



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**

**2,6-Dimethoxy-3,5-pyridinediamine HCl**

COLIPA N° A101

Adopted by the SCCP during the 5<sup>th</sup> plenary meeting  
of 20 September 2005

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Opinion on 2,6-Dimethoxy-3,5-pyridinediamine HCl

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**1. BACKGROUND**

Submission I on 2,6-Dimethoxy-3,5-pyridinediamine dihydrochloride was submitted to the Scientific Committee on Cosmetics (SCC) in May 1993, followed by Submission II in December 1994. Submission III was submitted, in January 1999, to Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) which adopted an opinion on 23 June 1999.

The above mentioned substance is regulated under reference number 25 of Annex III, Part 2 (List of substances provisionally allowed) of the Cosmetics Directive 76/768/EEC.

Submission IV 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://pharmacos.eudra.org/F3/cosmetic/doc/HairDyeStrategyInternet.pdf>) within the framework of the Cosmetics Directive 76/768/EEC.

**2. TERMS OF REFERENCE**

1. *Is 2,6-Dimethoxy-3,5-pyridinediamine dihydrochloride safe for use in hair dye formulations taken into account the data provided ?*
2. *Does the Scientific Committee on Consumer Products (SCCP) recommend any restrictions with regard to the use of 2,6-Dimethoxy-3,5-pyridinediamine dihydrochloride in 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-Dimethoxy-3,5-pyridinediamine HCl

**3.1.1.2. Chemical names**

3,5-Pyridinediamine, 2,6-dimethoxy-, dihydrochloride (CA INDEX NAME, 9CI)  
2,6-Dimethoxy-3,5-pyridinediamine dihydrochloride (IUPAC)

**3.1.1.3. Trade names and abbreviations**

Pyridinblau

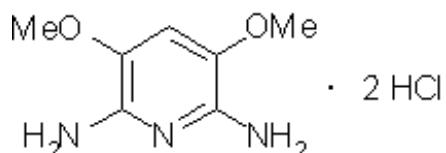
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## 3.1.1.4. CAS / EINECS number

CAS: 56216-28-5

EINECS: 260-062-1

## 3.1.1.5. Structural formula



## 3.1.1.6. Empirical formula

Formula: C<sub>7</sub>H<sub>11</sub>N<sub>3</sub>O<sub>2</sub>.2HCl

## 3.1.2. Physical form

Powder

Batch 91061020: Crystalline, black coloured solid

Batch LGH240383/1: Brown coloured solid (*information provided together with analytical results*)

## 3.1.3. Molecular weight

Molecular weight: 242.11

## 3.1.4. Purity, composition and substance codes

## Purity and impurities

Chemical	Content	
	Batch No. 91060120 (R96007367)	LGH240383/1
2,6-dimethoxy-3,5-pyridinediamine (freebase)	By NMR: 60.8 % (w/w) By HPLC at 210 nm: 93.1 area% 254 nm: 86.3 area% 315 nm: 98.1 area%	By NMR: 60.8 % (w/w) By HPLC at 210 nm: 97.9 area% 254 nm: 94.4 area% 315 nm: 99.5 area% Free base determined by HPLC: 62.7 % (w/w)
2,6-dimethoxy-3-nitropyridine	12 ppm	Not detected, LOD 5 ppm
2,5-dimethoxypyridine	Not detected, LOD* 29 ppm	Not detected, LOD 29 ppm
3-amino-2,6-methoxypyridine	Not detected, LOD 95 ppm	Not detected, LOD 95 ppm
3,5-diamino-2-methoxypyridine	Not detected, LOD 11 ppm	Not detected, LOD 11 ppm
2,6-dihydroxypyridine	Not detected, LOD 11 ppm	Not detected, LOD 11 ppm
Chloride	28.4 % (w/w)	28.8 % (w/w)
Water	0.5 % (w/w)	0.2 % (w/w)

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<b>Chemical</b>	<b>Content</b>	
	<b>Batch No. 91060120 (R96007367)</b>	<b>LGH240383/1</b>
Loss on drying	0.23 % (w/w)	0.02 % (w/w)
Residue on ignition	0.02 % (w/w)	0.07 % (w/w)
<b>Mass balance</b>	<b>Approximately 91%</b>	<b>Approximately 94 %</b>

\*LOD: Limit of detection.

It is further reported that:

- both samples are contaminated with an unknown ammonium compound and another indefinite component;
- HPLC analysis of batch 91060120 revealed multiple unidentified signals (HPLC-peaks) close to dead volume of the HPLC system. These signals were significantly less for the other batch analysed;
- a small amount of ethanol was present in the batchLGH240383/1.

It was concluded that the two batches of 2,6-Dimethoxy-3,5-pyridinediamine dihydrochloride were comparable but not identical.

### Deduced Raw material specification by the applicant

With the above mentioned information (see 3.1.4), following specification for the raw material of 2,6-Dimethoxy-3,5-Pyridinediamine HCl was established:

HPLC quantitative (free base): > 60 weight %

2,6-Dimethoxy-3-nitropyridine\*: < 100 ppm

Chloride: > 25%

Solvent content: < 1%

Ash: < 1 %

\*The limit for each impurity is based on an internal raw material contaminant risk assessment following a literature search for toxicity data. The limit represents the maximum acceptable amount of the respective contaminant in hair dye formulations that can be considered as not to pose an unacceptable risk to human health.

