



Scientific Committee on Consumer Safety

SCCS

# OPINION ON

# Hydroxyethyl-2-nitro-p-toluidine

## COLIPA n° B75


The SCCS adopted this opinion at its 11th plenary meeting

of 21 June 2011

About the Scientific Committees

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

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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ISSN 1831-4767 ISBN 978-92-79-30716-4

Doi:10.2772/97851 ND-AQ-11-009-EN-N

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Keywords: SCCS, scientific opinion, hair dye, B75, hydroxyethyl-2-nitro-p-toluidine, directive 76/768/ECC, CAS 100418-33-5, EC 408-090-7

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on hydroxyethyl-2-nitro-p-toluidine, 21 June 2011

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## 1. BACKGROUND

Submission I was submitted to the Commission in March 1992. The first opinion of the Scientific Committee on Cosmetic Products and Non-food Products intended for Consumers (SCCNFP) on Hydroxyethyl-2-nitro-p-toluidine was adopted on 23 June 1999. Submission II on the above substance was submitted to the SCCNFP in 14 January 2002. On 18 March 2003, a second opinion on that substance was adopted by the SCCNFP (SCCNFP/0635/03, final).

The above mentioned substance is listed in Annex III, Part 2 (List of substances provisionally allowed) under reference number 10 of the Cosmetics Directive 76/768/EEC.

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

## 2. TERMS OF REFERENCE

1. *On the basis of provided data the Scientific Committee on Consumer Safety (SCCS) is asked to assess the risk to consumer when Hydroxyethyl-2-nitro-p-toluidine is used in oxidative and non-oxidative hair dye formulations at a maximum on-head concentration of 1%.*

2. *Does the SCCS recommend any further restrictions with regard to its use in cosmetic products?*

## 3. OPINION

### 3.1. Chemical and Physical Specifications

#### 3.1.1. Chemical identity

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

Hydroxyethyl-2-nitro-p-toluidine

##### 3.1.1.2. Chemical names

Ethanol, 2-[(4-methyl-2-nitrophenyl)amino]- (CA Index Name, 9CI)
2-(4-methyl-2-nitroanilino)ethanol (IUPAC)
1-Methyl-3-nitro-4-(2´-hydroxyethyl)-aminobenzene
4-(2´-hydroxyethyl)-3-nitro-toluidine
1-(2´-hydroxyethyl)-amino-4-methyl-nitrobenzene
4-methyl-2-nitro-(β-hydroxyethyl)-aniline
4-(2'-hydroxyethyl)-amino-3-nitromethylbenzene

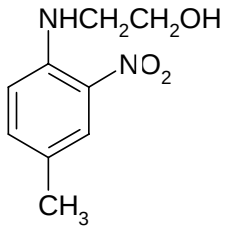
##### 3.1.1.3. Trade names and abbreviations

Jarocol HNT
Methylgelb

##### 3.1.1.4. CAS / EC number

CAS: 100418-33-5
EC: 408-090-7

##### 3.1.1.5. Structural formula




##### 3.1.1.6. Empirical formula

Formula: $C_9H_{12}N_2O_3$

#### 3.1.2. Physical form

Red crystalline powder

#### 3.1.3. Molecular weight

Molecular weight: 196.214 g/mol

### 3.1.4. Purity, composition and substance codes

Purity and impurities in various batches of Hydroxyethyl-2-nitro-p-toluidine

| **Description** | **Batch No.** | | | | | |
|---|---|---|---|---|---|---|
| | **B3/89** | **BRA 1/389** | **BRA 1/315** | **BRA 1/177** | **BRA 1/285** | **6718 Fass 10/20** |
| **NMR content, % (w/w)** | 99.9 | 98.3 | 98.4 | 94.7 | 99.8 | 96.8 |
| **HPLC purity, area%** | | | | | | |
| **210 nm** | 99.5 | 98.7 | 98.6 | 92.0 | 99.0 | - |
| **254 nm** | 99.8 | 98.8 | 99.7 | 99.3 | 99.6 | 99.8 |
| **460 nm** | 100.0 | 99.9 | 99.9 | 100.0 | 99.9 | 99.9 |
| **HPLC content, % (w/w)** | 97.9 | 96.9 | 97.9 | 91.9 | 98.1 | 96.8 |
| **Content of 4-methyl-2-nitroaniline, ppm** | 93 | 126 | 141 | 425 | Ca. 17[a] | 483 |
| **Water content, % (w/w)** | 0.06 | 0.06 | 0.05 | Not determined* | 0.01 | <0.01 |
| **Loss on drying, % (w/w)** | 0.13 | 0.13 | 0.12 | Not determined* | Not determined* | 0.2 |
| **Ash, % (w/w)** | 0.01 | 0.01 | 0.01 | Not determined* | 0.01 | 0.13 |

[a] limit of detection 17 ppm
* not determined due to lack of substance.

### 3.1.5. Impurities / accompanying contaminants

See 3.1.4

### 3.1.6. Solubility

Water: 0.351 g/L, pH 6.4 (OECD 105)
Acetone/water (1:1): ≥100 g/L
DMSO: ≥100 g/L
Ethanol: ≥100 g/L

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

Log $P_{ow}$ : 2.1 (20°C) (EU-A.8)

### 3.1.8. Additional physical and chemical specifications

Organoleptic properties: Red crystals
Melting point: 79.5°C
Boiling point: Decomposition at 259°C
Flash point: Relative self-ignition temperature (EU A.16) >105°C
Vapour pressure: 2.83 x $10^{-6}$ hPa (20°C)
Density: 1.32 g/$cm^3$ (20°C)
Viscosity: /
pKa: /
Refractive index: /

### 3.1.9. Stability

The stability of the test object (Methylgelb, WR 20883, batch: 6718, barrel 10/20 from OSL) in water was monitored over a total time period of seven days using HPLC-chromatography at the 460 nm detection wavelength. During the test procedure all stock solutions were stored at ambient temperature and protected from light.

The stability of hydroxyethyl-2-nitro-p-toluidine in a 0.03% solution in water was demonstrated to be acceptable (variation in concentration <3%).
Hydroxyethyl-2-nitro-p-toluidine in a ca. 10% solution in DMSO was stable (variation <7%) for seven days.
A typical hair dyeing formulation (Kolestone) containing 0.088% methylgelb was mixed with 1:1 with 6% hydrogen peroxide for checking the stability of methylgelb up to 45 min in the mixture. After 15, 30 and 45 min a portion of the mixture was analysed by HPLC-DAD using a standard methylgelb. The recovery of methylgelb from the mixture was shown to be ≥96%.

Comment
The hair dye formulation used for the stability testing contained only 088% (0.044% after mixing) methylgelb, while the marketed product may contain up to 2% methylgelb (1% after mixing with hydrogen peroxide).

**General comments on physico-chemical properties**

- Hydroxyethyl-2-nitro-p-toluidine is a secondary amine, and thus it is prone to nitrosation. The nitrosamine content in the test material is not reported.
- Stability of Hydroxyethyl-2-nitro-p-toluidine in typical hair dye formulations was not reported.

## 3.2. Function and uses

Hydroxyethyl-2-nitrotoluidine is used as a direct dye in hair dye formulations at a maximum concentration of 1%.

According to the applicant, Hydroxyethyl-2-nitrotoluidine is used as a non-reactive dye in oxidative hair dye formulations at a maximum concentration of 1%, after dilution with the oxidative agent.

## 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 per dose and sex; mouse: 10 per dose and sex
Test substance: Hydroxyethyl-2-nitro-p-toluidine
Batch: BRA1/285 (not given in study report, but in Submission III summary)
Purity: 99.6 area%, HPLC; 254 nm (not in study report, but stated in Submission III summary)
Doses: Single oral gavage. Rat: 900, 1700 and 2500 mg/kg bw
Mouse: 1000, 1500, 2000 and 2500 mg/kg bw
Vehicle: 10% gum Arabic solution
Observation period: 14 days
GLP: /
Study period: 11 January – 24 April 1985

This study was done in 1985. The protocol did not follow OECD Guideline 401, but is described as 'in analogy to Appraisal of the safety of chemicals in foods, drugs and cosmetics'. A Quality Assurance Statement, dated 12 September 1991, was included. The dose range was determined by a preliminary mouse study that indicated a median lethal dose between 1000 and 2000 mg/kg bw.

The animals were checked daily for clinical signs and deaths. Body weights were recorded on study Day 1, day 7 and Day 14. Post-mortems of all animals were conducted. Mortalities (Table below) occurred within 24h of administering the test substance.

| **Rat** | **Dose mg/kg bw** | **900** | **1700** | **2500** | |
|---|---|---|---|---|---|
| male | | 2/6 | 3/6 | 6/6 | |
| female | | 1/6 | 3/6 | 6/6 | |
| **Mouse** | **Dose mg/kg bw** | **1000** | **1500** | **2000** | **2500** |
| male | | 2/10 | 4/10 | 7/10 | 10/10 |
| female | | 0/10 | 4/10 | 6/10 | 10/10 |

Hydroxyethyl-2-nitro-p-toluidine caused reduced activity and orange coloration of the urine and extremities. Both effects had disappeared after 24 h.
No overt signs of toxicity were observed in survivors. Body weight increased at all doses. No post-mortem changes were noted.

Using the Spearman-Karber method, $LD_{50}$ were calculated as

| | | | | |
|---|---|---|---|---|
| **Rat** | male | 1564 mg/kg bw | female | 1436 mg/kg bw |
| **Mouse** | male | 1600 mg/kg bw | female | 1750 mg/kg bw |

Though this study does not follow Guideline 401, (1981), the results indicate that the acute oral toxicity of Hydroxyethyl-2-nitro-p-toluidine is low.

