EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

# OPINION ON

# 6-Methoxy-2-methylamino-3-aminopyridine HCl

COLIPA n° A130

Scientific Committees
on consumer products
on emerging and newly identified health risks
on health and environmental risks

The SCCP adopted this opinion at its 15th plenary of 15 April 2008

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.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

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 to be cited as: SCCP (Scientific Committee on Consumer Products), Opinion on 6-methoxy-2-methylamino-3-aminopyridine HCl, 15 April 2008

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## 1. BACKGROUND

Submission I and II for 6-methoxy-2-methyl-2,3-pyridinediamine were submitted by COLIPA[1] in September 1994 and in December 2001 respectively.

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) expressed its opinion n° SCCNFP/0643/03 at the 23rd plenary meeting of 18 March 2003 with the conclusion:

* *The NOAEL has to be defined based on a subacute or subchronic toxicity study according to current guidelines including investigations on haemato-toxicity and thyroid function.*
* *A study on percutaneous absorption according to the Notes of Guidance (SCCNFP/0321/00);*
* *data on the genotoxicity/mutagenicity following the SCCNFP-opinion "Proposal for a Strategy for Testing Hair Dye Cosmetic Ingredients for their Potential of Genotoxicity/Mutagenicity", doc. n° SCCNFP/0566/02 of 4 June 2002, and in accordance with the Notes of Guidance, regularly updated by the SCCNFP (doc. n° SCCNFP/0321/00).*

According to the current submission III, submitted by COLIPA in July 2005, 6-methoxy-2-methyl-2,3-pyridinediamine, its hydrochloride or its dihydrochloride are used as a precursor for hair colours. It reacts with primary intermediates to form the final dye. The reaction can be accelerated by addition of an oxidizing agent. The substance is used as an ingredient in hair dye formulations which may or may not contain a hydrogen peroxide based developer mix up to a final concentration on the scalp of 1.0% (calculated for the hydrochloride). Under intended conditions of use the exposure is terminated 30 minutes after application of the mixture to the hair by shampooing and thoroughly rinsing with water.

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 6-methoxy-2-methyl-2,3-pyridinediamine and its salts safe for use as an ingredient in any hair dye formulation at a concentration on-head of maximum 1.0% taking into account the scientific data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of 6-methoxy-2-methyl-2,3-pyridinediamine and its salts in hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

## 3. OPINION

This risk assessment relates only to 6-methoxy-2-methylamino-3-aminopyridine HCl. No data was submitted on other salts.

### 3.1. Chemical and Physical Specifications

#### 3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

6-Methoxy-2-methylamino-3-aminopyridine HCl (INCI)

3.1.1.2. Chemical names

2,3-Pyridinediamine, 6-methoxy-N2-methyl-
2,3-Pyridinediamine, 6-methoxy-N2-methyl-, dihydrochloride
2-Methylamino-3-amino-6-methoxypyridine dihydrochloride
3-Amino-2-methylamino-6-methoxypyridine dihydrochloride
3-Amino-6-methoxy-2-(methylamino)pyridine dihydrochloride
6-methoxy-N2-methylpyridine-2,3-diamine dihydrochloride

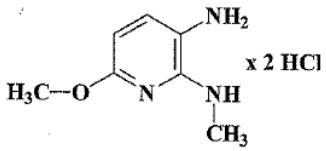
3.1.1.3. Trade names and abbreviations

HC Blue No. 7
Oxidinblau
Pyridinblau
Ro 730
3-AMMP
COLIPA A 130

3.1.1.4. CAS / EINECS number

| | | |
|---|---|---|
| CAS: | 90817-34-8 (HCl) | 83732-72-3 (dihydrochloride) |
| EINECS: | | 280-622-9 (dihydrochloride) |

3.1.1.5. Structural formula




3.1.1.6. Empirical formula

Formula: $C_7H_{11}N_3O$ . 2 HCl

#### 3.1.2. Physical form

Fine grey-violet powder

### 3.1.3. Molecular weight

Molecular weight: 153.18 (free base)
226.11 (dihydrochloride)

### 3.1.4. Purity, composition and substance codes

**Batch A2090110**

| | |
|---|---|
| Identification: | NMR, IR, UV/Vis |
| Purity: | 92% (w/w) by NMR |
| | 99.2% (HPLC peak area at 241 nm), 99.3% (HPLC peak area at 314 nm) |
| Chloride: | 31% (w/w) |
| Water: | 7.2% (w/w) |
| Sulphated ash: | 0.1% (w/w) |

**Batch R96003970**

| | |
|---|---|
| Identification: | UV/Vis (200-600 nm) |
| Purity: | 81% (w/w) using Batch A2090110 as standard |
| | 92.5% (HPLC peak area at 241 nm), 98.5% (HPLC peak area at 314 nm) |
| Chloride: | 28.4% (w/w) |
| Water: | 6.7% (w/w) |
| Sulphated ash: | 0.1% (w/w) |

### 3.1.5. Impurities / accompanying contaminants

**Batch A2090110**

15 impurities of approximately 0.01-0.16% (HPLC peak area) were found. The UV/Vis spectra (200-600 nm) of the impurities are provided, but they are not chemically characterised.
Heavy metal content: Pb < 20; Sb and Ni < 10; As and Cd < 5; Hg < 1 ppm

**Batch R96003970**

25 impurities of approximately 0.01-2.8% (HPLC peak area) were found. The UV/Vis spectra (200-600 nm) of the impurities are provided, but they are not chemically characterised.
Heavy metal content: Pb < 20; Sb and Ni < 10; As and Cd < 5; Hg < 1 ppm

### 3.1.6. Solubility

| | |
|---|---|
| Water: | > 100 g/l at room temperature |
| Ethanol: | 1 - 10 g/l at room temperature |
| DMSO: | > 100 g/l at room temperature |

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

Log $P_{ow}$: 0.7 (calculated Syracuse Vers. 1.66)

### 3.1.8. Additional physical and chemical specifications

| | |
|---|---|
| Melting point: | / |
| Boiling point: | decomposition at > 300 °C |
| Flash point: | / |
| Vapour pressure: | / |
| Density: | / |
| Viscosity: | / |
| pKa: | / |

| | |
|---|---|
| Refractive index: | / |
| pH: | / |
| UV_Vis spectrum | absorption at 241 nm and 314 nm ($\lambda_{max}$) |

### 3.1.9. Stability

The substance was stable for 2 hours at concentrations of 5 to 10 mg/ml in water (variation <10%).

**General Comments to physico-chemical characterisation**

* There are many unidentified impurities in concentrations up to 2.8% (HPLC peak area).
* Log $P_{ow}$: calculated values cannot be accepted as estimates of the true physical constant without justification.
* Stability of 6-methoxy-2-methylamino-3-aminopyridine HCl in solvents other than water and in the marketed products is not provided
* 6-Methoxy-2-methylamino-3-aminopyridine HCl is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

## 3.2. Function and uses

6-Methoxy-2-methylamino-3-aminopyridine and its hydrochloride is used as a precursor for hair colours. It reacts with primary intermediates to form the final dye-stuff. The reaction can be accelerated by addition of an oxidizing agent (e.g. hydrogen peroxide), but can also be achieved by air oxidation.

The final concentration of 6-methoxy-2-methylamino-3-aminopyridine HCl on head can be up to 1.0% (calculated for hydrochloride salt, corresponding to 0.68% calculated for the free base).