#### 3.1.5. Impurities / accompanying contaminants

See 3.1.4

#### 3.1.6. Solubility

Water solubility: not detectable as substance was unstable under test conditions (20°C, pH 0.30) (EU - A.6) (Reference: 10 / OP 88575)

Water solubility (QSAR): 1.0 exp.+6 mg/l (25°C), WSKOWWIN v.1.41

Water: > 10 % (w/w) (pH 1.3)

Acetone/water 1:1: 10 % (w/w) (pH 1)

DMSO: degradation

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Ethanol: 5.1 % (w/w)

*All water solubility data was presented in the same dossier (Submission IV)! pH values are different than those given 3.1.9 and in 3.18*

**3.1.7. Partition coefficient (Log P<sub>ow</sub>)**

Log P<sub>ow</sub> : 0.91 at pH 7.51 (room temperature) (EU - A.8)

**3.1.8. Additional physical and chemical specifications**

Organoleptic properties: /

Melting point: not detectable, decomposition at 170°C

Boiling point: not applicable

Flash point: Self ignition temperature 379°C

Vapour pressure: 3.3 exp-7 hPa (20°C)

Density: 1.361 (20°C)

Viscosity: /

pKa: 3.59 and 1.05 (calculated, Pallas Software)

Refractive index: /

pH-value: 1.8 (5% aqueous solution)

**3.1.9 Stability**

- Approximately 10% (w/v) in DMSO: complete degradation within 7 days at room temperature (t= 0h: 100.0%; 6h: 84.2%; 2d: 41.5%; 7d: 0%)
- 10% (w/v, pH 3) in acetone/water 1:1: linear degradation to 84% within 7 days at room temperature (t = 0h: 100.0%; 6h: 100.6%; 2d: 85.3%; 7d: 83.7%)
- 10% (w/v, pH 3) in water: degradation to approx. 90% within 2 days (t = 0h: 100.0%; 6h: 101.3%; 2d: 90.7%; 7d: 91.4%)
- Stable in formulation for 2 months at 20°C (t = 0h: 0.465%; 2 months: 0.456)

**General comments to Chemical and physical specifications**

- Analysis of the two batches of 2,6-Dimethoxy-3,5-pyridinediamine HCl has revealed that both of these contained 6-9% unknown impurities.
- Degradation products of the test substance are not reported

**3.2. Function and uses**

2,6-Dimethoxy-3,5-pyridinediamine HCl is used as an oxidative hair colouring agent (precursor). The intended maximum on-head concentration is 0.25 %.

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**3.3. Toxicological Evaluation****3.3.1. Acute toxicity****3.3.1.1. Acute oral toxicity****Acute oral toxicity study in rats and mice**

Guideline: /  
Species/strain: Wistar rats, CF1 mice  
Group size: Rats: 6 per sex and dose  
Mice: 10 females  
Test substance: 2,6-Dimethoxy-3,5-pyridinediamine HCl  
Batch No.: Not indicated  
Purity: Not indicated  
Doses: 100, 175, 250 and 325 mg/kg bw  
Observation period: 14 days  
GLP: /

A 2.5 and 5% solution of 3,5-diamino-2,6-dimethoxypyridine, dihydrochloride in aqua dest. were administered once via stomach tube to CF1 mice (10 females) and Wistar rats (6/sex) at 4 concentrations. During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals.

**Results**

The test substance caused reduced activity. Based on the observed mortality rates the following LD<sub>50</sub>-values were calculated:

LD<sub>50</sub> rat female: 212.5 mg/kg bw  
LD<sub>50</sub> rat male: 187.5 mg/kg bw  
LD<sub>50</sub> mouse female: 212.5 mg/kg bw

Ref.: 14

**3.3.1.2. Acute dermal toxicity**

No data submitted

**3.3.1.3. Acute inhalation toxicity**

No data submitted

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## 3.3.2. Irritation and corrosivity

## 3.3.2.1. Skin irritation

**Primary skin irritation in guinea pigs**

Guideline: /  
 Species/strain: White guinea pigs, strain Pirbright (SPF)  
 Group size: 6 females  
 Test substance: 2,6-dimethoxy-3,5-pyridinediamine HCL, (3 % aqueous dilution)  
 Batch: /  
 Purity: /  
 Application: Repeated open application on the clipped flank region 3 times daily for two consecutive days, exposure time 20 min each  
 GLP: Not in compliance

A 3 % aqueous dilution of 2,6-dimethoxy-3,5-pyridinediamine HCL was applied onto the clipped flank region of 6 albino guinea pigs, three-times daily for 2 consecutive days. The skin was not covered, but animals were fixed in order to avoid contact with the treated area (3 x 4 cm) following application. The skin was evaluated according to the Draize-scheme for erythema and oedema after each application and once daily for three days after the last application.

**Results**

No skin reactions at all were observed at any observation time point. In addition, no clinical signs or unusual behaviour of the treated animals was noted. Consequently, a 3 % dilution of 2,6-dimethoxy-3,5-pyridinediamine HCL revealed no indication of irritant properties to the guinea pig even after repeated application.