Ref.: 13, submission III
(ref 1, submission I)

#### 3.3.1.2. Acute dermal toxicity

Guideline: OECD 402 (1987)
Species/strain: Rat, Sprague Dawley Him: OFA SPF

| | |
|---|---|
| Group size: | 5 per dose and sex |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | B 3/89 |
| Purity: | 99.8%, (HPLC at 254 nm) |
| Doses: | 2000 mg/kg bw |
| Observation period: | 14 days |
| GLP: | in compliance |
| Study period: | July 1990 |

Hydroxyethyl-2-nitro-p-toluidine, moistened with water, was applied on a patch to the clipped skin (30 cm²). The test area was covered with tape. After 24 h exposure the test substance was wiped off. Clinical observations were made for 1, 10, and 30 min, 1, 2, 4, 6 hours and then at least twice daily for 14 days. Body weights were recorded on study Day 1, Day 7 and Day 14. Post-mortems of all animals were conducted.
There were no mortalities. Three animals of each sex displayed signs of general malaise (chromodacryorrhoea and ruffled fur) during the first 2 days. Body weight gain decreased in female rats over the 14 days.
Hydroxyethyl-2-nitro-p-toluidine indicated an $LD_{50}$ greater than 2000 mg/kg bw.

Ref.: 14, submission III
(ref 2, submission I)

#### 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 (1981) |
| Species/strain: | Rabbit, New Zealand White |
| Group size: | 3 females |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | B 3/89 |
| Purity: | 98% (study report): 99.8% (HPLC at 254nm) Submission III |
| Application: | Occlusive application of 0.5 g of test substance to ~ 6 $cm^2$ intact dorsal skin |
| Application time: | 4 h |
| GLP: | in compliance |
| Study period: | June 1990 |

The test substance was applied on a patch to a clipped area of dorsal skin. After 4 h, the residual test substance was wiped off. At 1, 24, 48 and 72 hours after patch removal, dermal irritation was scored and other local and systemic signs were examined.

Results
No general toxic effects were noted. Hydroxyethyl-2-nitro-p-toluidine had no irritant or corrosive effect on the intact rabbit skin at any time point in this study.

Ref.: 15, submission III
(ref 5, submission I)

#### 3.3.2.2. Mucous membrane irritation

| | |
|---|---|
| Guideline: | OECD 405 (1987) |
| Species/strain: | Rabbit, New Zealand White |
| Group size: | 3 females |

| | |
|---|---|
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | B 3/89 |
| Purity: | 98% (study report): 99.8% (HPLC at 254nm) Submission III |
| Application: | 0.1 ml of test substance into conjuctival sac |
| GLP: | in compliance |
| Study period: | June 1990 |

0.1 ml (~ 25-40 mg) Hydroxyethyl-2-nitro-p-toluidine was placed in the conjunctival sac of the left eye of each animal after pulling the lower lid away from the eyeball. The lids were gently held together for about one second to prevent loss. The right eye remained untreated and served as reference control.
The ocular reaction was assessed according to the numerical scoring system according to the EEC guideline 83/467 at approximately 1, 24, 48, 78 and 168 h after application.

Results
Minimal oedema of the conjunctivae was observed in one rabbit 1 h p.a. and minimal redness of the conjunctivae in another animal at 24 h p.a. No further irritant effects were noted at any reading time.

Conclusions
Hydroxyethyl-2-nitro-p-toluidine is considered to be not irritating to rabbit eye under experimental conditions.

Ref.: 16, submission III
(ref 4, submission I)

In Submission 1, a study in guinea pigs indicated that 1.5% Hydroxyethyl-2-nitro-p-toluidine in the conjunctival sac did not cause irritation.

(ref 3, submission I)

### 3.3.3. Skin sensitisation

**Local Lymph Node Assay**

| | |
|---|---|
| Guideline: | OECD 429 (2002) |
| Species/strain: | Mice CBA/J |
| Group size: | 5 females |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | 6718 FASS 10/20 |
| Purity: | 99.8% (HPLC at 254nm) |
| Concentrations: | 0, 0.5, 1.5, 5.0 and 10.0% (w/v) |
| Vehicles: | DMSO or water/acetone (1:1) mixed with olive oil (3:1) |
| Positive control: | p-phenylenediamine (1% in DMSO) |
| GLP: | In compliance |
| Study period: | 6 January – 24 June 2004 |

25 µl of 0 (vehicle only), 0.5, 1.5, 5.0 and 10.0% (equal to maximum solubility) in DMSO or in water/acetone (1:1) mixed with olive oil (3:1) or p-phenylenediamine (1% in DMSO) was applied to the mouse ear for 3 consecutive days.
Animals were checked daily before and after dosing for clinical signs and morbidity or death. Body weight was checked on Day –1 and 5. On Day 5, the mice were given an intravenous injection (phosphate buffer with 23.9 µCi $^{3}H$ methyl thymidine). Five hours later, all mice were killed by $CO_2$ inhalation and the draining lymph nodes were rapidly weighed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [$^{3}H$] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm was determined and the stimulation index was calculated, comparing with concurrent vehicle controls.

Results
There were no clinical signs or deaths. Minor body weight loss (in fewer than 10% animals) was not considered to be treatment related.
The mean stimulation index was not affected by Hydroxyethyl-2-nitro-p-toluidine treatment at any concentration or by either vehicle.

| | stimulation index | |
|---|---|---|
| | **DMSO** | **water/acetone/olive oil** |
| **Positive control PPD 1%** | 12.5 | - |
| **Hydroxyethyl-2-nitro-p-toluidine** | | |
| **0.5%** | 1.1 | 1.3 |
| **1.5** | 1.4 | 1.0 |
| **5.0** | 1.0 | 1.3 |
| **10.0** | 0.9 | 1.0 |

Hydroxyethyl-2-nitro-p-toluidine did not induce a biologically relevant immune response at any concentration with either vehicle and the stimulation index at each concentration was comparable with the vehicle controls. The $EC_3$ could not be calculated as the stimulation index was below 3.

The study authors did not consider Hydroxyethyl-2-nitro-p-toluidine as a skin sensitizer under these test conditions.

Ref.: 17, submission III

Comment
The highest concentration tested (10%) was too low for hazard identification. Therefore, a sensitising potential cannot be excluded.

In Submission 1, a Magnusson-Kligman study in guinea pigs was reported. Intradermal topical induction with 0.5% Hydroxyethyl-2-nitro-p-toluidine in water and challenge at 0.25% were performed. Slight erythema was observed at challenge (1/20 test animals; 2/20 controls) while no primary skin irritation occurred. Hydroxyethyl-2-nitro-p-toluidine was not considered a skin sensitizer under these test conditions.

Ref.: ref 7, submission I

### 3.3.4. Dermal / percutaneous absorption

**Submission IV, 2010**

**Percutaneous absorption *in vitro* (non-oxidative conditions)**

| | |
|---|---|
| Guideline: | OECD guideline 428 (2004) |
| Tissue: | Human skin (abdomen or breast; thickness: 380-400 µm) |
| Number of chambers: | 9 (from 4 donors) |
| Method: | Automated PTFE flow-through chambers |
| Chamber integrity | tritiated water |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine tested at a concentration of 1.0% in a typical hair dye formulation under non-oxidative conditions (DTF0744021AF01). |
| Batch: | 6718 Fass 10/20 |
| Purity: | 99.8% at 254 nm by HPLC |
| Area Dose: | 20 mg formulation/cm² |
| Receptor: | phosphate buffered saline with sodium azide (ca. 0.1% w/v) |
| Solubility: | 0.27% in water |
| Stability: | >2 weeks |
| Detection: | Liquid scintillation counting |

GLP: In compliance
Date: May 2010

The skin absorption of Hydroxyethyl-2-nitro-p-toluidine at the maximum concentration intended for hair colorants (1.0%), was investigated with human skin (abdomen and breast (400 µm)). An area dose of 20 mg/cm² of the final formulation (representing 0.2 mg/cm² of the dye) was applied once to the skin (0.64 cm²) in a commercial non-oxidative hair dye formulation in the presence of hydrogen peroxide.

Thirty minutes after substance application, the test item was removed by washing the skin three times with 0.32 ml water, then once with 0.32 ml washing solution (2% (v/v) sodium dodecyl sulphate) and again ten times with water. The skin surface was dried with three tissue swabs. The tissue swabs were retained in scintillation vials, mixed with methanol (1 ml) and scintillation fluid (10 ml) and analysed by liquid scintillation counting. The water and SDS solution were pooled in one pre weighed skin wash vial per skin sample and methanol (10 ml) was added. Duplicate weighed aliquots (1 ml) were removed from each skin wash vial, and measured by LC-MS/MS.
Receptor fluid was collected in 30 min fractions from 0 to 1 h post dose and hourly fractions from 1 to 6 h post dose and then in 2 hourly fractions from 6 to 72 h post dose. All receptor fluid samples were analysed by LC-MS/MS.
At termination of the experiment, the skin was washed as described above. The stratum corneum was removed with 20 successive tape strips. Each tape was placed into a separate vial and the sample analysed by LC-MS/MS.
The remaining epidermis and dermis were heat-treated and the epidermis was mechanically separated from the dermis. The dye content was determined in both skin compartments after extraction with methanol and analysed by LC-MS/MS.
To ensure the stability of Hydroxyethyl-2-nitro-p-toluidine in different matrices, extended solvent storage stability was tested in receptor fluid, skin wash solution, and solvents used for skin extracts, tape strips and tissue swap samples.

