EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

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

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

Hydroxyethyl-2-nitro-p-toluidine

COLIPA N° B75

Adopted by the SCCP
by written procedure on date 17 March 2006

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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, 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 Products (SCCP) is asked to assess the risk to consumer when Hydroxyethyl-2-nitro-p-toluidine is used in cosmetic products.*

2. *Does the SCCP 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-toluene
1-(2´-hydroxyethyl)-amino-4-methyl-nitrobenzene
4-methyl-2-nitro-(β-hydroxyethyl)-aniline

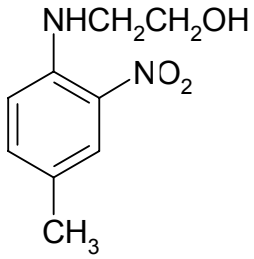
3.1.1.3. Trade names and abbreviations

Jarocol HNT, Methylgelb

3.1.1.4.	CAS / EINECS number

CAS : 100418-33-5
EINECS : /
ELINCS : 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

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

Description	Batch No.					
	B3/89	**BRA 1/389**	**BRA 1/315**	**BRA 1/177**	**BRA 1/285**	**6718 Fass 10/20**
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

Stability at room temperature

Approx. 10 % (w/v) in DMSO : t = 0h: 100%, 6 h: 95.1%, 2 d: 93.8%, 7 d: 96.9%
10% (w/v) in acetone/water 1:1 : t = 0h: 100%, 6 h: 62.2%, 2 d: 54.4%, 7 d: 51.7%
0.18% (w/v) in water, pH 6.4 : t = 0 h: 100%, 6 h: 51.2%, 2 d: 41.6%, 7 d: 36.1%

0.18% *(0.18 g/100 ml or 1.8 g/L)* aqueous solution of the dye is not in conformity with the water solubility of the test material determined by OECD method (see 3.1.6).

No raw data on stability of the test substance, reported above, is provided. The above reported degradation of the test substance in aqueous solution is not in agreement with the stability of the 0.35g/L aqueous solution for 6 days, reported in the study on solubility testing.

General comments on physico-chemical properties

- 0.18% aqueous solution of the dye is not in conformity with the water solubility of the test material (0.351 g/L) determined by OECD method.
- No data is provided for the stability of the dye in marketed products. However, the applicant declares that hydroxyethyl-2-nitrotoluidine is stable under the conditions used in formulation.
- 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.

In the B75 Opinion (SCCNFP/0183/99), a formulation 'fC' describes the use of test substance in combination with dialkanolamine. The formulation also contains HC Blue 1, which is classified by IARC as Carcinogenic 2B.

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:	/ (not in study report, but in Submission III as BRA1/285)
Purity:	/ (not in study report, Submission III as 99.6 area%, HPLC; 254 nm)
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: /

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 I, ref 1)

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

Hydroxyethyl-2-nitro-p-toluidine, moistened with water, was applied on a patch to the clipped skin (30 cm^2). 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 I, ref 2)

3.3.1.3. Acute inhalation toxicity

No data

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

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 I, ref 5)

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:	Occlusive application of 0.5 g of test substance to ~ 6 cm^2 intact dorsal skin
Application time:	4 h
GLP:	in compliance

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 I, ref 4)

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.

(submission I, ref 3)

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

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 23.9µCi ^{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 [H^3] 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 effected 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 were comparable with the vehicle controls. The EC_3 could not be calculated as the stimulation index were below 3.

Hydroxyethyl-2-nitro-p-toluidine was not considered a skin sensitizer under these test conditions.

Ref.: 17

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.: on request 6 (as non GLP)
(submission I, ref 7)

3.3.4. Dermal / percutaneous absorption

3.3.4.1. 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:	concentration 0.63 % 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
5 diffusion cells, contact 30 min. diffusion during 72 hours
5 diffusion cells, contact 30 min., 3 repeated application at 24 hours intervals, diffusion during 72 hours
GLP: In compliance

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
Skin absorption of hydroxyethyl-2-nitro-p-toluidine, 0.63 %, is not at 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 the mixture is available.
The stratum corneum is not isolated from the viable epidermis, 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

3.3.4.2. 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: - formulation without hydrogen peroxide 1 % - formulation with hydrogen peroxide 0.50% - 3.33% solution in DMSO in water 5/4.
Schedule:	single cutaneous application of 30 minutes.
Observation:	72 h
GLP:	not in compliance

^{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 Submission I, under the ref.: 8. No additional data was provided.
The study does not comply with modern Guidelines.

Ref.: 19
(submission I, ref 8)

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% carbomethylcellulose in distilled water (CMC)
Dosing period:	28 days
Observation period:	Satellite recovery group (control and high dose) 28 + 14 days
GLP:	In compliance

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.
Post mortem showed tissue staining particularly fat 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 red in one animal from the both the mid and high dose male groups. Kidney weight was affected but there was no dose response or consistency. There were no treatment related findings.
The NOAEL was deduced to be 240 mg/kg bw/day from this study.

Ref.: 20

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:	-(not specified in study but given as BRA 1/315, Submission III)
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:	/

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 groups.
Haematological chemistry data did not show dose-related changes. In the 90 mg/kg bw group, one female showed hyaline casts during urine investigation at week 13.
Post-mortem, the 90 mg/kg bw 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 group. The incidence of lobular structure of the liver increased in the control and the lower dose groups, but was less frequent at the top dose. There were no histopathological treatment-related changes observed.