Ref.: 15

**Primary skin irritation in rabbits**

Guideline: /  
 Species/strain: Female Albino rabbit, strain New Zealand  
 Group size: 3 animals  
 Test substance: 2,6-dimethoxy-3,5-pyridinediamine HCL  
 Batch: /  
 Purity: /  
 Application: 0.5 g (moistened with saline solution, pH 4-5) applied for 4 h  
 GLP: Not in compliance

0.5 g 2,6-dimethoxy-3,5-pyridinediamine HCL moistened with saline solution was applied onto the back skin (area about 6.25 cm<sup>2</sup>) of 3 female White New Zealand rabbits and kept in contact for 4 hours under occlusive conditions. The effects on the skin were evaluated and scored according the Draize scheme 1, 24, 48 and 72 h after application.

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**Results**

No general toxic effects were noted during the entire study period. No skin reactions were noted at any observation time point after application of the undiluted material under occlusive conditions following 4 hour contact to rabbit skin.

Ref.: 16

**Conclusions**

No indication of a skin irritant potential of 2,6-dimethoxy-3,5-pyridinediamine HCL tested at a 3 % aqueous solution in a repeated application assay in guinea pigs was noted. In an in vivo study in rabbits, undiluted 2,6-dimethoxy-3,5-pyridinediamine HCL did not cause any signs of skin irritation following contact for 4 hours under occlusive test conditions.

**3.3.2.2. Mucous membrane irritation**

Guideline: /

Species/strain: White guinea pigs, strain Pirbright (SPF)

Group size: 6 females

Test substance: 2,6-dimethoxy-3,5-pyridinediamine HCL; (3 % aqueous solution)

Batch: /

Purity: /

Application: 0.1 ml, permanent contact

GLP: Not in compliance

0.1 ml of a 3 % aqueous dilution of 2,6-dimethoxy-3,5-pyridinediamine HCL was applied into the conjunctival sac of the left eye of 6 female guinea pigs; the right eye served as control. The eyes were not rinsed and evaluated and scored according to the Draize scoring system ½, 1, 2, 3, 4, 6, and 7 h after application. A further reading by means of fluorescein-instillation took place at 24 h.

**Results**

No eye irritant effects were noted at any observation time point after instillation of 2,6-dimethoxy-3,5-pyridinediamine HCL as a 3 % aqueous dilution into the eyes of guinea pigs for permanent eye contact in any of the 6 tested animals. Thus, an irritation index of 0 was obtained in this study.

Based on the findings reported above, a 3 % aqueous dilution of 2,6-dimethoxy-3,5-pyridinediamine HCL is evaluated as not-irritating to guinea pig eyes under the test conditions.

Ref.: 17

**3.3.3. Skin sensitisation****Maximisation (Magnusson and Kligman) Test**

Guideline: OECD 406 (1992)

Species/strain: Female guinea pigs, strain Dunkin-Hartley

Group size: 20 animals for treatment and 10 for negative (vehicle) and positive control group, each

Test substance: 2,6-dimethoxy-3,5-pyridinediamine HCL

Batch: 910600120

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Purity:	86.3 % (HPLC, 254 nm)
Concentrations:	Intradermal induction: 1 % test substance in distilled water and in FCA Dermal induction: 75 % in sterile water, occluded, pre-treatment with 10 % sodium lauryl sulfate in petrolatum Challenge: 75 % test substance in distilled water, occluded
GLP:	In compliance

The dermal sensitisation potential was evaluated by the Magnusson-Kligman Maximisation method in guinea pigs, strain Dunkin-Hartley.

An intra-dermal and a topical range-finding study were conducted. Two animals were treated intra-dermally at six sites with concentrations ranging from 1 to 50 % of the test item in sterile water and skin reactions were evaluated according to the Draize scheme 5 days later. 5 animals were treated dermally under occlusive conditions at two flanks with concentrations ranging from 2 to 75 % of the test item in sterile water. Reading took place 24 and 48 hours after application.

In the main study, 20 animals were intradermally induced on day 1 with 0.1 ml of a 1 % dilution of 2,6-Dimethoxy-3,5-pyridinediamine HCL in sterile water. Freund's complete adjuvant was injected in parallel. Six days later the animals were topically pre-treated with 10 % sodium lauryl sulfate in petrolatum to cause a slight inflammation and enhance potential absorption. The next day (day 8), 75 % of 2,6-Dimethoxy-3,5-pyridinediamine HCL in sterile water (0.5 ml) was applied under occlusive conditions for 24 h. Animals were challenged on day 22 and day 29 (rechallenge) by applying about 0.2 ml of a 75% dilution of 2,6-Dimethoxy-3,5-pyridinediamine HCL in sterile water under occlusive patch conditions for 24 h.

Approximately 24 and 48 hours after the challenge/rechallenge phase, the test sites were evaluated for signs of elicited sensitisation. As the black staining of the treated sites may have obscured the evaluation, the skin fold thickness was determined in this study. The same procedures were carried out on the concurrent vehicle control group except that the solutions of the test article were replaced by sterile water (vehicle control). Data of the positive control Mercaptobenzothiazole (2% in Alembicol D and 50% in Acetone for intradermal and epidermal induction; 25% in Acetone for challenge and re-challenge) was reported to demonstrate the validity and sensitivity of the assay. The body weight of the animals was determined on days 1, 25 and 32.

