genotoxic (mutagenic/clastogenic) in the mouse lymphoma assay. 2,6-Dihydroxy-3,4-dimethylpyridine induced micronuclei in

OPINION ON 2,6-DIHYDROXY-3,4-DIMETHYLPYRIDINE

Chinese hamster V79 cells *in vitro* in the absence and the presence of metabolic activation. It was considered to be genotoxic in this *in vitro* test system when tested up to cytotoxic test item concentrations. The positive effect was clear and was obtained with a relatively low concentration of the test item. 2,6-Dihydroxy-3,4-dimethylpyridine did not induce micronuclei following application of toxic doses as determined by the *in vivo* micronucleus test with bone marrow cells of the mouse. Therefore, it was considered to be negative in the micronucleus assay. As the *in vitro* genotoxic/mutagenic effects were not expressed *in vivo*, 2,6-Dihydroxy-3,4-dimethylpyridine may be considered as non-mutagenic/non-genotoxic *in vivo*.

Carcinogenicity

No data submitted

4. CONCLUSION

Based on the information provided, the SCCP is of the opinion that the use of 2,6-Dihydroxy-3,4-dimethylpyridine itself as an oxidative hair dye substance at a maximum concentration of 1.0% in the finished cosmetic product (after mixing with hydrogen peroxide) does not pose a risk to the health of the consumer, apart from its sensitising potential.

2,6-Dihydroxy-3,4-dimethylpyridine itself is not mutagenic *in vivo*.

However, Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

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