## 3.3. Toxicological Evaluation

### 3.3.1. Acute toxicity

#### 3.3.1.1. Acute oral toxicity

| | |
|---|---|
| Guideline: | / |
| Species/strain: | Rat Wistar, Mouse, CF1 |
| Group size: | Rat: 6 dose/sex, Mouse: 10 female per dose |
| Test substance: | 3-Amino-2-methylamino-6-methoxypyridine dihydrochloride |
| Batch: | / |
| Purity: | / |
| Dose: | Rat: 600, 700, 800, and 900 mg/kg bw<br>Mouse: 625, 700, 775, 850, and 925 mg/kg bw |
| Route: | gavage |
| Exposure: | 14-day observation |
| GLP: | / |
| Date: | 14 May – 23 August 1982 |

The test predated GLP and the OECD guideline. The test substance was dissolved in water at 5, 10 or 20% depending on the dose. Over two weeks, the animals were observed daily for clinical-toxicological signs and death. Body weights were recorded weekly. Post-mortems of all animals were conducted.

Results
The test substance caused reduced activity, piloerection, diarrhoea, and death (exitus). No macroscopic pathological changes were observed. The $LD_{50}$ was calculated as 650 mg/kg bw for female rats, 700 mg/kg bw for male rats, and 813 mg/kg bw for female mice.

Ref.: 5

Comment
The results of this study are regarded as sufficiently valid. Repetition of the acute oral toxicity test is not scientifically justifiable.

#### 3.3.1.2. Acute dermal toxicity

No data submitted

#### 3.3.1.3. Acute inhalation toxicity

No data submitted

### 3.3.2 Irritation and corrosivity

#### 3.3.2.1. Skin irritation

| | |
|---|---|
| Guideline: | OECD 404 |
| Species: | New Zealand White strain |
| Group: | 3 females |
| Substance: | 3-AMMP |
| Batch: | / |
| Purity: | / |
| Dose: | 0.5g; semi-occlusive application |
| Vehicle: | moistened with water |
| GLP: | in compliance |
| Date: | 1 – 16 February 1999 |

Three female animals were used in the test. Each animal served as its own control. Approximately 24 hours prior to the treatment, the dorsal fur was shaved, to expose an area of about 100 cm².
The test compound was examined under semi-occlusive conditions. An aliquot of 0.5 g of the moistened test substance was applied to the intact shaved back skin of each animal. The patch was removed four hours after occlusive contact.
Animals were examined for signs of erythema, eschar and oedema formation. The skin reactions were assessed approx. 1 hour, 24, 48 and 72 hours and 7 days after termination of the exposure.

Results
The undiluted test substance produced slight erythema. In one animal 3 brown spots with crust formation were seen. The irritation was reversible within 7 days after exposure.

Conclusion
Under the conditions of the study, the undiluted 3-AMMP was slightly irritating to the rabbit skin.

Ref.: 6

### 3.3.2.2. Mucous membrane irritation

Guideline: /
Species: New Zealand white rabbits
Group: 6 males
Substance: 3-amino-2-methylamino-6-methoxy-pyridine
Batch: /
Purity: /
Dose: 0.1 mL of 5% preparation
Vehicle: water containing 2% carboxymethylcellulose
GLP: not in compliance
Date: February 1988

The equivalent of 0.1 ml of 3-amino-2-methylamino-6-methoxy-pyridine as a 5% solution (in 2% carboxymethylcellulose and 0.5% Cremophor, pH 9) was instilled into the conjunctival sac of one eye of the test animals. The substance remained in permanent contact with the eyes and was not rinsed. The other eyes served as controls.
The eye irritation reactions were scored approx. 1 hour, 24, 48 and 72 hours after instillation of the test solution.

Results
The instillation of a 5% 3-amino-2-methylamino-6-methoxy-pyridine solution into the eyes did not result in any effects on the cornea, iris or conjunctivae at any time point in any of the treated rabbits.

Conclusion
Under the conditions of the study, a 5% 3-amino-2-methylamino-6-methoxy-pyridine solution was not irritating to the rabbit eye.

Ref.: 7

## 3.3.3. Skin sensitisation

**Local Lymph Node Assay (LLNA)**

Guideline: OECD 429
Species: mice; CBA/CaOlaHsd strain
Group: 3 dose groups and 1 vehicle control group; 4 females each
Substance: A130
Batch: A2090110
Purity: 99.5%
Dose: 2.5%, 5% and 10%
Vehicle: ethanol:water (7:3 v/v)
Control: negative: vehicle
positive: α-hexylcinnamaldehyde
GLP: in compliance
Date: 28 April – 12 May 2004

The dermal sensitization properties of A130 were investigated in mice of the CBA/CaOlaHsd strain. Three dose groups and a control group (receiving the vehicle only) of four female mice each were chosen. The test item was topically applied to the dorsal surface of the ears to analyse the sensitization activity by measuring the proliferative response of lymph node cells.

A homogenous solution of the test item in a mixture of ethanol:water (7:3 v/v) was made shortly before each dosing. The highest non-irritating test item concentration was found in a pre-test with two mice. Based on these test results, 2.5%, 5% and 10% solutions were

chosen for the main study. The vehicle was chosen due to the chemical reactivity/instability of the test substance with other organic solvents like acetone or dimethylformamide.

Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with the different test item concentrations. The application volume, 25 µl, was spread over the entire dorsal surface of each ear lobe once daily for three consecutive days. The control group was treated with the vehicle exclusively. Five days after the first topical application, all mice were administered with radio-labelled thymidine ($^3$HTdR) by intravenous injection via the tail vein.
Approximately five hours after $^3$HTdR application all mice were killed. The draining lymph nodes were excised and pooled for each experimental group. After preparation of the lymph nodes, disaggregation and overnight precipitation of macromolecules, these precipitations were re-suspended and transferred to scintillation vials.

The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of $^3$H-methyl thymidine using scintillation counting. The proliferative response of lymph node cells is expressed as the ratio of $^3$HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index).

A test item is regarded as a sensitizer if the exposure to at least one concentration resulted in an at least 3-fold increase in incorporation of $^3$HTdR compared with concurrent controls, as indicated by the stimulation index (S.I.).

Results
The Stimulation Index (S.I.) showed a dose relationship:

| Test Item Concentration | **S.I.** |
|---|---|
| 2.5% (w/v) | 1.3 |
| 5% (w/v) | 2.5 |
| 10% (w/v) | 6.4 |

The EC 3 value was calculated to be 5.6%.

An appropriate reference (α-hexylcinnamaldehyde in acetone:olive oil (4:1)) was used as positive control to demonstrate the sensitivity of the test system. The EC3 was 11.7%.

Conclusion
Based on the criteria of the test system, A130 was found to be a moderate skin sensitizer when tested in ethanol:water (7:3 v/v) in mice and had an EC3 of 5.6%. The positive control was peri-contemporaneous.

Ref.: 8

### 3.3.4. Dermal / percutaneous absorption

| | |
|---|---|
| Guideline: | OECD 428 |
| Tissue: | pig skin; dermatomed to 0.40mm |
| Group size: | 3 donors (sex not specified) |
| Diffusion cells: | 6 static glass chambers per formulation; 2.54 cm² |
| Skin integrity: | transdermal electrical resistance |
| Test substance: | A130 |
| Batch: | A2090110 |
| Purity: | 99.5% |
| Radiolabel | [$^{14}$C]-A130 |
| Radiolabel batch | 050517 |
| Radiolabel purity | >98% |

| | |
|---|---|
| Test item: | 1.0% A130 in a) basic cream + developer mix with hydrogen peroxide; b) basic cream + developer mix without hydrogen peroxide; c) aqueous solution. |
| Doses: | 20 mg formulation per cm² pig skin |
| Receptor fluid: | phosphate buffered saline (pH 7.4). |
| Solubility receptor fluid: | 330 mg/ml in water |
| Stability: | / |
| Method of Analysis: | liquid scintillation counting |
| GLP: | in compliance |
| Date: | 21 – 28 June 2005 |

The dermal absorption/percutaneous penetration of [$^{14}$C]-A130 of standard hair dye formulations was studied on the clipped excised skin of three young pigs.