## Results

### Range-finding study (intradermal application)

Moderate to severe erythema were noted at 1, 2 and 5 %. Necrosis was observed at the test concentrations of 10, 20 and 50 % in sterile water in both test animals.

Based on these findings, 1 % dilution of the test substance was used for the intradermal application in the main study.

### Range-finding study (topical application)

No effects were noted up to the highest test concentration of 75 % in sterile water. However, the evaluation of skin reactions was complicated due to the black staining of the skin.

Based on these results the highest test concentration (75 %) was used for both the epicutaneous induction and challenge. In addition, the skin was pre-treated with 10 % lauryl sulfate in petrolatum to cause skin irritation.

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**Main study**

Body weight development was not affected by the treatment.

Discrete or patchy erythema (score 1) were observed during the epidermal challenge for 3 animals in the treated group at the 48-hour reading only. No reactions were noted in the control group at the 24- and the 48-hour reading. However, the evaluation of skin effects was complicated due to the black staining at the application sites. Therefore, the skin fold thickness was also evaluated. A slight increase was noted at the substance treated sites as compared to the vehicle treated sites. The findings were considered equivocal and a rechallenge, also applying a 75 % dilution in sterile water, was assessed 1 week later.

At the rechallenge again, no reactions were noted in the vehicle-control group. However, in the treated group 11 (55 %) and 9 (45 %) animals revealed skin reactions at the 24 and 48 hour readings, respectively. The skin fold thickness revealed similar findings as noted for the first challenge.

**Conclusion**

2,6-dimethoxy-3,5-pyridinediamine HCL is evaluated to be a skin-sensitiser.

Ref.: 18

**Local Lymph Node Assay (LLNA)**

Guideline:	OECD 429 (2002); US National Institute of Health publications No. 99-4494 (1999)
Species/strain:	Mice CBA/J
Group size:	5 females
Test substance:	2,6-dimethoxy-3,5-pyridinediamine HCL
Batch:	91060120
Purity:	86.3 % (HPLC, 254 nm)
Concentrations:	0.5, 1.5, 5.0 and 10.0 % (w/v) in DMSO and in aqua/acetone (1:1) mixed with olive oil (4:1)
GLP:	In compliance

The skin sensitising potential of 2,6-dimethoxy-3,5-pyridinediamine HCL was investigated in CBA/J mice by measuring the cell proliferation in the draining lymph nodes after topical application on the ear.

25 µl of 0 (vehicles only), 0.5, 1.5, 5 and 10 % of 2,6-dimethoxy-3,5-pyridinediamine HCL in DMSO or in a mixture of aqua/acetone (1:1) with olive oil (4:1) (equal to the maximum solubility) were applied to the surface of the ear of five female CBA/J mice per group for three consecutive days. After application, the ears were dried by means of a hair dryer for about 5 minutes. As positive control, p-phenylenediamine (PPD) at 1 % in DMSO was investigated in parallel under identical test conditions.

Animals were checked for morbidity/mortality at least once daily. Observation for clinical signs was done daily before and at least once after dosing. Body weight was determined on day -1 and on day 5.

On day 5, mice received an intravenous injection of 250 µl phosphate buffered saline containing 24.0 µCi of [ $H^3$ ] methyl thymidine. Approximately five hours later, the mice were sacrificed by CO<sub>2</sub>-inhalation and the draining auricular lymph nodes were removed and weighed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the

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radioactivity was determined (incorporation of [ $\text{H}^3$ ] 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

No abnormal clinical signs or mortality were observed throughout the study period. Body weight development was not affected by the treatment.

The mean stimulation indices were affected in a dose-dependent manner by the treatment with 2,6-dimethoxy-3,5-pyridinediamine HCL. With the test item in DMSO, mean stimulation indices of 1.2, 3.6, 4.5 and 4.2 were obtained for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively. An EC<sub>3</sub> value (equal to the concentration inducing a stimulation index of 3) of 1.25 % was calculated.

In the second vehicle (aqua/acetone/olive oil), the indices were 1.2, 1.3, 2.7 and 3.5 for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively. Thus, only at the highest test concentration the trigger value of 3 was just exceeded. An EC<sub>3</sub> value of 6.88 % was calculated from these findings.

The responses noted in both groups are considered positive and indicate a skin sensitising potential of 2,6-dimethoxy-3,5-pyridinediamine HCL.

The positive control (PPD, 1 % in DMSO) caused a stimulation index of 10 which demonstrated the sensitivity of the test system used.

## Conclusion

2,6-dimethoxy-3,5-pyridinediamine HCL induced a biologically relevant immune response in local lymph nodes after dermal application to the mouse ear with both vehicles tested. EC<sub>3</sub> values of 1.25 % and 6.88 % were calculated for DMSO and aqua/acetone/olive oil, respectively. The concurrent positive control demonstrated the sensitivity of the assay.

Based on these findings 2,6-dimethoxy-3,5-pyridinediamine HCL is evaluated to be a skin-sensitiser under the described test conditions.