Results
The total recovery was within the range of 100 ± 15% of the applied dose for 9 skin samples regarding the total dye, and therefore confirmed the validity of the test. Therefore, the following results are based on 9 samples of skin from 4 donors.

| | Cell Number and Donor Number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cell 1 | *Cell 2* | Cell 3 | Cell 5 | Cell 6 | Cell 7 | Cell 9 | Cell 10 | *Cell 11* | *Cell 13* | Cell 14 | Cell 15 | | |
| | 0320 | *0320* | 0320 | 0317 | 0317 | 0317 | 0313 | 0313 | *0313* | *0298* | 0298 | 0298 | Mean | SD |
| Skin Wash 30 min | 197.22 | *155.70* | 199.39 | 167.87 | 200.03 | 193.61 | 201.08 | 166.97 | *159.07* | *150.14* | 181.63 | 172.25 | 186.67 | 14.50 |
| Tissue Swab 30 min | 3.01 | *1.76* | 1.03 | 2.85 | 7.03 | 2.13 | 2.12 | 0.38 | *2.13* | *3.66* | 1.53 | 0.50 | 2.29 | 2.01 |
| Pipette Tips 30 min | 1.17 | *0.12* | 0.02 | 0.10 | 0.03 | 0.55 | 0.20 | 0.04 | *0.04* | *0.22* | 0.02 | *0.01 | 0.24 | 0.39 |
| Dislodgeable Dose 30 min | 201.40 | *157.58* | 200.45 | 170.82 | 207.09 | 196.29 | 203.39 | 167.39 | *161.24* | *154.02* | 183.18 | 172.76 | 189.20 | 15.68 |
| Skin Wash 72 h | 0.42 | *0.88* | 0.33 | 0.41 | 0.38 | 0.63 | 0.47 | 0.87 | *0.38* | *0.28* | 0.42 | 0.54 | 0.50 | 0.17 |
| Tissue Swab 72 h | *0.02 | *0.12* | *0.00 | 0.18 | 0.40 | 0.04 | *0.00 | 0.13 | *0.04* | *0.02* | 0.04 | 0.24 | 0.12 | 0.14 |
| Pipette Tips 72 h | °0.00 | *°0.00* | °0.00 | °0.00 | °0.00 | °0.00 | °0.00 | °0.00 | *°0.00* | *°0.00* | °0.00 | °0.00 | 0.00 | 0.00 |
| Cell Wash | 0.11 | *0.08* | °0.01 | 0.34 | 0.54 | *0.05 | *0.01 | 0.15 | *0.13* | *0.63* | 0.79 | 0.35 | 0.26 | 0.27 |
| Dislodgeable dose 72 h | 0.54 | *1.09* | 0.34 | 0.93 | 1.32 | 0.73 | 0.48 | 1.15 | *0.55* | *0.93* | 1.25 | 1.13 | 0.88 | 0.37 |
| Total Dislodgeable Dose | 201.94 | *158.67* | 200.79 | 171.75 | 208.42 | 197.02 | 203.87 | 168.54 | *161.79* | *154.96* | 184.43 | 173.90 | 190.07 | 15.51 |
| Stratum Corneum 1-5 | *0.00 | *°0.01* | *0.01 | 0.01 | 0.01 | 0.04 | *0.00 | *0.00 | *°0.01* | *0.03* | 0.01 | 0.01 | 0.01 | 0.01 |
| Stratum Corneum 6-10 | °0.00 | *°0.01* | *0.00 | *0.01 | *0.01 | 0.02 | *0.00 | *0.01 | *°0.00* | *0.01* | *0.00 | *0.01 | 0.01 | 0.00 |
| Stratum Corneum 11-15 | *0.00 | *°0.00* | *0.00 | *0.00 | *0.01 | 0.01 | *0.00 | °0.00 | *°0.00* | *°0.01* | *0.00 | *0.00 | 0.00 | 0.00 |
| Stratum Corneum 16-20 | *0.00 | *°0.00* | *0.00 | *0.00 | *0.00 | 0.01 | *0.00 | °0.00 | *°0.00* | *°0.00* | *0.00 | *0.00 | 0.00 | 0.00 |
| Stratum Corneum | 0.01 | *0.02* | 0.01 | 0.02 | 0.03 | 0.08 | 0.01 | 0.01 | *0.01* | *0.05* | 0.02 | 0.02 | 0.02 | 0.02 |
| Unexposed Skin | *0.01 | *0.06* | *0.01 | *0.00 | °0.00 | °0.00 | 0.04 | °0.00 | *°0.02* | *0.03* | *0.03 | *0.01 | 0.01 | 0.01 |
| Total Unabsorbed | 201.96 | *158.75* | 200.80 | 171.78 | 208.45 | 197.10 | 203.92 | 168.55 | *161.82* | *155.04* | 184.48 | 173.92 | 190.11 | 15.51 |
| Epidermis | *0.00 | *°0.01* | *0.01 | *0.01 | *0.01 | *0.01 | *0.01 | °0.00 | *°0.01* | *°0.02* | *0.01 | *0.01 | 0.01 | 0.00 |
| Dermis | *0.02 | *°0.02* | *0.00 | 0.05 | *0.01 | *0.03 | *0.01 | °0.00 | *°0.01* | *0.09 | 0.07 | 0.06 | 0.03 | 0.03 |
| Receptor Fluid | 2.32 | *2.85* | 2.12 | 2.33 | 1.97 | 3.02 | 3.03 | 2.17 | *2.61* | *3.87* | 5.67 | 5.10 | 3.08 | 1.37 |
| Receptor Rinse | °0.00 | *°0.00* | °0.00 | *0.00 | *0.00 | *0.00 | °0.00 | °0.00 | *°0.00* | *°0.00* | *0.00 | *0.00 | 0.00 | 0.00 |
| Total Absorbed | 2.32 | *2.85* | 2.12 | 2.33 | 1.97 | 3.03 | 3.03 | 2.17 | *2.61* | *3.87* | 5.67 | 5.10 | 3.08 | 1.37 |
| Dermal Delivery | 2.34 | *2.88* | 2.13 | 2.38 | 1.99 | 3.07 | 3.05 | 2.18 | *2.63* | *3.99* | 5.75 | 5.17 | 3.12 | 1.39 |
| Mass Balance | 204.30 | *161.62* | 202.94 | 174.16 | 210.44 | 200.17 | 206.97 | 170.73 | *164.45* | *159.03* | 190.23 | 179.10 | 193.23 | 15.12 |

Cells 2, 11 and 13 rejected as mass balance outside 100±15%.
°= Value not quantifiable, limit of detection value entered.
*= Value less than the lower limit of quantification. Extrapolated value entered.

The majority of the applied dose of Hydroxyethyl-2-nitro-p-toluidine was rinsed off from the skin surface representing 95.27%.

A mean amount of 3.08 ± 1.37 µg/cm² (1.55 ± 0.68% of the applied dose) Hydroxyethyl-2-nitro-p-toluidine passed through the skin and was recovered in the receptor fluid during the 72 hour exposure.

Conclusion
Under the described test conditions (non-oxidative hair dye formulation), a total amount of 3.12 µg/cm² Hydroxyethyl-2-nitro-p-toluidine is obtained by summing up the amounts present in receptor fluid and in the epidermis and dermis. Consequently, this amount is considered as bioavailable.

Ref.: 1 (subm IV)

Comment
The study was well conducted and the mean+1SD may be used in calculation of MOS. Under non-oxidative conditions, the amount of hydroxyethyl-2-nitro-p-toluidine bioavailable from a hair dye formulation containing 1% of it is 4.45 µg/cm².

**Percutaneous absorption *in vitro* (oxidative conditions)**

| | |
|---|---|
| Guideline: | OECD guideline 428 (2004) |
| Tissue: | Human skin (abdomen or breast; thickness: 380-400 µm) |
| Number of chambers: | 10 (from 4 donors) |
| Method: | Automated PTFE flow-through chambers |
| Chamber integrity: | tritiated water. |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine tested at a concentration of 1.0% in a typical hair dye formulation under oxidative conditions (DTF0744021AF01). |
| Batch: | 6718 Fass 10/20 |
| Purity: | 99.8% at 254 nm by HPLC |
| Area Dose: | 20 mg formulation/cm² |
| Receptor: | phosphate buffered saline with sodium azide (ca. 0.1% w/v) |
| Solubility: | 0.27% in water |
| Stability: | >2 weeks |
| Detection: | Liquid scintillation counting |
| GLP: | In compliance |
| Date: | May 2010 |

The skin absorption of Hydroxyethyl-2-nitro-p-toluidine at the maximum concentration intended for hair colorants (1.0%) was investigated with human skin (abdomen and breast (400 µm)). An area dose of 20 mg/cm² of the final formulation (representing 0.2 mg/cm² of the dye) was applied once to the skin (0.64 cm²) in a commercial oxidative hair dye formulation in the presence of hydrogen peroxide.

Thirty minutes after substance application, the test item was removed by washing the skin three times with 0.32 ml water, then once with 0.32 ml washing solution (2% (v/v) sodium dodecyl sulphate) and again ten times with water. The skin surface was dried with three tissue swabs. The tissue swabs were retained in scintillation vials, mixed with methanol (1 ml) and scintillation fluid (10 ml) and analysed by liquid scintillation counting. The water and SDS solution were pooled in one pre weighed skin wash vial per skin sample and methanol (10 ml) was added. Duplicate weighed aliquots (1 ml) were removed from each skin wash vial, and measured by LC-MS/MS.
Receptor fluid was collected in 30 min fractions from 0 to 1 h post dose and hourly fractions from 1 to 6 h post dose and then in 2 hourly fractions from 6 to 72 h post dose. All receptor fluid samples were analysed by LC-MS/MS. However, two occasions of measurements were

done, since in the first dosing occasion, the results from only 4 cells were acceptable due to incomplete fraction collections. Therefore, a second dosing occasion was required, testing a further 8 samples. In total 12 skin samples were used.
At termination of the experiment, the skin was washed as described above. The stratum corneum was removed with 20 successive tape strips. Each tape was placed into a separate vial and the sample analysed by LC-MS/MS.
The remaining epidermis and dermis were heat-treated and the epidermis was mechanically separated from the dermis. The dye content was determined in both skin compartments after extraction with methanol and analysed by LC-MS/MS.

To ensure the stability of Hydroxyethyl-2-nitro-p-toluidine in different matrices, extended solvent storage stability was tested in receptor fluid, skin wash solution, and solvents used for skin extracts, tape strips and tissue swap samples.