The skin integrity of frozen (at -20 °C) skin discs was checked by measuring the trans-dermal electrical resistance. The intact, clipped excised pig skin of the flanks area was exposed for 30 minutes to the test substance in the basic hair dyeing formulation without occlusion.

Shortly before topical application to skin the basic cream was mixed (1:1) with the developer mix with and without hydrogen peroxide as study A and B, respectively. Additionally, a third formulation C was produced by dissolving A130 (traced with [$^{14}$C] radio-labelled material) in water. The nominal concentration of A130 in all three final application formulations was 1.0%.

The composition of the basic cream and the developer mix with and without hydrogen peroxide is shown in the tables below.
The dermal absorption/percutaneous penetration of the test substance was investigated for the open application of about 20 mg formulation per cm² pig skin. Therefore, the resulting dose of the test substance was approx. 0.23 mg/cm² skin. Skin discs of 2.54 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with a 3% Teepol® solution.
Each of the two formulations and the solution were analysed with six replicates for adsorbed, absorbed and penetrated amount of the test substance. The receptor fluid used was phosphate buffered saline (pH 7.4).

| **Ingredient of basic cream** | **Concentration in %** |
|---|---|
| A130 | 2.00 |
| Toluene-2,5-diamine (COLIPA A 005) | 1.08 |
| Hydrenol D | 9.35 |
| Texapon NSO-UP | 15.00 |
| Dehyton K | 12.50 |
| Lorol techn. | 2.20 |
| Eumulgin B2 | 0.75 |
| Sodium sulphite | 0.20 |
| Ammonium sulfate | 0.40 |
| Ascorbic acid | 0.20 |
| Citric acid | for pH adjustment |
| Ammonia | for pH adjustment |
| Water | ad 100 |
| | pH 10.0 |

This formulation was traced with [$^{14}$C] radio-labelled A130 shortly before application.

| **Ingredient of developer mix** | **with $H_2O_2$ in %** | **without $H_2O_2$ in %** |
|---|---|---|
| Dipicolinic acid | 0.10 | 0.10 |
| Sodium pyrophosphate, acid | 0.03 | 0.03 |
| Turpinal SL | 1.50 | 1.50 |
| Texapon NSO-UP | 2.00 | 2.00 |

| Ingredient of developer mix | with $H_2O_2$ in % | without $H_2O_2$ in % |
|---|---|---|
| Ammonia, 25% | for pH adjustment | for pH adjustment |
| Tartaric acid | for pH adjustment | for pH adjustment |
| Aculyn 33 | 15.00 | 15.00 |
| Hydrogen peroxide (50% $H_2O_2$ solution) | 12.00 | - |
| Water | ad 100 | ad 100 |
| | pH 3.8 | pH 3.8 |

In the static system, samples of the receptor fluid were drawn before the application of the test substance formulation and 0.5, 2, 4, 6, 24, 29 and 48 hours after application. The removed volume was replaced by fresh receptor fluid.

Results
The quantities that had penetrated during the 30 minute exposure to A130 containing formulations and within the 48 hours after application are shown in the following table. Both the amounts absorbed and penetrated were taken as systemically available.

| ANALYSED SAMPLE | Formulation A with $H_2O_2$ | | Formulation B without $H_2O_2$ | | Solution C in water | |
|---|---|---|---|---|---|---|
| | [% of dose] | [µg/cm²] | [% of dose] | [µg/cm²] | [% of dose] | [µg/cm²] |
| Skin rinsings | 102.62 | - | 97.31 | - | 96.11 | - |
| Adsorption (stratum corneum) | 0.554 | 1.25 | 0.344 | 0.775 | 0.442 | 1.00 |
| **Not Bio-available** | 103.18 | - | 97.65 | - | 96.55 | - |
| Absorption (epidermis/dermis) | 1.01 | 2.27 | 1.37 | 3.09 | 0.437 | 0.989 |
| Penetration (receptor fluid) | 0.707 | 1.59 | 1.57 | 3.54 | 0.444 | 1.00 |
| **Bio-available** | 1.72 | 3.87 | 2.94 | 6.62 | 0.881 | 1.99 |
| **Total recovery / mass balance** | 105 | - | 101 | - | 97.6 | - |

Cream formulation <u>with</u> $H_2O_2$

| | Chamber number | | | | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| | 1 | 6 | 10 | 15 | 21 | 24 | | |
| **Flange** | 0.102 | 0.163 | 0.148 | 0.124 | 0.064 | 0.088 | 0.115 | 0.037 |
| **Donor Chamber** | 0.194 | 0.397 | 0.450 | 0.363 | 0.609 | 0.258 | 0.378 | 0.146 |
| **Wash** | 232.3 | 227.4 | 227.2 | 217.7 | 237 | 241.7 | 230.6 | / |
| **Stratum corneum** | 0.725 | n/s * | 2.43 | 1.95 | 1.43 | 0.959 | 1.25 | 0.876 |
| **Epidermis / dermis** | 1.90 | 5.01 | 2.35 | 2.45 | 1.03 | 0.915 | 2.27 | 1.49 |
| **penetrated** | 1.41 | 2.58 | 1.50 | 2.42 | 0.880 | 0.779 | 1.59 | 0.757 |
| **Bio-available** | **3.31** | **7.59** | **3.85** | **4.87** | **1.91** | **1.694** | **3.86** | **2.17** |

* Epidermis tore during first tape strip

Cream formulation <u>without</u> $H_2O_2$

| | Chamber number | | | | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| | 3 | 7 | 12 | 16 | 22 | 26 | | |
| **Flange** | 0.481 | 0.273 | 0.283 | 0.244 | 0.108 | 0.131 | 0.253 | 0.134 |
| **Donor Chamber** | 0.101 | 0.377 | 0.163 | 0.792 | 0.521 | 0.182 | 0.356 | 0.265 |
| **Wash** | 219.4 | 211.7 | 219.0 | 221.7 | 219.8 | 222.4 | 219.3 | / |
| **Stratum corneum** | 0.260 | 2.59 | 0.417 | 0.378 | 0.483 | 0.517 | 0.775 | 0.895 |
| **Epidermis / dermis** | 1.69 | 4.07 | 3.28 | 4.64 | 2.77 | 2.07 | 3.09 | 1.14 |
| **penetrated** | 3.62 | 4.74 | 3.22 | 4.77 | 2.59 | 2.27 | 3.54 | 1.06 |
| **Bio-available** | **5.31** | **8.81** | **6.50** | **9.41** | **5.36** | **4.34** | **6.62** | **2.05** |

Test substance in water

| | Chamber number | | | | | Mean | SD |
|---|---|---|---|---|---|---|---|
| | 5 | 13 | 18 | 23 | 27 | | |
| **Flange** | 0.327 | 0.152 | 0.030 | 0.194 | 0.094 | 0.159 | 0.112 |
| **Donor Chamber** | 0.230 | 0.498 | 0.193 | 0.297 | 0.333 | 0.310 | 0.118 |
| **Wash** | 220.6 | 213.0 | 217.5 | 212.0 | 222.8 | 217.0 | 5.2 |
| **Stratum corneum** | 0.253 | 1.59 | 0.378 | 1.83 | 0.958 | 1.00 | 0.703 |
| **Epidermis / dermis** | 0.782 | 1.39 | 0.404 | 1.55 | 0.812 | 0.989 | 0.474 |
| **penetrated** | 0.761 | 1.19 | 0.841 | 1.21 | 1.01 | 1.00 | 0.203 |
| **Bio-available** | **1.543** | **2.58** | **1.245** | **2.76** | **1.822** | **1.989** | **0.677** |

In conclusion, in this in vitro dermal penetration study the amount of A130 systemically available from:

1. a standard cream formulation with hydrogen peroxide was found to be 3.9 ± 2.17 µg/cm$^2$ (range 1.69 to 7.59 µg/cm$^2$) or 1.72% (range 0.75 to 3.36%).
2. a standard cream formulation without hydrogen peroxide was found to be 6.62 ± 2.05 µg/cm$^2$ (range 4.34 to 9.41 µg/cm$^2$) or 2.93% (range 1.9 to 4.18%).