Ref.: 20

### 3.3.4. Dermal / percutaneous absorption

#### 3.3.4.1. Percutaneous absorption *in vitro*

Not available.

Ref.: 21

#### 3.3.4.2. Percutaneous absorption *in vivo*

Guideline: /

Species/strain: Sprague Dawley rats Him:OFA, SPF

Group size: 3 animals per sex and treatment group

Test substance:  $^{14}\text{C}$ -2,6-dimethoxy-3,5-pyridinediamine HCL (ring-labelled)

Radioactive purity: > 97 %, specific activity: 49.1  $\mu\text{Ci}/\text{mg}$

Batch: /

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Doses:	Solution in water: 3.3%, 0.304 g/animal; 1.13 mg/cm <sup>2</sup> . Commercial formulation without peroxide: 1.0 %, 1 g/animal, 1.11 mg/cm <sup>2</sup> ; with peroxide: 0.5 % 1 g/animal, 0.55 mg/cm <sup>2</sup>
Treatment period:	Single cutaneous application in water and as part of a formulation with and without hydrogen peroxide (0.5 h contact); total study period 72 h under occlusive conditions
GLP:	In compliance

<sup>14</sup>C-2,6-dimethoxy-3,5-pyridinediamine HCL was applied dermally to groups of three male and three female Sprague Dawley, Him:OFA (SPF) rats (body weight about 205 g at the day of application). The application area was 9 cm<sup>2</sup> and the test substance was applied at concentrations of 3.33 % in water and of 1 % without and 0.5% with hydrogen peroxide, respectively, in a commercial formulation (30 min contact time). The mean dosages of the dyestuff applied were 1.13 mg/cm<sup>2</sup>, 1.11 mg/cm<sup>2</sup>, and 0.55 mg/cm<sup>2</sup>, respectively. Application was done under anaesthesia.

Thereafter, the test substance was scraped off (formulation only) and the skin was rinsed with a 3 % shampoo formulation (about 100 ml) and warm water. After rinsing, the area was covered with gauze and by an air permeable plastic truncated cone to further prevent licking of the treated area during the 72 h in the metabolism cages.

Urine and faeces were collected daily (0-24, 24-48 and 48-72 h after administration) from the metabolic cages.

Animals were killed 72 hours after the application and the application sites, blood and numerous organs were taken and analysed for radioactivity. The radioactivity in the remaining carcass was also determined after complete removal of the skin.

## Results

Total recovery of the applied radioactivity was good with recovery rates of 97.9 to 100 % for all test groups.

The majority of the applied dose (96.3 to 99 % of the applied amount) was recovered in the dressings and the washing solutions. The amount of radioactivity remaining at the application site (skin) for the water solution represented 0.98 % and 1.47 % of the applied dose for males and females, respectively. The respective figures for formulations with and without hydrogen peroxide were 1.02 % and 0.66 % and 0.93 % and 0.32 % for males and females, respectively.

Absorbed radioactivity was mainly excreted via urine for the commercial formulations with (0.12 % males, 0.12 % females) and without hydrogen peroxide (0.42 % males, 0.19 % females) as well as for the water solution (0.51 % males, 0.22 % females). The elimination was fast, since 90 – 95 % of the total amount eliminated via urine was excreted within the first 24 hours. Excretion via faeces was of less importance representing 17 to 22 % of the absorbed dose. The figures for the formulation with hydrogen peroxide were 0.033 % for males and 0.021 % for females. For the groups with peroxide and the water solution the respective figures were 0.094 % for males and 0.029 % for females as well as 0.163 % for males and 0.041 % for females.

The remaining amount of <sup>14</sup>C detected in the carcass 3 days after application was low, representing 0.009 %, 0.01 % and 0.014 % of the applied dose for the formulations without and with hydrogen peroxide and the water solution, respectively. The concentrations in the organs were correspondingly low with comparable residue levels and organ distribution. Furthermore, no relevant differences were noted between males and females.

The mean amounts found in urine, faeces, residual carcass and for a worst case assumption the total skin including the application site were considered as bioavailable. This calculation results

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in a cutaneous absorption of 0.997 % equal to 5.4 µg/cm<sup>2</sup> for a commercial formulation with hydrogen peroxide applied under typical use conditions. The respective figures for a formulation without hydrogen peroxide was 1 % equal to 11.13 µg/cm<sup>2</sup> and is 1.71 % equal to 19.14 µg/cm<sup>2</sup> for the water solution, respectively.

### Conclusion

When applied dermally to rats in a commercial formulation in the presence of hydrogen peroxide 0.158 % of <sup>14</sup>C-2,6-dimethoxy-3,5-pyridinediamine HCL was absorbed if the content in urine, faeces and carcass without skin is considered. Excretion takes place predominantly via urine. Excretion via urine is fast, as 90 to 95 % excreted within the first 24 hours. Low tissue residue levels were noted. When applied in a formulation without hydrogen peroxide, dermal absorption was increased to 0.37 %. As no data are available from an *in vitro* study with pig skin, the results obtained in the rat study *in vivo* will be used for risk assessment.