Results
The total recovery was within the range of 100 ± 15% of the applied dose for 10 skin samples regarding the total dye. Therefore, the following results are based on 10 samples of skin from 4 donors.

| | Cell Number and Donor Number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Cell 37* | *Cell 38* | Cell 39 | Cell 42 | Cell 58 | Cell 59 | Cell 60 | Cell 63 | Cell 64 | Cell 65 | Cell 68 | Cell 69 | | |
| | *0320* | *0320* | 0320 | 0317 | 0313 | 0313 | 0313 | 0298 | 0298 | 0298 | 0317 | 0317 | Mean | SD |
| Skin Wash 30 min | *155.55* | *163.35* | 176.05 | 177.12 | 184.25 | 170.59 | 173.96 | 181.87 | 175.92 | 165.57 | 168.05 | 170.80 | 174.42 | 5.88 |
| Tissue Swab 30 min | *1.78* | *1.47* | 2.77 | 2.97 | 2.68 | 1.32 | 0.77 | 1.88 | 0.68 | 8.59 | 1.24 | 2.55 | 2.54 | 2.29 |
| Pipette Tips 30 min | *0.08* | *0.11* | 0.04 | 0.42 | 0.17 | 0.20 | 0.09 | 0.21 | 0.17 | 0.21 | 0.13 | 0.25 | 0.19 | 0.10 |
| Dislodgeable Dose 30 min | *157.41* | *164.94* | 178.86 | 180.51 | 187.10 | 172.11 | 174.82 | 183.95 | 176.76 | 174.37 | 169.41 | 173.60 | 177.15 | 5.48 |
| Skin Wash 72 h | *0.28* | *0.30* | 0.29 | 0.60 | 0.20 | 0.14 | 0.24 | 0.21 | 0.38 | 0.85 | 0.14 | 1.22 | 0.43 | 0.36 |
| Tissue Swab 72 h | **0.01* | **0.03* | *0.02 | 0.07 | 0.04 | *0.00 | *0.01 | 0.14 | 0.05 | 0.30 | *0.01 | 0.08 | 0.07 | 0.09 |
| Pipette Tips 72 h | °*0.00* | °*0.00* | °0.00 | *0.00 | °0.00 | °0.00 | °0.00 | °0.00 | *0.00 | °0.00 | °0.00 | °0.00 | 0.00 | 0.00 |
| Cell Wash | *0.49* | *0.11* | 0.07 | 0.10 | 0.34 | 0.64 | 1.19 | 0.22 | 0.09 | 0.33 | 0.07 | 0.45 | 0.35 | 0.35 |
| Dislodgeable dose 72 h | *0.77* | *0.44* | 0.39 | 0.77 | 0.59 | 0.79 | 1.44 | 0.58 | 0.52 | 1.48 | 0.22 | 1.74 | 0.85 | 0.52 |
| Total Dislodgeable Dose | *158.18* | *165.38* | 179.24 | 181.28 | 187.69 | 172.90 | 176.25 | 184.53 | 177.28 | 175.85 | 169.63 | 175.34 | 178.00 | 5.38 |
| Stratum Corneum 1-5 | *0.01* | *0.02* | 0.01 | 0.13 | 0.01 | *0.00 | 0.01 | 0.01 | *0.00 | 0.03 | 0.01 | *0.00 | 0.02 | 0.04 |
| Stratum Corneum 6-10 | *0.01* | *0.01* | *0.01 | 0.05 | *0.01 | *0.00 | *0.00 | *0.00 | *0.00 | 0.01 | *0.01 | *0.00 | 0.01 | 0.02 |
| Stratum Corneum 11-15 | **0.00* | *0.01* | *0.01 | 0.03 | °0.00 | *0.00 | *0.00 | °0.00 | *0.00 | 0.01 | °0.00 | *0.00 | 0.01 | 0.01 |
| Stratum Corneum 16-20 | **0.00* | **0.01* | 0.01 | 0.03 | °0.00 | °0.00 | °0.00 | °0.00 | °0.00 | *0.00 | °0.00 | N.S. | 0.01 | 0.01 |
| Stratum Corneum | *0.03* | *0.05* | 0.03 | 0.24 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.06 | 0.02 | 0.01 | 0.04 | 0.07 |
| Unexposed Skin | °*0.00* | °*0.00* | *0.02 | *0.02 | °0.00 | °0.00 | °0.00 | *0.01 | *0.00 | °0.00 | °0.00 | °0.00 | 0.01 | 0.01 |
| Total Unabsorbed | *158.22* | *165.43* | 179.30 | 181.54 | 187.71 | 172.91 | 176.27 | 184.55 | 177.29 | 175.91 | 169.65 | 175.35 | 178.05 | 5.39 |
| Epidermis | **0.00* | **0.02* | *0.01 | 0.05 | °0.00 | °0.00 | °0.00 | °0.00 | °0.00 | *0.00 | °0.00 | N.S. | 0.01 | 0.02 |
| Dermis | *0.06* | *0.07* | 0.11 | 0.18 | 0.05 | 0.07 | 0.14 | 0.05 | 0.06 | 0.06 | *0.01 | 0.05 | 0.08 | 0.05 |
| Receptor Fluid | *3.20* | *3.10* | 3.98 | 3.51 | 3.47 | 5.72 | 6.22 | 3.98 | 4.06 | 3.76 | 1.92 | 2.95 | 3.96 | 1.24 |
| Receptor Rinse | °*0.00* | °*0.00* | °0.00 | 0.00 | °0.00 | *0.00 | *0.00 | °0.00 | °0.00 | *0.00 | °0.00 | °0.00 | 0.00 | 0.00 |
| Total Absorbed | *3.20* | *3.10* | 3.98 | 3.51 | 3.47 | 5.72 | 6.22 | 3.98 | 4.06 | 3.76 | 1.92 | 2.95 | 3.96 | 1.24 |
| Dermal Delivery | *3.26* | *3.19* | 4.10 | 3.74 | 3.53 | 5.79 | 6.36 | 4.03 | 4.12 | 3.83 | 1.93 | 3.00 | 4.04 | 1.27 |
| Mass Balance | *161.48* | *168.62* | 183.40 | 185.28 | 191.24 | 178.70 | 182.63 | 188.58 | 181.41 | 179.74 | 171.58 | 178.35 | 182.09 | 5.58 |

Cells 37 and 38 rejected as mass balance outside 100±15%.
°= Value not quantifiable, limit of detection value entered.
*= Value less than the lower limit of quantification. Extrapolated value entered.
NS = not sampled.

The majority of the applied dose of Hydroxyethyl-2-nitro-p-toluidine was rinsed off from the skin surface representing 96.11%. A maximum amount of 3.96 ± 1.24 µg/cm² (2.14 ± 0.72% of the applied dose) Hydroxyethyl-2-nitro-p-toluidine passed through the skin and was recovered in the receptor fluid during the 72 hour exposure.

Conclusion
Under the described test conditions (oxidative hair dye formulation), a total amount of 4.04 µg/cm² Hydroxyethyl-2-nitro-p-toluidine is obtained by summing up the amounts present in receptor fluid and in the epidermis and dermis. Consequently, this amount is considered as bioavailable.

Ref.: 1 (subm. IV)

Comment
The study was well conducted and the mean+1SD may be used in calculation of MOS. Under oxidative conditions, the amount of hydroxyethyl-2-nitro-p-toluidine bioavailable from a hair dye formulation containing 1% of it is 5.20 µg/cm².

**Percutaneous penetration *in vitro***

| | |
|---|---|
| Guideline: | OECD–Draft Guideline "Skin absorption: in vitro method" (1999) |
| Tissue: | porcine back skin (thickness: 1000 µm) |
| Method: | flow through diffusion chambers |
| Test substance: | hydroxyethyl-2-nitro-p-toluidine tested in a commercial hair dye formulation N°: 73910030100. |
| Batch: | I901003 |
| Purity: | not documented |
| Stability: | warranted for 30 months at room temperature |
| Concentration: | 0.63%<br>0.626 mg/cm² tested as part of a hair dye formulation |
| No. of chambers: | 5 diffusion cells contact 30 min. diffusion during 24 hours<br>5 diffusion cells, contact 30 min. diffusion during 72 hours<br>5 diffusion cells, contact 30 min., 3 repeated application at 24 hours intervals, diffusion during 72 hours |
| GLP: | In compliance |
| Study period: | 23 November 1999 – 28 February 2000 |

Percutaneous absorption was investigated with pig skin dermatomed (1000 µm thick). The integrity of the skin was monitored at the beginning of the experiment using tritiated water. Dye was applied to the skin in a commercial hair dye formulation (0.626 mg/cm²). The receptor solution (physiological phosphate buffer containing NaCl and antibiotics and 3% ethanol) was pumped through the receptor chamber at a rate of 2.5 ml/h. Six chambers per experimental group were investigated (5 diffusion cells treated, 1 diffusion cell as a negative control).

Thirty minutes after the substance application, the test item was removed by washing the skin in six steps: twice with 0.5 ml water, then once with 0.5 ml washing solution (shampoo-formulation) and again three times 0.5 ml with water. The washing solutions were combined and the amount of dye was determined by HPLC. The receptor fluid was sampled and analyzed by HPLC. At termination of the experiment, the skin was separated as two parts: "upper skin" (stratum corneum and upper stratum germinativum) and "lower skin" (lower stratum germinativum and upper dermis). Then the skin samples were extracted and the dye content quantified.

Results
The majority of hydroxyethyl-2-nitro-p-toluidine remained on the skin surface representing 96.77 ± 2.48% to 106.33 ± 3.23% of the applied dose.
The total skin content (24 hours/1 application) is 0.088 ± 0.043% of the dose (548 ± 266 ng/cm²), the receptor fluid content is 0.199 ± 0.180% of the dose (1246 ± 1126 ng/cm²)
The total skin content (72 hours/1 application) is 0.096 ± 0.032% of the dose (601 ± 199 ng/cm²), the receptor fluid content is 0.313 ± 0.056% of the dose (1961 ± 350 ng/cm²)
The total skin content (72 hours/3 applications) is 0.228 ± 0.358% of the dose (4278 ± 6731 ng/cm²), the receptor fluid content is 0.194 ± 0.026% of the dose (3644 ± 484 ng/cm²)

Conclusion
In this experiment, hydroxyethyl-2-nitro-p-toluidine was used at a concentration of 0.63%, which is below the maximum concentration intended for hair colorants (1%).
The study was conducted with a mixture of two hair dyes, hydroxyethyl-2-nitro-p-toluidine and HC Blue 12, in the same formulations. This procedure is not acceptable because no information concerning the behaviour of this mixture is available.
The stratum corneum was not isolated from the viable epidermis; therefore the amount of dye present in the skin and bioavailable cannot be evaluated.