Ref.: 18

Comment
As too few donors and chambers were used, the bioavailability of A130 in an oxidising formulation containing 1.0% A130 should be considered as Amax 7.59 µg/cm² and in a non-oxidising formulation Amax 9.41 µg/cm². These values may be used for calculating the MOS.

## 3.3.5. Repeated dose toxicity

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

Guideline: OECD 407 (1995)
Species/strain: Rat, Wistar (Han Brl)
Group size: 5 per dose/sex
Test substance: A 130
Batch: A2090110
Purity: 99.5% (HPLC peak area)
Dose: 0, 80, 160, and 360 mg/kg bw/day
Vehicle: Water, bidistilled
Route: Gavage, 10 ml/kg bw
Exposure: 28 day
GLP: /
Date: 21 July 2004 – 7 Oct 2004

Due to high susceptibility to oxidation of the test substance, extended investigations were necessary. Acceptable stability (± 10%) of the doses was up to 2 hours from preparation, when stirred slowly to dosages under nitrogen.
During the study, the animals were checked for clinical signs and mortality at least once daily during treatment. Food consumption and body weights were performed weekly over the acclimatization and treatment periods. Analysis of thyroid hormone plasma levels was performed at necropsy. At the end of the study, the pathology of the animals was investigated.

Results
No deaths occurred during the study period. Hypersalivation was noted in all dose groups that was described as excessive salivation from week 3. Food consumption was reduced in all dose groups especially during week 1. In week 2, a dose related reduction in food intake was seen in males at all doses. These were not statistically significant. Body weight gain was reduced in mid and high dose males and in high dose females.

Thyroid hormone levels were measured as T3/T4 in plasma. No difference between T3 levels were noted between any of the male dosed groups (0.52 – 0.56 ng/ml; control 0.51 ng/ml) In females, the control level was 0.48 ng/ml, compared with 0.60 – 0.74 ng/ml for the dosed animals.

T4 levels showed greater variability. The mean and range are shown in the table below.

| | **Mean ± SD** | **Range** (ng/ml) |
|---|---|---|
| **Males** | | |
| control | 28.34 ± 2.32 | 25.18- 31.07 |
| low dose | 33.71 ± 3.69 | 29.95 – 39.45 |
| mid dose | 37.74 ± 6.45 | 30.30 – 47.37 |
| high dose | 32.58 ± 5.59 | 26.55 – 40.18 |
| **Females** | | |
| control | 33.00 ± 9.59 | 22.77- 45.89 |
| low dose | 36.24. ± 5.52 | 31.04- 42.69 |
| mid dose | 34.49 ± 6.44 | 25.49 – 42.44 |
| high dose | 36.44 ± 11.46 | 26.19- 52.55 |

The study authors considered that the treatment did not influence T3 and T4 levels.
No treatment related gross lesions were noted. Increased liver weights were seen in mid and high dose males and high dose females. The thymus weight was decreased in all treated animals. At the high dose in both sexes, the kidneys showed a higher incidence and severity of tubular basophilia. No other lesions were considered to be due to the test substance.
Based on this study, the NOAEL (No Observed Adverse Effect Level) was defined as 80 mg/kg bw/day.

Ref.: 14

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

| | |
|---|---|
| Guideline: | OECD 408 (1998) |
| Species/strain: | Rat, Wistar (Han Brl) |
| Group size: | 10 per dose/sex, recovery group 5 per dose/sex control and high dose |
| Test substance: | A 130 |
| Batch: | A2090110 |
| Purity: | 99.5% (HPLC peak area) |
| Dose: | 0, 50, 100, and 200 mg/kg bw/day |
| Vehicle: | Water, bidistilled |
| Route: | Gavage, 10 ml/kg bw |
| Exposure: | 90 day |
| Recovery group: | + 28 days |
| GLP: | in compliance |
| Date: | 10 September 2004 – 2 March 2005 |

The dose level selection was based on the results of a range finding 28-day study from dose 0, 80, 160, 360 mg/kg bw/day in Wistar rats (ref 14 - RCC study no. 854670, non-GLP Study). The dosages were kept under nitrogen and stirred slowly to reduce contact with oxygen and used within 2 hours of preparation. The pH was adjusted to 5.0 (with NaOH).

During the study, the animals were checked for clinical signs and mortality at least once daily during treatment and recovery periods. Food consumption, body weights and more detailed clinical observations were performed weekly over the acclimatization, treatment and recovery periods. Water consumption was monitored once weekly and in weeks 1, 5, 12 for 24 h. Functional observational battery tests were performed at week 13. At the end of the study, the pathology of the animals was investigated. The animals of the recovery groups were additionally examined during the 4-week treatment-free period.

Results
No deaths, no effects on food consumption and no evidence of eye toxicity were noted throughout the study period. Increased salivation was noted at all doses in males and in mid and high dose females. Deep yellow coloured urine was also seen in all but the low dose males. Water consumption increased in the high dose animals, associated with increased urine volume that had slightly lower density. Body weight gain was reduced in mid and high dose males, but was returning to the control levels by the end of the recovery period. Reduced mean hind limb grip strength for mid and high dose males, and also reduced forelimb grip strength in high dose males were noted at week 13, but this was attributed to the lower body weights.
Haematological changes noted were an increase in the reticulocyte count and maturity index in the mid and high dose groups of both sexes; the total count continued to remain elevated after the recovery period. Blood coagulation, including higher platelet counts and prothrombin time, showed minimal effects that were completely reversed during recovery.
Mid and high dose males and high dose females showed significantly altered lipid metabolism, including increased levels of cholesterol, triglycerides, and phospholipids after 90 days dosing, this returned to the normal range during recovery period.
Statistically significant changes in sodium (-), potassium (+), calcium (+, males only) and chloride (-, females only) were seen at all doses after 90 days dosing. This suggested a disturbed electrolyte or water- balance. These effects were reversed during recovery, except for an elevated potassium level persisting in high dose females.
High dose males only showed statistically significant increased levels for total protein, albumin, and inorganic phosphorous by the termination of dosing.
Other statistically significant changes were the depressed values for GLDH (at all dose levels) and ASAT (high dose only) but the toxicologically relevance was unclear. Increased urine pH in high dose animals were considered associated with the excretion of the test substance or its metabolites.
Absolute and relative liver weights were affected in both sexes at all dose levels (males low: +10%/+14%), mid: +9%/+17%, high: +20%/+34%; females low: +14%/+13%, mid: +18%/+26%, high +37%/+46%). These higher weights were confirmed by the adaptive changes in the liver histology. Centrilobular hypertrophy of the hepatocytes was observed in all treated males and in 6/10 low dose and in all mid and high dose females. The degree of severity varied from minimal to slight at the low dose, slight to moderate at the mid dose and moderate at the high dose. This hypertrophy was probably due to mixed function oxidase induction. After 4 weeks recovery, although there appeared to be to a reduction in the severity, minimal or slight centrilobular hypertrophy was seen in all males and in 2/5 females at the high dose.