Ref.: 25

#### 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
Species/strain:	Wistar rats, HanIbm: WIST(SPF)
Group size:	10 animals per sex and dose, no recovery group
Test Substance:	2,6-Dimethoxy-3,5-pyridinediamine HCl in de-ionised water
Batch No.:	LGH 240383/1
Dose level:	0, 5, 15 and 35 mg/kg bw/day
Route:	Oral, gavage
Exposure period:	13 weeks
GLP:	in compliance

2,6-Dimethoxy-3,5-pyridinediamine HCl was administered, by gavage, once daily to 4 groups Sprague Dawley CFY rats (10/sex) for 90 days. The test substance was administered at dosage levels of 5, 15 or 35 mg/kg bw. The control group received the vehicle (distilled water) only. All animals were sacrificed at the end of the study. All animals were observed daily for mortality, clinical signs and water consumption. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic examination was performed. Blood samples were withdrawn from 5 males and 5 females of each test group for haematological and clinical chemistry investigations, during week 12. Organ weights were measured and macroscopy and histopathology was performed, on all animals.

### Results

6 animals died during the study (1 control and 5 high dosed animals), due to accidentally maldosing. In 2 of 5 high dosed mortalities, deaths were considered to be treatment-related (marked body weight loss; day 51 and 79). A dose-related decrease in body weight gain and food consumption was observed in the mid and high dose group (significant decreased in the high

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dose groups). Reduced pupillary reflex was noted in 2 high dose females and 1 female of the same group showed a permanent mydriasis.

In the mid and high dose groups, significantly decreased blood glucose and creatinine levels were observed. Significantly decreased absolute liver and kidney weights were observed in the high dose males and mid and high dose females. Enlarged cervical lymph nodes were seen in 6 mid and 3 high dose animals. Treatment-related changes in the liver (necrosis, mononuclear cell foci), lungs (pneumonitis) and oesophagus (epithelial hyperplasia/hyperkeratosis) were seen in the mid and high dose animals. The dose level without adverse effects was 5 mg/kg bw.

**Remark**

The test substance has limited stability in water.

Ref.: 26

3.3.5.3. Chronic (> 12 months) toxicity
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No data

3.3.6. Mutagenicity / Genotoxicity
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3.3.6.1. Mutagenicity / Genotoxicity <i>in vitro</i>
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**Bacterial gene mutation assay**

Guideline: OECD 471  
 Species/strain: *Salmonella typhimurium*, TA98, TA100, TA102, TA1535, TA1537  
 Replicates: Three independent tests (one with TA1537 only)  
 Test substance: 2,6-Dimethoxy-3,5-pyridinediamine HCl (A101)  
 Batch No.: 91060120  
 Purity: 86.3 area% (HPLC: 254 nm) (according to submission)  
           94.1 area% (HPLC: 240 nm) (according to test report)  
           93.1 area% (HPLC: 340 nm) (according to test report)  
 Concentrations: 3.0 - 2500 µg/plate without metabolic activation  
                   10.0 - 5000 µg/plate with metabolic activation  
 GLP: Quality Assurance Statement included

2,6-Dimethoxy-3,5-Pyridinediamine HCl (A 101) has been investigated for the induction of gene mutations in *Salmonella typhimurium*. Liver S9 fraction from rats induced with phenobarbital/β-naphthoflavone was used as the exogenous metabolic activation system. The concentration range was based on pre-experiments with all tester strains. Negative and positive controls were in accordance with the OECD guideline.

**Results**

Precipitation of the test substance was observed at 2500 µg/plate and above in the absence and presence of S9-mix in experiment I, and in the presence of S9-mix in experiment II. 2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) did not induce gene mutations in this bacterial gene mutation test. An isolated increase in the mutation frequency in TA1537 in the first experiment (333 and 100 µg/plate in the presence of S-9 mix) was not reproducible in an independent repeat experiment and no mutagenic effect was seen in the pre-incubation assay.

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Therefore, the isolated positive effect does not have biological relevance. Some negative and positive controls exceeded the historical control range. However, these deviations do not compromise the test which can be considered as being appropriate. It can be concluded that 2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) is not mutagenic in the bacterial gene mutation assay under these test conditions.

Ref.: 27

A previous submission contained results from a bacterial gene mutation test performed with only three *Salmonella typhimurium* strains (TA97, TA98, TA100). A101 was tested in the absence and presence of S9-mix at concentrations up to 3000 µg/plate. No mutagenic effect was measured under these test conditions.

***In vitro micronucleus test***

Guideline:	OECD 487 (draft)
Cells:	Peripheral human blood lymphocytes (blood pooled from two donors)
Replicates:	2 independent tests with and without S9 mix
Test substance:	2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101)
Batch No.:	91060120
Purity:	86.3 area% (HPLC, 254 nm) (according to submission) 96.3% (by NMR) (according to test report)
Concentrations:	<p>Experiment I: 49.27 – 98.30 µg/ml without metabolic activation; treatment for 24 h (24 h after stimulation)</p> <p>8.44 – 20.62 µg/ml without metabolic activation; treatment for 3 h (24 h after stimulation)</p> <p>Experiment II: 45.33 – 85.29 µg/ml without metabolic activation; treatment for 24 h (48 h after stimulation)</p> <p>25.17 – 49.15 µg/ml with metabolic activation; treatment for 3 h (48 h after stimulation)</p>
GLP:	Quality Assurance Statement included

2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) has been investigated for induction of micronuclei in human lymphocytes *in vitro*. Duplicate cultures were prepared from pooled blood of two healthy male donors. The Cytochalasin B modification of the test was used and micronuclei were scored in binucleated cells. Liver S9 fraction from Aroclor1254-induced rats was used as the exogenous metabolic activation system. Reduction in the replication index (RI) was taken as a measure for cytotoxicity. Negative and positive controls were in accordance with the OECD guideline.