The huge variation obtained within the data clearly demonstrates that this way of measuring does not provide a suitable skin penetration evaluation for the risk assessment of hydroxyethyl-2-nitro-p-toluidine.
The study is considered inadequate.

Ref.: 18, submission III

**Percutaneous absorption *in vivo***

| | |
|---|---|
| Guideline: | / |
| Species: | Rat, Sprague-Dawley: OFA, SPF |
| Group: | 3 per dose/sex |
| Test substance: | $^{14}C$ labelled 4-(2'-hydroxyethyl)-amino-3-nitromethylbenzene |
| Batch: | / |
| Purity: | radiochemical purity 97% |
| Doses/Applications: | Application area 3 x 3 $cm^2$. Three preparations used:<br>- formulation without hydrogen peroxide 1%<br>- formulation with hydrogen peroxide 0.50%<br>- 3.33% solution in DMSO in water 5/4. |
| Schedule: | single cutaneous application of 30 minutes. |
| Observation: | 72 h |
| GLP: | not in compliance |
| Study period: | April 1986 |

$^{14}C$ labelled sA, included in two different hair dye formulations (fA and fB) or dissolved in DMSO/water (5/4) at a concentration of 3.33%, was applied to the clipped dorsal skin of three male and three female Sprague Dawley rats. After 30 min. the substance was washed off with shampoo, water and absorbent cellulose tissue. Rinsing was continued until the rinsing water and tissues were free of colour. The skin was covered with gauze for 72 h, after which the animals were killed. Radioactivity of rinsings treated skin, urine, faeces, organs (13) and carcass was estimated by liquid scintillation counting.

Results
The majority of the applied $^{14}C$ (97.8 to 99.7%) was removed from the skin by rinsing after the cutaneous treatment. The mean $^{14}C$ content of the skin at the application site was 0.29% (fA), 0.55% (fB) and 0.18% (sA solution) of the applied $^{14}C$. The mean percutaneous absorptions were 0.21% for fA and 0.24% for fB. The absorption of sA in DMSO/water was significantly higher: 0.69% of the applied $^{14}C$. Excretion: After cutaneous application means of 0.21% (fA), 0.23% (fB) and 0.70% (sA in DMSO/water) of the applied $^{14}C$ were recovered in urine and faeces within 72 h. 80 to 85% of the absorbed amount of sA was excreted in urine and 14 to 19% in faeces. 85 to 93% of the totally absorbed amount was excreted in the first 24 h after application. Carcass: The remaining mean amounts of $^{14}C$ in the carcass 72 h after application were near the detection limit and varied from 0.0025% to 0.0042% of the administered dose. Organs: 72 h after application mean concentrations of $^{14}C$ were near or below the detection limits in all organs. The detection limits were about 0.00002%/g for large organs, 0.00025%/g for small organs. Relatively highest concentrations were noted in fat (fB), thyroid (fA), liver (fA, sA solution), skin (sA solution), spleen (fB) and kidney (sA solution,). No accumulation of $^{14}C$ was observed.

Conclusion
The *in vivo* percutaneous absorption study ref.: 19 (dated August 1986), has been previously evaluated in the SCCNFP opinion of 1993 (ref. 8). No additional data was provided.
The study does not comply with modern Guidelines.

Ref.: 19, submission III
(ref 8, submission I)

## 3.3.5. Repeated dose toxicity

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

**Repeated Dose (28 days) oral toxicity**

| | |
|---|---|
| Guideline: | OECD 407 (1981) |
| Species/strain: | Rat, Wistar; Crl: (Wi)/Br (SPF) |
| Group size: | 5 per dose and sex |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | M266/4279, Faß 70/22 |
| Purity: | 100%, HPLC |
| Doses: | 0, 80, 240 and 720 mg/kg bw/day oral gavage, 7days/week |
| Vehicle: | 0.5% carboxymethylcellulose in distilled water (CMC) |
| Dosing period: | 28 days |
| Observation period: | Satellite recovery group (control and high dose) 28 + 14 days |
| GLP: | In compliance |
| Study period: | 25 March – 6 May 1992 |

The test substance suspensions were made daily immediately before dosing. The daily amounts needed were suspended in CMC. The stability of the suspensions of the test substances was unknown.
The animals were checked daily for clinical signs, behavioural changes and deaths. Body weights and food consumption were recorded weekly. Ophthalmological examinations were performed before treatment and on day 28.
Clinical biochemistry and haematology were performed on study days –1 and 28, and in the satellite group on day 42. Urine was collected between study days –1/0 and 27/28, and in the satellite group on day 41/42.
Post-mortems of all animals were conducted. Adrenals, kidneys and liver were weighed, together with the testes in the males. Various tissues were fixed for further examination. The histopathology of the adrenals, kidneys, liver, heart and spleen from the control and high dose groups was examined. In addition, histopathology of all gross lesions was also performed.

Results
The homogeneity of the test solutions of Hydroxyethyl-2-nitro-p-toluidine was found to deviate less than 5%. The concentrations were between 94 –103% of nominal values, so the nominal values were used throughout the study.
There were no treatment-related deaths. In the high dose group there was increased salivation and soft faeces. All treated animals had stained fur, tails and bedding.
Body weights of all treated groups were comparable to the control group, even in the high dose females that had decreased food consumption during dosing and increased food consumption in males in the satellite group recovery period.
In the high dose group, males showed a decrease in mean corpuscular haemoglobin concentration (MCHC) and lymphocytes but increased neutrophils. Females showed a decrease in haemoglobin concentration (Hb) and erythrocytes. There was a statistically significant decrease in the haematocrit values in the mid-dose females but only a slight decrease in the high dose females.
In the high dose group, males showed a statistically significant increase in calcium, alkaline phosphatase, GOT, GPT and total bilirubin and decreases in glucose and cholesterol, whereas in the females there was only a slight increase in alkaline phosphatase. By the end of the recovery period, GOT and GPT were still statistically significant elevated in males.
Urinalysis showed low pH in both sexes. However, it was statistically significant lower in males. This persisted till the end of the recovery period. The urine was intensively stained (dark yellow to dark red). This indicated renal excretion of the dye. This colouration made it impossible to use test sticks for determination of all scheduled parameters [mid dose: 2/5 male and 1/5 female; high dose 4/5 male and 3/5 female] and for determination of

ketones, urobilinogen and bilirubin [low dose: 2/5 male and 1/5 female; mid dose: 4/5 male and 5/5 female; high dose: 5/5 male and 5/5 female]. Submission III stated that normal urine parameters were not altered, but there was no evidence in the study report.
Post mortem showed tissue staining, particularly of fat and especially in the high dose group. Statistically significant changes at the high dose were lower absolute adrenal weights in females and relative liver weights in males. The thymus was stained red in one animal from both the mid and high dose male groups. Kidney weight was affected but there was no dose response or consistency. There were no other treatment related findings.
The NOAEL was deduced to be 240 mg/kg bw/day from this study.

Ref.: 20, submission III

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

**Sub-chronic (90 days) oral toxicity**

| | |
|---|---|
| Guideline: | OECD 408 (1981) |
| Species/strain: | Rat, Sprague Dawley Crl:CD (SD) BR |
| Group size: | 10 per dose and sex |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | BRA 1/315 (not in study report, but stated in Submission III summary) |
| Purity: | 99.7%, HPLC |
| Doses: | 0, 10, 45 and 90 mg/kg bw/day oral gavage, 7days/week |
| Vehicle: | distilled water |
| Dosing period: | 13 weeks |
| GLP: | / |
| Study period: | 30 July – 31 October 1985 |

The test substance suspensions were made freshly daily before dosing. During the dosing period, the test suspensions were constantly stirred and used within 2 h of preparation. On the basis of this, checking the stability of the test substance suspensions were considered unnecessary by the study authors.
The animals were checked twice daily for clinical signs, behavioural changes and deaths. Body weights and food consumption were recorded weekly. Ophthalmological examinations were performed before and at the end of dosing period.
Clinical biochemistry, haematology and urinalysis were performed on study day –1 and during week 13 in the control and high dose animals.
Post-mortems of all animals were conducted. Adrenals, brain, heart, kidneys, liver, ovaries, pituitary, spleen, testes and thyroid were weighed. Various tissues were fixed for further examination. The histopathology of a wide range of tissues was performed from the control and high dose groups, in addition, to all gross lesions noted.

Results
No deaths occurred. Bedding of treated animals showed dose-related orange-yellow staining, but no mention of staining of fur and tail. There was a slight reduction of both overall body weight gain and food consumption in males of the 10 and 90 mg/kg bw/day groups.
Haematological chemistry data did not show dose-related changes. In the 90 mg/kg bw/day group, one female showed hyaline casts during urine investigation at week 13.
At post-mortem, the 90 mg/kg bw/day group showed slight decrease in absolute and relative liver weights compared with the controls. Macroscopically, the kidneys in the treated groups showed a loamy colour with deposits and dilatation of renal pelvis in the 90 mg/kg bw/day group. There were no histopathological treatment-related changes observed.

Ref.: 21, submission III
(ref 9, submission I)

Comment

The study authors suggested that the NOAEL could be deduced as 90 mg/kg bw/day. Based on the effects on the kidney, the SCCS considered the NOAEL should be 45 mg/kg bw/day.