There was a higher incidence of hemosiderin deposits in the red pulp of the spleen in the treated animals compared with controls (controls: 1/10 males, 6/10 females; mid dose: 7/10 males, 8/10 females; high dose: 6/10 males, 10/10 females, respectively). However in females, there is often a minimal presence of hemosiderin as background. After 4 weeks of recovery, all high dose males and females of group 4 still showed minimal/slight or moderate (one female) hemosiderin deposits.
In males, the kidneys showed a higher incidence and severity of tubular basophilia (controls 4/10; low and mid dose; 4/10, high dose 7/10). At the high dose, the distribution was multifocal and minimal to moderate in the cortex, while in controls, distribution was minimal, focally and mostly unilateral. This was not resolved by the end of the recovery period at the high dose (3/5 male). In females, tubular basophilia was seen only at the high dose (3/10) and in the control (1/10)
Macroscopically, the thyroid had a black discoloration in mid and high dose animals that persisted through the recovery period in high dose animals. Microscopically, there was follicular cell hypertrophy in all high dose animals and in 9/10 mid dose males. This change, slight in males and minimal or slight in females, was associated with a thickening of the colloid (not reported in the table of individual animal findings) that probably correlated with the black discoloration reported at necropsy. All males and 3/5 females of the high dose

recovery group still showed minimal to slight hypertrophy. No macroscopic or microscopic changes were noted at the low dose.
In the pituitary, minimal to moderate hypertrophy of the TSH (thyroid stimulating hormone) cells was observed in all mid and high dose males and minimal to slight hypertrophy in 2/10 mid and 9/10 high dose females. An increase in the pituitary basophilic cells, shown by PAS/Orange G staining, corroborated the TSH-producing cells hypertrophy (TSH-producing cells belong to the subpopulation of basophilic cells). In the recovery group, minimal to slight hypertrophy was still seen in 4/5 males and all females.
The remaining microscopic findings were within the range and severity of spontaneous background lesions that may be observed in rats of this strain and age in this laboratory and considered to be of no toxicological significance.

Conclusion
The study authors considered that the centrilobular hypertrophy of the hepatocytes, the result of mixed function oxidase activation, in turn, lead to the indirect perturbation of the pituitary-thyroid axis. Follicular hypertrophy of the thyroid gland, with increased turnover of thyroxine (T4), is associated with activation of the thyroid gland through the increased secretion of TSH by the pituitary, particularly in rats.
The study authors considered the NOAEL (No Observed Adverse Effect Level), based on pathology only, to be 50 mg/kg bw/day.

Ref.: 15

Comment
The SCCP considered that the lowest dose, 50 mg/kg bw/day, was not a NOAEL, but a LOAEL (lowest-observed-adverse-effect-level), due to the observed liver and biochemical effects.

### 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 Reverse Mutation Test**

| | | |
|---|---|---|
| Guideline: | OECD 471 | |
| Species/strain: | *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102 | |
| Replicates: | triplicates in 3 independent experiments both in the presence and absence of metabolic activation (experiment 1A without S9-mix only). | |
| Test substance: | A130 | |
| Solvent: | deionised water | |
| Batch: | A2090110 | |
| Purity: | 99% | |
| Concentrations: | Experiment I: | 3, 10, 33, 100, 333, 1000 and 2500 µg/plate without and with S9-mix |
| | Experiment IA: | 25, 50, 100, 150, 200 and 300 µg/plate without S9-mix, TA100 only |
| | Experiment II: | 3, 10, 33, 100, 333, 1000 and 2500 µg/plate without S9-mix |
| | Experiment II: | 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate with S9-mix |
| Treatment: | Experiment I: | direct plate incorporation with 48 h incubation, without and with S9-mix |

| | | |
|---|---|---|
| | Experiment II: | pre-incubation method with 60 minutes pre-incubation and 48 h incubation, without and with S9-mix. |
| GLP: | In compliance | |
| Date: | 13 April – 19 May 2004 | |

A130 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment on toxicity measuring a putative reduction in the number of revertant colonies and/or thinning of the bacterial background lawn in strains TA98 and TA100 both without and with S9-mix for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate. The pre-experiment was reported as part of experiment I. Experiment I was performed with the direct plate incorporation method, experiment II with the pre-incubation method. Negative and positive controls were in accordance with the guideline.

Results
Up to the highest dose investigated precipitation of the test compound was not observed. Strong toxic effects evident as a reduction in the number of revertants were observed in experiment I without S9-mix at 1000 µg/plate and above in TA1535, TA1537, TA98 and TA102 and at 333 µg/plate and above in TA100; with at S9-mix at 1000 µg/plate and above in TA1535, TA98 and TA100 and at 2500 µg/plate in TA1537 and TA102. In experiment IA strong toxic effects were seen at 300 µg/plate. In experiment II strong toxic effect were observed at 333 µg/plate and above without and at 1000 µg/plate and above with S9-mix in all strains. A dose dependent increase in revertant colonies was found in strains TA1535 and TA98 in the absence of metabolic activation. The observed reduction in revertant colonies at the highest concentrations may be caused by overlapping toxic effects. The test compound did not induce an increase in the number of revertant colonies in the other strains at any concentration tested both in the presence or absence of metabolic activation

Conclusion
Under the experimental conditions used A130 was mutagenic in this gene mutation tests in TA98 and TA1537 without metabolic activation.

Ref.: 9

### ***In Vitro*** **Mammalian Cell Gene Mutation Assay (*tk* locus*)***

| | | |
|---|---|---|
| Guideline: | OECD 476 | |
| Cells: | L5178Y Mouse lymphoma cells | |
| Replicates: | duplicate cultures in 2 independent experiments | |
| Test substance: | A130 | |
| Solvent: | deionised water | |
| Batch: | A2090110 | |
| Purity: | 99.5 % | |
| Concentrations: | Experiment I: | 4.5, 9, 18, 36 and 54 µg/ml without S9-mix<br>18.1, 36.3, 72.5 and 145 µg/ml with S9-mix |
| | Experiment II: | 0.8, 1.5, 3, 6 and 9 µg/ml without S9-mix |
| Treatment | Experiment I: | 4 h treatment without and with S9-mix; expression period 72 h; selection period of 10-15 days |
| | Experiment II: | 24 h treatment without S9-mix only; expression period 48 h; selection period of 10-15 days |
| GLP: | in compliance | |
| Date: | 24 November 2004 – 31 January 2005 | |

A130 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test

concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth and precipitation. In the main test, cells were treated for 4 h (experiment I) or 24 h (experiment II) followed by an expression period of 72 h (4 h treatment) or 48 h (24 h treatment) to fix the DNA damage into a stable *tk* mutation. Toxicity was measured in the main experiments as relative suspension growth and/or relative total growth of the treated cultures relative to that of the solvent control cultures. The number of colonies was counted manually; colony size distribution was determined in the controls and in all treated concentrations of A130. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test, precipitation was observed at 575 µg/ml and above in the absence and presence of S9-mix at both treatment intervals. Analysable concentrations with the appropriate levels of toxicity (10-20% survival) were never reached in both experiments since the highest concentrations tested always demonstrated toxicity levels below 10% survival and the second highest concentrations (far) above the 20% survival.
In one culture of experiment I without S9-mix, a more or less dose dependent increase was seen in the mutant frequency (MF). The increase remained well within the range of the historical control data for negative and solvent controls and was not seen in culture II of experiment I. Therefore, this increase was considered not biologically relevant. In experiment II an increase in the MF was found in both parallel cultures at 6 µg/ml without S9-mix. However, the absolute values of the MF were rather low and remained within the range of the historical control data for negative and solvent controls. This increase was also considered not biologically relevant.