**Results**

2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) did not induce micronuclei in experiment I in the absence of S9-mix (treatment 24 h after stimulation). However, clearly increased micronucleus frequencies were measured in experiment II without metabolic activation (treatment 48 h after stimulation). There was no induction of micronuclei in both experiments in

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the presence of S9-mix. The difference between the two experiments in the absence of S9-mix cannot readily be explained and no attempts were made to further clarify these conflicting results. This test led to an equivocal result, but in its present state indicates that the test compound has a potential to induce micronuclei. The origin of the induced micronuclei (clastogenic vs. aneugenic events) has not been investigated.

Ref.: 29

***In vitro mammalian cell gene mutation test***

Guideline:	Not mentioned
Cells:	L5178Y mouse lymphoma cells (TK <sup>+/−</sup> )
Replicates:	one tests with and without S9 mix; not replicated
Test substance:	2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101)
Batch No.:	LGH 240383/1
Purity:	94.4 area% (HPLC, 254 nm) (according to the submission) 99.5% (recrystallized) (according to the test report)
Concentrations:	8 - 100 µg/ml without and with metabolic activation
GLP:	Quality Assurance Statement included

2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) has been investigated for induction of gene mutations at the TK-locus in L5178Y mouse lymphoma cells after exposure for 24 hours without and for 4 hours with metabolic activation. Liver S9 fraction from Aroclor1254-induced rats was used as the exogenous metabolic activation system. Test concentrations were based on the level of toxicity in pre-experiments. Appropriate negative and positive controls were included.

**Results**

In the main study, marked toxicity (reduced cell growth) was observed but colony forming was obviously not affected. Under these conditions no biologically relevant induction of mutant cells was measured in the absence or presence of S9-mix. In the absence of S9-mix, 100 µg/ml led to an increased mutant frequency. However, the increase was small (about 50%) and occurred under conditions of strong cytotoxicity (6% growth). The positive controls showed clearly increased mutant frequencies.

Under the experimental conditions used, 2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) was not mutagenic in mammalian cells (L5178Y mouse lymphoma cells) *in vitro*. However, the test performance was incomplete and does not allow a final statement.

Ref.: 28

A previous submission contained results from another TK<sup>+/−</sup> mammalian gene mutation assay (fluctuation test). A101 was tested in the absence and presence of S9-mix at concentrations up to 100 µg/ml and did not induce gene mutations under these conditions.

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

Guideline:	OECD 474
Species/strain:	Mouse, NMRI
Group size:	10 males analyzed

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Test substance:	2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101)
Batch No.:	91060120
Purity:	86.3 area% (HPLC, 254nm) (according to the submission) 94.1 area% (HPLC, 240 nm) (according to the test report)
Dose levels:	43.75, 87.5 and 175 mg/kg bw (single dose, oral by gavage)
Sacrifice time:	24 hours and 48 hours (high dose only) after the treatment
GLP:	Quality Assurance Statement included

2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) has been investigated for induction of micronuclei in the bone marrow cells of mice. Dose selection was based on results in pre-experiments for toxicity. Negative and positive controls were in accordance with the OECD guideline.

### Results

Systemic distribution and bio-availability was indicated by general symptoms of toxicity and discoloured urine. The number of PCEs per 2000 cells was not reduced in the treated animals, indicating that there was no clear cytotoxic effect on bone marrow cells. The mean MNPCE frequencies were not significantly increased in any of the groups treated with the test substance. The positive control substance gave the expected result.

The study was conducted appropriately. 2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) did not induce chromosome aberrations or damage to the mitotic apparatus in bone marrow cells of mice after oral treatment under the test conditions used.

Ref.: 30

A previous submission contained results from an in vivo bone marrow micronucleus test with mice. A101 was tested at doses of 15, 50 and 150 mg/kg bw. The highest dose was evaluated 24, 48 and 72 hours after administration, the other doses only after 24 hours. A101 did not induce micronuclei und these test conditions.

A101 did also not induce DNA damage leading to increased DNA repair (UDS) in an previously submitted in vivo UDS-test with rats. Animals were treated with 10, 30 or 100 mg/kg bw and analysed 24 hours thereafter.