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

No data submitted

### 3.3.6. Mutagenicity / Genotoxicity

#### 3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

**Bacterial gene mutation assay**

| | |
|---|---|
| Guideline: | OECD 471 |
| Species/strain: | *Salmonella typhimurium*, TA98, TA100, TA102, TA1535, TA1537 |
| Replicates: | triplicate cultures in two independent tests |
| Test substance: | WR 20883 Methylgelb |
| Batch: | 6718 Fass 10/20 |
| Purity: | 99.8 area% (HPLC; 254 nm) |
| Vehicle: | DMSO |
| Concentrations: | 3, 10, 33, 100, 333, 1000, 2500 and 5000 μg/plate without and with metabolic activation |
| Treatment: | experiment I: plate incorporation test with 48 h incubation<br>experiment II: pre-incubation method with 60 minutes pre-incubation |
| GLP: | in compliance |
| Study period: | 22 January-12 February 2004 |

WR 20883 Methylgelb was investigated for the induction of gene mutation in *Salmonella typhimurium*. Liver S9 fraction from rats induced with phenobarbital/ß-naphthoflavone was used as the exogenous metabolic activation system. On the basis of a preliminary toxicity study, the maximum recommended concentration of 5000 μg/plate was used in the main study. Since in the pre-experiment evaluable plates at five concentrations or more are used in all strains, the pre-experiment is reported as main experiment I. Negative and positive controls were in accordance with the OECD guideline.

Results
In experiment I, precipitation of the test substance was observed from 2500 μg/plate up to 5000 μg/plate with and without S9-mix in all strains used. WR 20883 Methylgelb induced toxic effects (i.e. a reduced background growth and/or reduced frequency of revertants) in the absence and in the presence of S9-mix. Substantial increases in revertant colony numbers of any of the 5 tester strains were not observed following treatment with WR 20883 Methylgelb at any concentration level, neither in the presence or absence of S9-mix.

Conclusion
Under the experimental conditions used WR 20883 Methylgelb was not mutagenic in the bacterial gene mutation assay.

Ref.: 22, submission III

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

| | |
|---|---|
| Guideline: | OECD 476 |
| Cells: | L5178Y mouse lymphoma cells ($tk^{+/-}$) |
| Replicates: | 2 independent tests (experiment II without S9 mix) |
| Test substance: | WR 20883 Methylgelb |
| Batch: | 6718 Fass 10/20 |

| | | |
|---|---|---|
| Purity: | 99.8 area% (HPLC; 254 nm) | |
| Vehicle: | DMSO | |
| Concentrations: | experiment I: | 31.3, 62.5, 125, 250 and 375 μg/ml without and with metabolic activation |
| | experiment Ia: | 31.3, 62.5, 125, 250, 300 and 375 μg/ml without and with metabolic activation |
| | experiment II: | 31.3, 62.5, 125, 250 and 375 μg/ml without metabolic activation |
| Treatment: | experiment I and Ia: | 4 h without and with S9-mix; expression period 72 h, selection growth 10-15 days |
| | experiment II: | 24 h without S9-mix; expression period 48 h, selection growth 10-15 days |
| GLP: | in compliance | |
| Study period: | 15 December-01 March 2004 | |

WR 20883 Methylgelb has been investigated for induction of gene mutations at the *tk*-locus in L5178Y mouse lymphoma cells after exposure for 4 hours without and with metabolic activation (experiment I) and after exposure for 24 hours without S9-mix (experiment II). Liver S9 fraction from phenobarbital/ß-naphthoflavone-induced rats was used as the exogenous metabolic activation system. Test concentrations were based on the level of toxicity in an initial range-finding study. Negative and positive controls were in accordance with the OECD guideline.

Results
The required toxic range of 10-20% of the relative total growth was generally reached. In the first experiment without S9 mix, an increase in the mutant frequency was measured at a concentration of 250 µg/ml. However, no induction of mutants was observed in the parallel culture and in the culture treated with a higher concentration (375 µg/ml) causing very high toxicity (4% relative total growth). Moreover the data of the second culture with metabolic activation were not considered valid since the positive control failed to respond. Therefore experiment I was repeated.
A substantial biological relevant increase in the mutant frequency was not observed in any of the main experiments. No relevant shift of the ratio of small versus large colonies was recorded up to the maximum concentration of the test item.

Conclusion
Under the experimental conditions used, WR 20883 Methylgelb was not mutagenic in mammalian cells (L5178Y mouse lymphoma cells) *in vitro*.

Ref.: 24, submission III

**_In vitro_ micronucleus test**

| | | |
|---|---|---|
| Guideline: | OECD 487 (draft) | |
| Cells: | Peripheral human blood lymphocytes from two donors | |
| Replicates: | duplicate cultures in 2 independent tests | |
| Test substance: | Methylgelb (WR20883) | |
| Batch: | 6718 Fass 10/20 | |
| Purity: | 99.8% (HPLC, 254 nm) | |
| Vehicle: | DMSO | |
| Concentrations: | experiment 1: | 210.7, 263.3, 329.2 and 411.5 μg/ml without S9-mix<br>682.6, 718.5 and 756.3 μg/ml with S9-mix |
| | experiment 2: | 254.5, 349.2 and 479.0 μg/ml without S9-mix<br>750.0, 875.0 and 950.0 μg/ml with S9-mix |
| Treatment | 20 h treatment without S9-mix, harvest time 48 hours after the beginning of treatment | |

| | |
|---|---|
| | 3 h treatment without S9-mix, harvest time 48 hours after the beginning of treatment |
| GLP: | in compliance |
| Study period: | 2 June – 11 August 2004 |

Methylgelb (WR20883) 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 Aroclor 1254-induced rats was used as the exogenous metabolic activation system. Reduction in the replication index (RI) was taken as a measure for cytotoxicity. In experiment I, treatment started 24 hours after PHA stimulation. Cultures were treated for 20 hours without S9-mix or for 3 hours with S9-mix. Preparation time point was 48 hours after the start of the treatment. Experiment II was similar but treatment started 48 hours after mitogen stimulation. Negative and positive controls were in accordance with the OECD guideline.

Results
The highest concentrations tested caused the required reduction in the RI. Methylgelb (WR20883) did not induce an increase in cells with micronuclei in both experiments in the absence of S9-mix. There was a statistically significant increase in the cells with micronuclei in experiment I with S9-mix at two concentrations (718.5 and 756.3 µg/ml). However, the effect was small and within the range of historical negative controls and it was not reproducible in experiment II. Therefore, this equivocal effect is considered not to be biologically relevant.

Conclusion
Under the experimental conditions used, Methylgelb (WR20883) was not genotoxic (clastogenic or aneugenic) in this micronucleus test with cultured human peripheral lymphocytes.

Ref.: 23, submission III

### 3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

**Mouse bone marrow micronucleus test**

| | |
|---|---|
| Guideline: | OECD 474 (2001) |
| Species/strain: | Mouse, NMRI |
| Group size: | 5 males and 5 females |
| Test substance: | Methylgelb - 20883 |
| Batch: | 6718 |
| Purity: | 99.8% (HPLC; 254 nm) |
| Vehicle: | PEG 400 |
| Dose levels: | 187.5, 375, 750 mg/kg bw/day (three consecutive treatments by gavage, 24 hour interval) |
| Sacrifice time: | 24 hours after the last treatment |
| GLP: | in compliance |
| Study period: | 22 April-12 July 2002 |

Methylgelb – 20883 has been investigated for induction of micronuclei in the bone marrow cells of mice. Dose selection was based on the result of a pre-experiment for toxicity in which 2 mice were orally (on 3 consecutive days at 24 h intervals) exposed to 1000 mg/kg bw/day. The animals were examined for acute toxic symptoms at intervals of around 1, 2-4, 6, and 24 h after each administration of Methylgelb - 20883. In the main experiment mice were treated on 3 consecutive days at 24 h intervals (positive control only single treatment). The animals of the highest dose group were examined for acute toxic symptoms at intervals around 1, 2-4, 6 and 24 h after treatment. Bone marrow cells were collected 24h after final treatment. Toxicity and thus exposure of the target cells was determined by

measuring the ratio between polychromatic and total erythrocytes (PCE/TE ratio). Bone marrow preparations were stained and examined microscopically for the NCE/TE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline. The concurrent positive control group was sampled 24 hours after a single dose.

Results
In the pre-experiment for toxicity, exposure to 1000 mg/kg bw/day Methylgelb – 20883 resulted in the death of 1 male and 1 female mouse after the second treatment. Many animals of both 1000 and 750 mg/kg bw/day expressed toxic effects like reduction of spontaneous activity, abdominal position, eyelid closure, ruffled fur and apathy. These effects were strongest and observed in the highest number of animals after the second application. In the main experiment similar toxic effects were observed with an almost identical timing. Additionally, orange coloured urine was reported in treated animals 24 h after the first, from 6 h after the second and after the third treatment. The signs of toxicity and the discoloured urine indicated systemic distribution of the compound and thus exposure of the bone marrow. However, the PCE/TE ratio was not affected by the treatment.
In comparison to the concurrent vehicle controls there was no biologically relevant or statistically significant increase in the number of erythrocytes with micronuclei at any preparation interval and dose level. The positive control substance gave the expected result.

Conclusion
Under the experimental conditions used Methylgelb – 20883 did not induce micronuclei in erythrocytes of treated mice and, consequently, Methylgelb – 20883 was not genotoxic (clastogenic and/or aneugenic) in erythrocytes of mice.

Ref.: 25, submission III

### 3.3.7. Carcinogenicity

One publication giving only a summary of the results evaluating toxicity and carcinogenicity by skin painting with formulations of hair dyes is available. 60 male and 60 female Eppley Swiss mice were painted three times a week for 20 months with 0.05 ml of a mixture of different dyes containing 0.3% Hydroxyethyl-2-nitro-p-toluidine. No conclusions with regard to carcinogenicity could be drawn from this study.