Conclusion
Under the experimental conditions used, A130 was not mutagenic in the mouse lymphoma assay at the *tk*-locus.

Ref.: 10

Comment
A concentration with the appropriate level of toxicity (10-20% survival) was never reached in both experiments. The highest concentrations tested always demonstrated toxicity of <10% survival. Therefore, the SCCP considers this result as equivocal.

### 3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

**Mammalian Erythrocyte Micronucleus Test**

| | |
|---|---|
| Guideline: | OECD 474 |
| Species/strain: | NMRI mice |
| Group size: | 5 mice/sex |
| Test substance: | A130 |
| Batch: | A2090110 |
| Purity: | 99.5% |
| Dose level: | 0, 62.5, 125 and 250 mg/kg bw |
| Route: | intraperitoneal |
| Vehicle: | deionised water |
| Sacrifice times: | 24h and 48h (highest dose only) after the treatment. |
| GLP: | in compliance |
| Date: | 28 July – 7 October 2004 |

A130 has been investigated for the induction of micronuclei in bone marrow cells of mice. The test concentration was based on a preliminary study on acute toxicity. Two mice per sex were treated intraperitoneally with 100 – 300 mg/kg bw A130 and examined for symptoms of acute toxicity at 1, 2-4, 6, 24, 30 and 48 h. 250 mg/kg bw was selected as the maximum tolerated dose level. In the main experiment bone marrow cells were collected

24h and 48h (highest dose only) after dosing. Toxicity and thus exposure of the target cells was determined by counting the number of polychromatic erythrocytes per 2000 erythrocytes. In the main experiment the animals were examined for acute toxic symptoms at intervals around 1, 2-4, 6 and 24 h after treatment. Bone marrow preparations were stained and examined microscopically for cytotoxicity and micronuclei. In order to quantify the concentration of A130 in blood serum 3 male mice were treated with 250 mg/kg bw A130. 1 and 4 h after treatment the animals were sacrificed and blood collected. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment for toxicity, all animals treated with 250 mg/kg bw expressed toxic effects like reduction of spontaneous activity, abdominal position, eyelid closure and ruffled fur up to 48 h after treatment. Apathy was found up to 6 h after treatment. In the main experiment identical toxic effects were observed for the high dose group. Animals of the 125 mg/kg bw group showed reduction of spontaneous activity and ruffled fur up to 24 h and abdominal position up to 2-4 h; in the 62.5 mg/kg bw group these toxic effects further decreased. The toxic effects point to systemic availability of A130 which was also demonstrated by a decrease in the number of PCEs per 2000 erythrocytes at the highest concentration at both time points. Therefore, serum analysis was not performed.
Biologically relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found following treatment with A130 at any concentration at both time points.

Conclusion
Under the experimental conditions used A130 did not induce micronuclei in bone marrow cells of treated mice and, consequently, A130 is not clastogenic and/or aneugenic in bone marrow cells of mice.

Ref.: 11

Comment
Although blood serum of 3 male mice treated with 250 mg/kg bw A130 was collected to quantify the concentration of A130, this analysis was not performed. This is accepted since the systemic availability of A130 has been sufficiently demonstrated.

**Mammalian Bone Marrow Chromosome Aberration Test**

| | |
|---|---|
| Guideline: | OECD 475 |
| Species/strain: | HanBrl: WIST rats |
| Group size: | 5 rats/sex |
| Test substance: | SAT 010561 |
| Batch: | R96003970 |
| Purity: | 99.4% |
| Dose level: | 50 mg/kg bw |
| Route: | intraperitoneal |
| Vehicle: | 0.9% NaCl solution |
| Sacrifice times: | 24h and 48h after the treatment. |
| GLP: | in compliance |
| Date: | 28 August – 6 September 2001 |

A130 has been investigated for the induction of chromosomal aberrations in bone marrow cells of rats. The test concentration was based on a preliminary study on acute toxicity. Two mice per sex were treated intraperitoneally with 5 – 2000 mg/kg bw A130 and examined for symptoms of acute toxicity at 1, 2-3, and approximately 24, 48, 72 and 96 h. 50 mg/kg bw was selected as the maximum tolerated dose level. In the main experiment bone marrow cells were collected 24h and 48h after dosing. Toxicity and thus exposure of the target cells was measured with the mean mitotic index. Prior to sacrifice, the rats were injected with

colcemid (2.0 mg/kg bw) to arrest cells in metaphase. In the main experiment the animals were examined for acute toxic symptoms at intervals around 1, 2, 3, 24 and 48 h after treatment. Bone marrow preparations were stained and examined microscopically for cytotoxicity and chromosomal aberrations. Negative and positive controls were in accordance with the OECD guideline.

Results
All rats of the pre-experiment treated with 2000 mg/kg bw died. At 200 mg/kg bw rats showed ventral recumbency (up to 3 h), ruffled fur (up to 96 h) and hunched posture (up to 24 h); these toxic symptoms disappeared after 24 h in rats treated with 50 mg/kg bw and 5 mg/kg bw. In the main experiment the rats showed ruffled fur and lethargy up to 3 h after treatment. Thereafter these toxic effects disappeared. The toxic effects point to systemic availability of A130 which was also demonstrated by the decrease of the mitotic index at both time points. Biologically relevant increases in the number of bone marrow cells with chromosomal aberrations as compared to the concurrent vehicle controls were not found following treatment with A130 at any time point.

Conclusion
Under the experimental conditions an increase in cells with chromosomal aberrations due to treatment with A130 was not observed and, consequently, A130 is not clastogenic and/or aneugenic in bone marrow cells of rats.

Ref.: 12

**Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo***

| | |
|---|---|
| Guideline: | OECD 486 |
| Species/strain: | Wistar rats |
| Group size: | 3 male rats/group |
| Test substance: | C059 |
| Batch: | R96003970 |
| Purity: | > 99 % |
| Dose level: | 0, 250, 500 and 1000 mg/kg bw |
| Route: | gavage, once |
| Vehicle: | physiological saline |
| Sacrifice times: | 2-4 h and 12-16 h after dosing |
| GLP: | In compliance |
| Date: | 22 January – 25 March 2002 |

The test compound was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. The test concentrations were based on a pilot study in which 3 male rats per dose were treated with 1000 and 2000 mg/kg bw for 1 or 2 days. During this period mortality and physical condition were recorded. A pilot perfusion was performed to examine possible hepatotoxic effects. The highest dose selected for this UDS assay was 1000 mg/kg bw; 2 additional dose levels were selected using dilutions of the highest dose. Rats were treated *in vivo*. Hepatocytes for UDS analysis were collected at 2 - 4 h and 12 - 16 h after administration of the test compound. Hepatocytes were isolated by perfusion with the proteolytic enzyme collagenase. The obtained cell suspension was cleared from dead, endothelial and Kuppfer cells by centrifugation. After attachment of the remaining hepatocytes to cover slips, they were labelled for 4 h with 10 µCi/ml $^{3}$H-thymidine. Evaluation of autoradiography was done after 7 days exposure. UDS was measured by counting manually the number of grains above nuclei and above heavily labelled adjacent cytoplasm areas of the same size. The corrected nuclear grain count was calculated for each cell by subtracting cytoplasmic grain counts from nuclear grain counts. Unscheduled synthesis was determined in 50 randomly selected hepatocytes per cover slip and 2 cover slips per rat. Negative and positive controls were in accordance with the OECD guideline.