3.3.7. Carcinogenicity
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No data

3.3.8. Reproductive toxicity
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3.3.8.1. Two generation reproduction toxicity
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No data submitted

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**3.3.8.2. Teratogenicity**

Guideline:	/
Species/strain:	Wistar rat, strain Crl:Wi/Br (SPF)
Group size:	24 pregnant females per dose group
Test substance:	2,6-Dimethoxy-3,5-pyridinediamine HCl in deionised water
Batch:	8020/85
Purity:	No information available
Dose levels:	10, 25 and 45 mg/kg bw/day
GLP:	In compliance

2,6-Dimethoxy-3,5-pyridinediamine HCl was administered, by gavage, to 4 groups of 24 pregnant SPF-Albino Wistar rats (Crl:Wi/Br). The test substance was daily administered at dosage levels of 10, 25 or 45 mg/kg bw from day 5 to 15 of gestation. The control group received the vehicle only. All mated females were sacrificed at day 20 of gestation. The animals were observed daily for clinical signs. Individual body weights were recorded at days 0, 5, 10, 15 and 20. Food consumption was measured for the day-intervals 0-5, 5-15, 15-20 and 0-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for visceral imperfections (organic defects).

No animal died during the study. During treatment all treated females had blue discoloured urine (due to the test substance). The high dose females showed slightly increased activity and exhibited rough haircoat. Food consumption and body weight gain were significantly reduced in the high dose group over the entire gestation period and at 10 and 25 mg/kg bw during treatment. No irreversible structural changes were found.

The NOAEL of embryo/foetotoxicity was 45 mg/kg bw, for maternal toxicity no NOAEL was determined since even at the lowest dose of 10 mg/kg bw food consumption and body weight gain was decreased.

**Remark**

The test substance has limited stability in water.

Ref.: 33

**3.3.9. Toxicokinetics**

No data

**3.3.10. Photo-induced toxicity****3.3.10.1. Phototoxicity / photoirritation and photosensitisation**

No data

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**3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity**

No data

**3.3.11. Human data**

No data

**3.3.12. Special investigations**

No data

**3.3.13. Safety evaluation (including calculation of the MoS)****CALCULATION OF THE MARGIN OF SAFETY**

(2,6-Dimethoxy-3,5-pyridinediamine HCl)  
(Oxidative/permanent)

The maximum concentration of 0.5 % of 2,6-Dimethoxy-3,5-pyridinediamine HCl is mixed before use with H<sub>2</sub>O<sub>2</sub>. Thus the usage volume of 100 ml contains at maximum 0.25 %.

Maximum amount of ingredient applied	I (mg)	=	250
Typical body weight of human		=	60 kg
Maximum absorption through the skin	A (%)	=	0.37
Dermal absorption per treatment	I x A	=	0.925 mg
Systemic exposure dose (SED)	I x A/60	=	0.015
No observed adverse effect level (mg/kg)	NOAEL	=	5 mg/kg

<b>Margin of Safety</b>	<b>NOAEL / SED =</b>	<b>330</b>
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**3.3.14. Discussion**

2,6-dimethoxy-3,5-pyridinediamine HCL is used as a precursor in oxidative hair dye formulations at a maximum on-head concentration of 0.25 %.

*Toxicity*

The NOAEL of embryo/foetotoxicity was 45 mg/kg bw, for maternal toxicity no NOAEL was determined since even at the lowest dose of 10 mg/kg bw food consumption and body weight gain was decreased.

In a 90 day-study the dose level without adverse effects was 5 mg/kg bw.

*Irritation*

No skin irritating properties were noted at test concentrations of 3 % in guinea pigs and with the undiluted product in rabbits. No eye irritant effects were noted in guinea pigs at 3 %.

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*Sensitisation*

2,6-Dimethoxy-3,5-pyridinediamine HCl was evaluated to be a skin-sensitiser under the described test conditions (Maximisation test and LLNA).

*Percutaneous absorption*

The skin penetration rate of 2,6-dimethoxy-3,5-pyridinediamine HCl in a representative commercial hair dye formulation was determined in rats *in vivo*. For the calculation of the systemic exposure dose (SED), a penetration rate of 0.37 % was considered.

*Mutagenicity*

2,6-Dimethoxy-3,5-pyridinediamine HCl (A 101) led to an equivocal result in the *in vitro* micronucleus test. The mammalian cell gene mutation test (mouse lymphoma assay) did not indicate mutagenicity *in vitro* but was not appropriately performed. The general strategy for mutagenicity testing requires that the mutagenic potential has to be clearly assessed *in vitro* before performing any *in vivo* study. The test substance did not induce damage to chromosomes or the mitotic apparatus in the *in vivo* micronucleus test, suggesting that a mutagenic potential *in vitro* (possibly indicated by the micronucleus test) is not expressed *in vivo*.

Some previously submitted genotoxicity tests which did not meet the requirements of actual OECD guidelines did not indicate a genotoxic/mutagenic potential of A101 *in vitro* and *in vivo*.

#### **4. CONCLUSION**

The SCCP is of the opinion that the use of 2,6-Dimethoxy-3,5-pyridinediamine HCl itself as an oxidative hair dye at a maximum concentration of 0.25% in the finished cosmetic product (after mixing with peroxide) does not pose a risk to the health of the consumer, apart from its sensitising potential.

However:

- data on the stability of 2,6-Dimethoxy-3,5-pyridinediamine HCl in typical hair dye formulations should be provided, and
- studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP opinions and in accordance with its Notes of Guidance.

#### **5. MINORITY OPINION**

Not applicable

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Prof. T. Platzek (Chairman and rapporteur)  
Dr. S.C. Rastogi  
Prof. T. Sanner  
Prof. G. Speit  
Dr. J. van Engelen  
Dr. I.R. White