Ref.: 26, submission III
(ref 15, submission I)

### 3.3.8. Reproductive toxicity

#### 3.3.8.1. Two generation reproduction toxicity

No data submitted

#### 3.3.8.2. Teratogenicity

| | |
|---|---|
| Guideline: | OECD 414 (1984) |
| Species/strain: | Rat, Sprague Dawley Him:OFA (SPF) |
| Group size: | 24 pregnant female per dose |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | / (not specified in study but given as BRA 1/315, Submission III) |
| Purity: | / (not specified in study but given as 99.7%, HPLC, Submission III) |
| Doses: | 0, 10, 30 and 60 mg/kg bw/day oral gavage, 7days/week |
| Vehicle: | 0.5% CMC in distilled water |
| Dosing period: | Gestation Days (GD) 6 -15 |
| GLP: | / |
| Study period: | 25 March – 28 April 1985 |

The test substance suspensions were made daily before dosing.
The animals were checked daily for clinical signs, behavioural changes and deaths. Body weights were recorded GD 0, 6, 11, 16 and 20. Food consumption was measured for GD 0 – 6, 6 – 11, 11 –16, and 16 –20 and calculated for the entire study.
Post-mortems of all animals were conducted on GD 20. Adrenals, brain, heart, kidneys, liver, ovaries and intact uterus removed and examined for corpora lutea, implantation sites, the presence of resorption sites (early and late) and foetuses (live, dead and position). Live foetuses were weighed sexed and checked for gross malformations. Skeletal and visceral staining of 50% of the foetuses was examined. Placental weights were recorded.

Results
Maternal body weight gain and food consumption of the lowest dose group were slightly increased when compared with all other groups. Reproduction data showed no significant or dose related differences between the groups. Hydroxyethyl-2-nitro-p-toluidine did cause no maternal toxicity.
Foetal examination of the 60 mg/kg bw group showed significantly more foetuses with a dilatation of the oesophagus. This has no functional relevance. No further treatment related effects on the foetuses were observed.
Malformation frequencies were highest in the control group. No embryotoxic effects and no structural irreversible effects were observed.

Ref.: 27, submission III
(ref 14, submission I)

Comment
The data seemed scientifically sound, but since the highest dose did not cause either maternal or foetal toxicity the study has limited value. The stability of the test substance suspensions in 0.5% CMC in distilled water was not given.

### 3.3.9. Toxicokinetics

**Bioavailability across intestinal barrier in TC-7 (human intestinal epithelial) cells**

| | |
|---|---|
| Guideline: | / |
| Cells: | TC-7 (human intestinal epithelial) |
| Test substance: | Hydroxyethyl-2-nitro-p-toluidine |
| Batch: | 6718 Fass 10/20 |
| Purity: | not specified in study but given as 99.8%, HPLC 254 nm, submission III |
| Test concentration: | 50 µM |
| Vehicle: | HBSS buffer with 1% DMSO |
| Incubation: | 60 min |
| GLP: | / |
| Study period: | 25 March – 5 April 2004 |

There is no official guideline for this *in vitro* method but it was performed according to ECVAM recommendations. Two independent experiments were done.
The bioavailability of Hydroxyethyl-2-nitro-p-toluidine across the intestinal barrier was investigated at $37^0$C in shaken 96-well plates for 60 min. The permeability from the apical (pH 6.5) to basolateral (pH 7.4) side of the epithelium was measured by HLPC-MS/MS and the apparent permeability coefficient ($P_{app}$) was calculated for the two experiments. $^{14}$C-mannitol (~4µM) was used to show the integrity of the cell monolayers; only monolayers with a permeability of <2x $10^{-6}$ cm/sec were used. Propranolol, vincristine and ranitidine were used as concurrent reference material to show validity of the test system. Ranitidine that has 50% absorption in humans is used as the low permeability reference (FDA).

Permeability is classified in this laboratory: low $P_{app}$ <2x $10^{-6}$ cm/s,

| | |
|---|---|
| moderate | $P_{app}$ 2 – 20 x $10^{-6}$ cm/sec, |
| high | $P_{app}$ ≥20 x $10^{-6}$ cm/sec. |

Results
Recovery for both the reference substances and Hydroxyethyl-2-nitro-p-toluidine was from 83 –100%. The reference substances propranolol ($P_{app}$ 29.6 x $10^{-6}$ cm/sec) and ranitidine ($P_{app}$ 0.4 x $10^{-6}$ cm/sec) respectively with 90% and ~50% absorption in humans indicate the validity of the assay. Hydroxyethyl-2-nitro-p-toluidine was rated as having a high permeability with $P_{app}$ 93.6 x $10^{-6}$ cm/sec, which would indicate very good intestinal absorption.

Ref.: 28, submission III

### 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

In Submission III, it is stated that several human patch tests have been performed for formulations. No data was provided but it is stated that Hydroxyethyl-2-nitro-p-toluidine did not cause irritation.

### 3.3.12. Special investigations

No data submitted

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

**CALCULATION OF THE MARGIN OF SAFETY**

**Hydroxyethyl-2-nitro-p-toluidine**

**(Oxidative conditions)**

| | | | |
|---|---|---|---|
| **Absorption through the skin** | **A (mean + 1SD)** | **=** | **5.20 µg/cm²** |
| **Skin Area surface** | **SAS** | **=** | **580 $cm^2$** |
| **Dermal absorption per treatment** | **SAS x A x 0.001** | **=** | **3.02 mg** |
| **Typical body weight of human** | | **=** | **60 kg** |
| **Systemic exposure dose (SED)** | **SAS x A x 0.001/60** | **=** | **0.05 mg/kg bw/d** |
| **No Observed Adverse Effect Level (90 day study, oral, rat)** | **NOAEL** | **=** | **45 mg/kg bw/d** |
| **MOS** | | **=** | **890** |

**(Non-oxidative conditions)**

| | | | |
|---|---|---|---|
| **Absorption through the skin** | **A (mean + 1SD)** | **=** | **4.45 µg/cm²** |
| **Skin Area surface** | **SAS** | **=** | **580 $cm^2$** |
| **Dermal absorption per treatment** | **SAS x A x 0.001** | **=** | **2.58 mg** |
| **Typical body weight of human** | | **=** | **60 kg** |
| **Systemic exposure dose (SED)** | **SAS x A x 0.001/60** | **=** | **0.04 mg/kg bw/d** |
| **No Observed Adverse Effect Level (90 day study, oral, rat)** | **NOAEL** | **=** | **45 mg/kg bw/d** |
| **MOS** | | **=** | **1125** |

### 3.3.14. Discussion

*Physico-chemical properties*
Hydroxyethyl-2-nitro-p-toluidine is used as a direct dye in hair dye formulations, both with and without oxidative agent, at a final maximum concentration of 1%. Hydroxyethyl-2-nitro-p-toluidine is a secondary amine, and thus it is prone to nitrosation. The nitrosamine content in the test material is not reported.
The stability of methylgelb under oxidative conditions was shown over the period of 45 minutes. The hair dye formulation used for this stability testing contained only 088% (0.044% after mixing) methylgelb, while the marketed product may contain up to 2% methylgelb (1% after mixing with hydrogen peroxide).
The stability of Hydroxyethyl-2-nitro-p-toluidine in typical hair dye formulations is not reported.

*Irritation / sensitisation*
Hydroxyethyl-2-nitro-p-toluidine is not a skin and eye irritant. In the LLNA, the highest concentration tested (10%) did not elicit a SI >3. However, this concentration was too low for hazard identification and therefore, a sensitising potential cannot be excluded.

*Dermal absorption*
Under non-oxidative conditions, the amount of hydroxyethyl-2-nitro-p-toluidine bioavailable from a hair dye formulation containing 1% of it is 4.45 µg/cm².

Under oxidative conditions, the amount of hydroxyethyl-2-nitro-p-toluidine bioavailable from a hair dye formulation containing 1% of it is 5.20 µg/cm².

*General toxicity*
Hydroxyethyl-2-nitro-p-toluidine has low acute oral and dermal toxicity.
In repeated-dose studies, clinical signs of toxicity were minimal and the histological changes observed were reversible.
The NOAEL for general toxicity in the 90-day repeat-dose studies in rats was set at 45 mg/kg bw/day (see page 20). Based on the effects on the kidney in the 13 weeks oral toxicity study, a NOAEL of 45 mg/kg bw/day is used for the calculation of the MoS.
Hydroxyethyl-2-nitro-p-toluidine did not cause maternal toxicity or embryotoxic effects at the concentrations used in this study.
Hydroxyethyl-2-nitro-p-toluidine was rated as having a high permeability $P_{app}$ 93.6 x $10^{-6}$ cm/sec in human intestinal epithelial cells that would indicate very good intestinal absorption.

*Mutagenicity*
Overall, the genotoxicity of hydroxyethyl-2-nitro-p-toluidine is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Hydroxyethyl-2-nitro-p-toluidine is not mutagenic *in vitro*. It did not induce gene mutations in bacteria nor in cultured mammalian cells. It also did not induce an increase in the number of cells with micronuclei in human peripheral blood lymphocytes *in vitro*. In mice, exposure to hydroxyethyl-2-nitro-p-toluidine did not result in an increase in erythrocytes with micronuclei. Hydroxyethyl-2-nitro-p-toluidine can be considered to have no genotoxic potential and additional tests are unnecessary.
This statement is supported by some older genotoxicity tests presented in a former submission which suffered from shortcomings with regard to the test performance. However, all of these tests (Ames test, mouse lymphoma assay, *in vitro* UDS test and *in vivo* micronucleus test) were negative.

Carcinogenicity
No conclusion could be drawn from a skin painting experiment

## 4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of hydroxyethyl-2-nitro-p-toluidine with a maximum on-head concentration of 1.0% in oxidative and non-oxidative hair dye formulations does not pose a risk to the health of the consumer.

A possible sensitising potential of hydroxyethyl-2-nitro-p-toluidine cannot be excluded.