Results
At the 2-4 h time point clinical signs observed immediately after dosing were lethargy and irregular respiration; at 12-16 h time point lethargy only in rats from the high dose group. Prior to perfusion, at the 2-4 h time point lethargy, rough coat, salivation and hunched posture were observed, at 12-16 h only lethargy, rough coat and hunched posture in rats from the high dose group. The viability of the hepatocytes was at least 61% (at least 72% in controls) indicating no direct liver toxicity. The net nuclear grain count did not reveal biologically relevant positive responses at any of the dose levels tested at both samples times. The percentage of cells in repair (cells with net nuclear grain count > 5) also revealed no biologically relevant increase at any dose at both sampling times.

Conclusion
Under the experimental conditions used the test compound did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 13

Comment
According to submission III the investigated material is falsely identified as "C059". As also verified by the batch number and Henkel substance code the material tested is actually COLIPA A130

## 3.3.7. Carcinogenicity

No data submitted

## 3.3.8. Reproductive toxicity

### 3.3.8.1. Two generation reproduction toxicity

No data submitted

### 3.3.8.2. Teratogenicity

| | |
|---|---|
| Guideline: | OECD 414 (2001) |
| Species/strain: | Rat, Wistar Han BRL |
| Group size: | 22 per dose |
| Test substance: | COLIPA A 130 / SEG II / Ro 730 |
| Batch: | A2090110 |
| Purity: | 99.5% |
| Dose: | 0, 50, 100 and 200 mg/kg bw |
| Vehicle: | ultrapure water |
| Route: | Gavage, 10 ml/kg bw |
| Exposure: | Gestation day 6-20 |
| GLP: | in compliance |
| Date: | 6 October 2004 – 2 March 2005 |

Dosages were based on the results of the previously performed dose range-finding study (0, 100, 200 and 400 mg/kg bw/day). The animals were dosed within 2 hrs of preparation of the dosages. Up to dosing, the dosages were kept under nitrogen and stirred slowly to reduce contact with oxygen. The pH was adjusted to 5.0 (with NaOH). The mortality and the body weight gain were observed daily.
The mated females were housed individually, if the daily vaginal smear was sperm positive. A copulation plug was observed. The day of mating was designated gestation day (GD) 0. The dams were sacrificed on GD 21 by carbon dioxide asphyxiation and subjected to necropsy. The number of alive and dead foetuses, their distribution and site in the uterus, early and late resorption, implantation and number of *corpora lutea* was determined. The

weight of the foetuses, gravid uteri, uteri without foetuses, placentae and the sex of foetuses were recorded. Approximately one-half of the foetuses were selected at random and examined for visceral alterations. The remaining foetuses were examined for skeletal malformations, variations and retardation of the normal organogenesis after appropriate staining.

Results
All females survived. At mid and high dose, ruffled fur and signs of discomfort following dosage were noted on most treatment days.
Mean food consumption and body weight gain in the mid and high dose groups was dose-dependently reduced during the treatment period. The corrected body weight gain (corrected for gravid uterus weight) was similarly dose-dependently reduced.
Post-implantation losses and the mean number of foetuses per dam were unaffected by treatment with the test item at all dose levels. No test substance related effects on foetal sex ratios were noted in any group.
Mean foetal body weights were similar in all groups and gave no indication of a test substance related effect.
No abnormalities that were considered to be attributable to treatment with the test substance were noted during foetal external examination. No foetal visceral or skeletal/cartilage findings were noted that were test substance related.

Conclusion
Up to and including the highest dose of 200 mg/kg bw/day, 3-amino-2-methylamino-6-methoxypyridine HCl revealed no embryotoxic or teratogenic potential. Based on the results from this study the maternal NOEL was determined as 50 mg/kg bw/day and the foetal NOEL was determined as 200 mg/kg bw/day.

Ref.: 16, 17

### 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

No data submitted

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

**CALCULATION OF THE MARGIN OF SAFETY**

**(6-methoxy-2-methylamino-3-aminopyridine HCl**)
(oxidative / permanent)

| | | | |
|---|---|---|---|
| **Maximum absorption through the skin** | **A (µg/cm$^2$)** | **=** | **7.59 µg/cm²** |
| **Skin Area surface** | **SAS (cm$^2$)** | **=** | **700 cm$^2$** |
| **Dermal absorption per treatment** | **SAS x A x 0.001** | **=** | **5.31 mg** |
| **Typical body weight of human** | | **=** | **60 kg** |
| **Systemic exposure dose (SED)** | **SAS x A x 0.001/60** | **=** | **0.09 mg/kg bw** |
| **Lowest observed adverse effect level (90-day, rat, oral)** | **LOAEL** | **=** | **50 mg/kg bw** |
| **Margin of Safety** | **LOAEL / SED** | **=** | **555** |

**(6-methoxy-2-methylamino-3-aminopyridine HCl**)
(non-oxidative / semi-permanent)

| | | | |
|---|---|---|---|
| **Maximum absorption through the skin** | **A (µg/cm$^2$)** | **=** | **9.41 µg/cm²** |
| **Skin Area surface** | **SAS (cm$^2$)** | **=** | **700 cm$^2$** |
| **Dermal absorption per treatment** | **SAS x A x 0.001** | **=** | **6.59 mg** |
| **Typical body weight of human** | | **=** | **60 kg** |
| **Systemic exposure dose (SED)** | **SAS x A x 0.001/60** | **=** | **0.11 mg/kg bw** |
| **Lowest observed adverse effect level (90-day, rat, oral)** | **LOAEL** | **=** | **50 mg/kg bw** |
| **Margin of Safety** | **LOAEL / SED** | **=** | **455** |

Although the MoS calculation was based on an LOAEL, the obtained MoS was considered sufficient to support safe use of the substance in the indicated application.

### 3.3.14. Discussion

*Physico-chemical properties*
6-Methoxy-2-methylamino-3-aminopyridine HCl is used as a precursor for hair colours in oxidative hair dye formulations. Its final concentration on head can be up to 1.0% (calculated for hydrochloride salt, corresponding to 0.68% calculated for the free base).
6-Methoxy-2-methylamino-3-aminopyridine HCl is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

The batches, for which analytical data was provided, contained many unidentified impurities in concentrations up to 2.8% (HPLC peak area). Calculated Log $P_{ow}$ values cannot be accepted as estimates of the true physical constant without justification. The stability of 6-methoxy-2-methylamino-3-aminopyridine HCl in the marketed products is not provided.

*General toxicity*

The $LD_{50}$ for 6-methoxy-2-methylamino-3-aminopyridine HCl was calculated as 650 mg/kg bw for female rats, 700 mg/kg bw for male rats and 813 mg/kg bw for female mice.
The thyroid effects, seen at 80 mg/kg bw/day in the 28-day study, suggested that this was a possible target organ.
In the sub-chronic study, the lowest dose 50 mg/kg bw/day was considered a LOAEL (lowest observed adverse effect level), due to the observed liver and bio-chemical effects. The centrilobular hypertrophy of the hepatocytes, the result of mixed function oxidase activation, lead to indirect perturbation of the pituitary-thyroid axis, which was minimal at this dose.
The use of the LOAEL does not raise concerns about the safe use of 6-methoxy-2-methylamino-3-aminopyridine HCl given the high MoS.
No maternal toxicity or embryotoxic/teratogenic potential of 6-methoxy-2-methylamino-3-aminopyridine HCl was seen (maternal NOEL 50 mg/kg bw/day, foetal NOEL 200 mg/kg bw/day).