Hydroxyethyl-2-nitro-p-toluidine is a secondary amine, and thus prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

## 5. MINORITY OPINION

Not applicable

## 6. REFERENCES

*Submission I, II*

1. Weide, J. (10/5/1985) Akute orale Toxizitätsprüfung von 1-(2'-Hydroxyethyl)-amino-4-methyl-l-2-nitrobenzol. Biological laboratory of Wella AG. Darmstadt.
2. Klein, W. (19/1990) Acute dermal toxicity of "Methylgelb" in rats limit-test). Osterreichisches Forschugszentrum, Seibersdorf.
3. Weide, J. (4/9/1985) Augen- und Augenschleimhautverträglichkeit am Albinomeer-schweinchen mit 1-(2'-Hydroxethyl)-amino-4-methyl-2-nitro-benzol. Biological laboratory of Wella AG. Darmstadt.
4. Klein, W. (7/1990) Acute eye irritation/corrosion study with Methylgelb. Österreichisches Forschungszentrum, Seibersdorf.
5. Weide, J. (31/7/1985) Hautverträglichkeitsprüfung am Albinomeerschweinchen mit 1–(2'– Hydroxyethyl)–amino-4-methyl-2-nitrobenzol. Biological laboratory of Wella AG, Darmstadt.
6. Klein, W. (7/90) Acute dermal irritation/corrosion study with Methylgelb.Österreichlisches Forschungszentrum, Seibersdorf.
7. Sterner, W. and M. Fürst (21/l/1987) Methylgelb, Prüfung auf sensibilisierende Eigenschaften auf Meerschweinchen nach B. Magnusson und A.M. Kligman (gemäss OECD-Richtlinien); Projectno.2-5-809-86. IBR Forschungs GmbH, Walsrode.
8. Reindl, E. (8/1986) Percutaneous Absorption of 4-(2'-Hydroxyethyl)-Amino-3-Nitromethylbenzene ("Methylgelb"). Österreichisches Forschungszentrum, Seibersdorf.
9. Zühlke, U. (13/11/1986) Methylgelb: 13 week oral (gavage) chronic toxicity study in the rat. Hazleton Laboratories Deutschland, Münster.
10. Flasshoff , H.J. (9/3/1983) Mutagenitätsprüfung von LGH 22 12 83/2 im Ames-Salmonella/Mikrosomen-Plattentest. Labor für Biologische Analytik, Bad Kissingen.
11. Kennelly, J.C. (4/6/1985) Study to determine the ability of GHS 191184 to induce mutations to 6-thioguanine resistance in mouse lymphoma L5178Y cells using fluctuation assay. Microtest Research Limited, Heslington.
12. Kennelly, J.C. (25/2/1985) Study to determine the ability of GHS 191184 to induce unscheduled DNA synthesis in HeLa cells. Microtest Research Limited, Heslington.
13. Völkner, W. (29/4/1988) Sister chromatid exchange assay in bone marrow cells of the Chinese hamster with GHS 191184. Cytotest Cell Research GMBH & Co., Darmstadt.
14. Hruby, B. (8/1985) Teratogenicity study of 4-(2-hydroxyethyl)-amino-3-nitromethyl-benzene (Methylgelb) in rats. Österreichisches Forschungszentrum, Seibersdorf.
15. Jacobs, M.M. et al. (1984) Evaluation of the toxicity and carcinogenicity of hair dyes in Swiss mice. Drug and Chemical Toxicology, 7(6), 573-586.
16. Ippen, H. Allergenität von Methylgelb B75. Dermatological Hospital of the University of Göttingen, Göttingen, 29.07.1993
17. Sonnenschein, B.;Grötsch, W. In vivo micronucleus test of GHS 19 1184 in mice. Laboratory L+S GmbH Bad Bocklet, 16.1.1992

*Submission III*

1. Jonas, W.; Bestimmung des Verteilungskoeffizienten n-Oktanol/Wasser; NATEC; 03/1991
2. DIN Sicherheitsdatenblatt Methylgelb; BAYER; 17.01.1990
3. Jonas, W.; Bestimmung des Schmelzpunktes bzw. des Schmelzbereiches der Prüfsubstanz Methylgelb gemäß OECD- Prüfrichtlinie 102 bei Normaldruck; NATEC; 10/1990
4. Jonas, W.; Bestimmung des Siedepunktes oder der Zersetzungstemperatur Prüfsubstanz Methylgelb bei Normaldruck; NATEC; 05/1991

5. Jonas, W.; Addendum 1 : Determination of the Density (OECD Guideline 109) Bestimmung der Dichte der Prüfsubstanz Methylgelb bei 293 K gemäß OECD Richtlinie 109; NATEC; 10/1990
6. Jonas, W.; Bestimmung des Gesamtdampfdrucks der Prüfsubstanz Methylgelb bei 293 K gemäß OECD-Prüfrichtlinie 104; NATEC; 10/1990
7. Jonas, W.; Bestimmung der Oberflächenspannung wässriger Lösungen der Prüfsubstanz Methylgelb gemäß OECD-Prüfrichtlinie 115 bzw Richtlinie 79/831/EWG Anhang V, A.5.; NATEC; 10/1990
8. Jonas, W.; Bestimmung der Wasserlöslichkeit der Prüfsubstanz Methylgelb gemäß OECD-Prüfrichtlinie 105; NATEC; 02/1991
9. Jonas, W.; Bestimmung der Entzündlichkeit der Prüfsubstanz Methylgelb; NATEC; 01/1991
10. Lenz, Dr.; Explosive properties Methylgelb; BIOCHEM. 15.05.1992
11. Jonas, W.; Bestimmung der Selbstentzündlichkeit der Prüfsubstanz Methylgelb; NATEC; 12/1990
12. Opinion of the Scientific Committee on Cosmetology concerning B 75; SCCNFP; 23.06.1999
13. Weide, J.; Acute oral toxicity test of 1-(2-hydroxyethyl)-amino-4-methyl-2-nitrobenzene; WELLA AG; 25.09.1991
14. Klein, W.; Acute dermal toxicity test of "Methylgelb" in rats; SEIBERSDORF; 18.09.1990
15. Klein, W.; Acute dermal irritation/corrosion study with Methylgelb; SEIBERSDORF; 10.07.1990
16. Klein, W.; Acute eye irritation/corrosion study with Methylgelb; SEIBERSDORF; 10.07.1990
17. Christ, M.; Hydroxyethyl-2-nitro-p-toluidine WR 20883 - Local Lymph Node Assay; MDS PHARMA SERVICES; 14.06.2004
18. Python, M.-N.; Cutaneous Absorption of 4-(ethyl-(2-hydroxyethyl)-amino-N-(2-hydroxyethyl)-2-nitro-aniline-hydrochloride and 4-(2'-hydroxyethyl)-amino-3-nitrotoluene in a hair dye formulation through pig skin in vitro; COSMITAL; 23.03.2000
19. Reindl, E.; Percutaneous absorption of 4-(2-hydroxyethyl)-amino-3-nitromethylbenzene ("Methylgelb"); SEIBERSDORF; 08/1986
20. Klein, W.; 28-day Toxicity Study with "Methylgelb" in Rats; SEIBERSDORF; 20.07.1992
21. Zühlke, U.; Final Report Methylgelb 13 week oral (gavage) chronic toxicity study in the rat; HAZLETON; 13.11.1986
22. Sokolowski, A.; Salmonella typhimurium Reverse Mutation Assay with WR 20883 Methylgellb B075; RCC-CCR; 13.04.2004
23. Withwell, J.; Methylgelb (WR20883): Induction of micronuclei in cultured human peripheral blood lymphocytes; COVANCE;
24. Poth, A.; Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells with WR 20883 Methylgelb B075; RCC-CCR; 02.07.2004
25. Honarvar, N.; Micronucleus assay in bone marrow cells of the mouse with Methylgelb - 20883; RCC; 01.11.2002
26. Jacobs, M. M.; Burnett, C. M.; Penicnak, J. A.; Herrera, J. A.; Morris, W. E.; Shubik, P.; Apaja, M.; Granroth, G. ; Evaluation of the toxicity and carcinogenicity of hair dyes in swiss mice; DRUG CHEM. TOXICOL.; 1984
27. Hruby, R.; Teratogenicity study of 4-(2-hydroxyethyl)-amino-3-nitromethylbenzene in rats; SEIBERSDORF; 08/1985
28. McVean, M.; ADME: A-B permeability - Study of Hydroxyethyl-2-Nitro-p-Toluidine; CEREP; 13.04.2004
29. Simon, G. A.; Maibach, H. I.; The pig as an experimental animal model of percutaneous permeation in man: Qualitative and quantitative observations - an overview; SKIN PHARMACOL. PHYSIOL.; 2000

*Submission III, References provided upon request*

1. Kennelly, J. C.; Study to determine the ability of GHS 191184 to induce mutations to 6-Thioguanine resistance in mouse lymphoma L5178Y cells using a fluctuation assay; MICROTEST RESEARCH LTD.; 1985
2. Grötsch, W.; In vivo micronucleus test of GHS 191184 in mice; LABOR L+S GMBH; 1992
3. Grötsch, W.; Mutagenicity Evaluation of LGH 221283/2 in the Ames Salmonella/ Microsome Plate-Assay; LABOR L+S GMBH; 1983
4. Völkner, W.; SCE Assay in bone marrow cells of the chinese hamster with GHS 191184; CCR; 1988
5. Kennelly, J. C.; Study to determine the ability of GHS 191184 to induce UDS in HeLa cells; MICROTEST RESEARCH LTD.; 1-16 12.03.1985;
6. Sterner, W.; Methylgelb Prüfung auf sensibilisierende Eigenschaften am Meerschweinchen nach Magnusson und Kligmann; IBR; 1-20 21.01.1987;

*Submission IV, 2010*

1. Blackstock et al. (2010). The *In Vitro* Percutaneous Absorption of Hydroxyethyl-2-nitro-p-toluidine (B075, WR20883) in Typical Hair Dye Formulations Under Oxidative and Non-Oxidative Conditions Through Human Skin. Study No. 787702, Report No. 31175. Charles River, EH33 2NE UK