*Irritation / sensitisation*
Under the conditions of the study, the undiluted 6-methoxy-2-methylamino-3-aminopyridine HCl was slightly irritating to the rabbit skin. A 5% solution was not irritating to the rabbit eye.
Based on the criteria of the test system, 6-methoxy-2-methylamino-3-aminopyridine HCl was found to be a moderate skin sensitizer when tested in ethanol:water (7:3 v/v) in mice and had an EC3 of 5.6%.

*Dermal absorption*
As too few donors and chambers were used, the bioavailability of 6-methoxy-2-methylamino-3-aminopyridine HCl in an oxidising formulation containing 1.0% 6-methoxy-2-methylamino-3-aminopyridine HCl should be considered as Amax 7.59 µg/cm² and in a non-oxidising formulation Amax 9.41 µg/cm². These values may be used for calculating the MOS.

*Mutagenicity / genotoxicity*
Overall, the genotoxicity of 6-methoxy-2-methylamino-3-aminopyridine HCl is sufficiently investigated in valid genotoxicity tests for the three types of mutation: gene mutation, structural and numerical chromosome aberration. 6-Methoxy-2-methylamino-3-aminopyridine HCl did induce gene mutations in bacteria whereas this result was sufficiently not contradicted in mammalian cells. However, under *in vivo* conditions, the findings of the *in vitro* gene mutation assays were not confirmed in an *in vivo* UDS test.
Clastogenicity was tested in two *in vivo* tests either evaluating micronuclei in bone marrow cells of mice or chromosomal aberrations in bone marrow cells of rats. Both tests were negative.
Consequently, 6-methoxy-2-methylamino-3-aminopyridine HCl itself can be considered to have no genotoxic potential. However, appropriate tests with 6-methoxy-2-methylamino-3-aminopyridine HCl in combination with hydrogen peroxide have to be provided.

*Carcinogenicity*
No data submitted

## 4. CONCLUSION

This risk assessment relates only to 6-methoxy-2-methylamino-3-aminopyridine HCl. No data was submitted on other salts.

6-Methoxy-2-methylamino-3-aminopyridine HCl is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

The characterised batches of 6-methoxy-2-methylamino-3-aminopyridine HCl contained many unknown impurities in concentrations up to 2.8% (HPLC peak area), which should be identified.

6-Methoxy-2-methylamino-3-aminopyridine HCl itself has no mutagenic potential. 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.

The SCCP is of the opinion that, apart from the risks associated with the use of a moderate sensitiser, the use of 6-methoxy-2-methylamino-3-aminopyridine (free base or dihydrochloride) as an ingredient in any hair dye formulation, oxidative and non-oxidative, at a maximum concentration on the head of 0.68% as free base (1.0% as dihydrochloride) does not pose a risk to the health of the consumer.

## 5. MINORITY OPINION

Not applicable

## 6. REFERENCES

1. Meinigke, B. (2005) Raw Material Description – A 130. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500272
2. Meinigke, B. (2005) Dossier of hair dye A130 – Analysis of batch A2090110 used in toxicological tests. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0400271
3. Meinigke, B. (2005) Dossier of hair dye A130 – Analysis of batch R96003970 used in toxicological tests. Henkel KGaA, Düsseldorf, Report No. R 0500369
4. Meinigke, B. (2005) Comparison of analytical results of batches A2090110 and R96003970. Henkel KGaA, Düsseldorf, Report No. R 0500370
5. Weide, J. (1982). Akute orale Toxizitätsprüfung von 3-Amino-2-methylamino-6-methoxypyridin, Dihydrochlorid. Biological Laboratory, Wella AG Darmstadt, Germany, Archive Code at Henkel KGaA, Düsseldorf, Report No. R 9400377
6. Koopmans, M.J.E. and Daamen, P.A.M. (1989) Primary skin irritation/corrosion study with 3-AMMP in rabbits (4-hour semi-occlusive application). RCC NOTOX B.V., 's-Hertogenbosch, The Netherlands, internal study code: 1282/1750 (002521) Archive Code at Henkel KGaA, Düsseldorf, Report No. Ex 0184
7. Potokar, M. (1988) 3-Amino-2-methylamino-6-methoxypyridin, Prüfung der primären Schleimhautirritation am Kaninchenauge. Institut für Toxikologie, Henkel KGaA, Düsseldorf, Germany, Archive code at Henkel KGaA, Düsseldorf, Report No. TBD 880092

8. Wang-Fan, W. (2004) A 130: Local lymph node assay (LLNA) in mice (identification of contact allergens). RCC Ltd, Itingen, Switzerland, internal study code: 853988. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0400471
9. Sokolowski, A. (2004) Salmonella typhimurium reverse mutation assay with A 130. RCC Cytotest Cell Research GmbH, Rossdorf, Germany, internal study code 824105. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0400568
10. Wollny, H.-E. (2005) Cell mutation assay at the thymidine kinase locus ($TK^{+/-}$) in mouse lymphoma L5178Y cells with A 130, CCC Cytotest Cell Research GmbH, Rossdorf, Germany, internal study code 864504. Archive code at Henkel KGaA, Report No. R 0500136
11. Hornavar, N. (2004) Micronucleus assay in bone marrow cells of the mouse with A 130. RCC Cytotest Cell Research GmbH, Rossdorf, Germany, internal study code 838710. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500006
12. Arcelin, G. (2001) SAT 010561: Chromosome aberration assay in bone marrow cells of the rat. RCC Ltd, Füllinsdorf, Switzerland, internal study code 825838. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0101071
13. Meerts, I.A.T.M. (2002) Evaluation of DNA repair inducing ability of C 059* in male rat hepatocytes (in vivo rat hepatocyte DNA-repair assay). NOTOX B.V., 's-Hertogenbosch, The Netherlands, internal study code 342091. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0200335. * Note: throughout the report the investigated material is falsely identified as "C 059". As also verified by the batch number and Henkel substance code the material tested is actually COLIPA A 130
14. Bachmann, et al. (2004) A 130: 28-day oral (gavage) range-finding toxicity study in the Wistar rat. RCC Ltd, Toxicology, Itingen, Switzerland, internal study code 854670. Archive code at Henkel KGaA, Düsseldorf, Report No. 0401090
15. Bachmann, et al. (2005) A 130: 90-day oral (gavage) toxicity study in Wistar rats followed by a 4-week recovery period. RCC Ltd, Toxicology, Itingen, Switzerland, internal study code 854671. Archive code at Henkel KGaA, Düsseldorf, Report No. 0500127
16. Marbuger, A. and Flade, D. (2004) A 130: Dose range finding prenatal developmental toxicity study in the Han Wistar rat. RCC Ltd, Itingen, Switzerland, internal study code 854672. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0400815
17. Marbuger, A. and Flade, D. (2005) A 130: Prenatal developmental toxicity study in the Han Wistar rat. RCC Ltd, Itingen, Switzerland, internal study code 854673. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500124
18. Hadfield, N. (2005) A 130: In vitro penetration from three different vehicles through pig skin. Central Toxicology Laboratory, Cheshire, UK, internal Study No. JV1857. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500353