Comment
The lack of data on the loss of stability and hence possible alteration of the test substance suspensions over the dosing period was not investigated.
The study authors suggested that the NOAEL could be deduced as 90 mg/kg bw. Based on the effects on the kidney, the SCCP considered the NOAEL should be 45 mg/kg bw.

Ref.: 21
(submission I, ref 9)

3.3.5.3. Chronic (> 12 months) toxicity

No data available

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:	Two independent tests (plate incorporation and pre-incubation)
Test substance:	Hydroxyethyl-2-nitro-p-toluidine
Batch:	6718 Fass 10/20
Purity:	99.8 area% (HPLC; 254 nm)
Vehicle:	DMSO
Concentrations:	3 - 5000 µg/plate without and with metabolic activation
GLP:	in compliance

Hydroxyethyl-2-nitro-p-toluidine has been 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 study the concentrations tested in the main study reached the recommended maximum of 5000 µg/plate. 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. Hydroxyethyl-2-nitro-p-toluidine 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 but it did not significantly induce gene mutations in any of the tester strains.

Hydroxyethyl-2-nitro-p-toluidine is not mutagenic in the bacterial gene mutation assay.

Ref.: 22

***In vitro* micronucleus test**

Guideline:	OECD 487 (draft)
Cells:	Peripheral human blood lymphocytes (blood pooled from two donors)
Replicates:	2 independent tests with and without S9 mix
Test substance:	Hydroxyethyl-2-nitro-p-toluidine
Batch:	6718 Fass 10/20
Purity:	99.8% (HPLC, 254 nm)
Vehicle:	DMSO
Concentrations:	210 – 910 µg/ml without and with metabolic activation
GLP:	in compliance

Hydroxyethyl-2-nitro-p-toluidine 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. Negative and positive controls were in accordance with the OECD guideline. 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.

Results
Hydroxyethyl-2-nitro-p-toluidine did not induce micronuclei in both experiments in the absence of S9-mix. There was a statistically significant increase in the frequencies of 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. The highest concentrations tested caused a clear reduction (more than 50%) in the RI.
In conclusion, under the experimental conditions used, Hydroxyethyl-2-nitro-p-toluidine did not lead to a biologically relevant induction of micronuclei in cultured human peripheral lymphocytes.

Ref.: 23

***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:	Hydroxyethyl-2-nitro-p-toluidine
Batch:	6718 Fass 10/20
Purity:	99.8 area% (HPLC; 254 nm)
Vehicle:	DMSO
Concentr. tested:	31.3 - 375 µg/ml without and with metabolic activation
GLP:	in compliance

Hydroxyethyl-2-nitro-p-toluidine 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 a initial range-finding study. Negative and positive controls were in accordance with the OECD guideline.

Results
In the first experiment without S9 mix, an increase in the mutant frequency was measured at a concentration of 250 µg/ml which also induced strong toxic effects (18% relative total growth). However, no induction of mutations 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). No induction of mutations was seen in the test with metabolic activation and in experiment II (24 hours treatment without S9-mix). Due to problems with the positive control (with S9-mix) in experiment I, the whole experiment was repeated and revealed a negative result up to the highest concentrations (300 and 350 µg/ml) which clearly induced toxicity (about 20% and 5% relative total growth, respectively).

ConclusionHydroxyethyl-2-nitro-p-toluidine was not mutagenic in mammalian cells (L5178Y mouse lymphoma cells) *in vitro* under the experimental conditions used.

Ref.: 24

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474
Species/strain:	Mouse, NMRI
Group size:	5 males and 5 females analysed per group
Test substance:	Hydroxyethyl-2-nitro-p-toluidine
Batch:	6718 Fass 10/20
Purity:	99.8% (HPLC; 254 nm)
Vehicle:	PEG 400
Dose levels:	187.5, 375, 750 mg/kg bw (three consecutive treatments by gavage, 24 hour interval)
Sacrifice time:	24 hours after the last treatment

GLP: in compliance

Hydroxyethyl-2-nitro-p-toluidine has been investigated for induction of micronuclei in the bone marrow cells of mice. Dose selection was based on results from pre-tests for toxicity. 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
The highest dose (750 mg/kg) caused the same signs of toxicity as in the preliminary experiment. This effect and the fact that the urine was discoloured may indicate systemic distribution of the compound and exposure of the bone marrow. However, the PCE/NCE ratio was not affected by the treatment. The mean micronucleated PCE frequencies were not significantly increased in any of the groups treated with the test substance. The positive control substance gave the expected result.
The study was conducted appropriately but the study authors specified that the stability of Hydroxyethyl-2-nitro-p-toluidine in PEG 400 was not indicated by the sponsor. Hydroxyethyl-2-nitro-p-toluidine did not induce chromosome aberrations or damage to the mitotic apparatus in bone marrow cells of mice after oral treatment under the test conditions used.

Ref.: 25

Conclusion
Hydroxyethyl-2-nitro-p-toluidine is not mutagenic *in vitro*. It did not induce gene mutation in bacteria and in cultured mammalian cells. It also did not induce chromosome aberrations in mammalian cells *in vitro*. The test substance did not induce damage to chromosomes or the mitotic apparatus in the *in vivo* micronucleus test. Thus, no relevant mutagenic potential was found for Hydroxyethyl-2-nitro-p-toluidine *in vitro* and *in vivo* under the test conditions used. 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.

3.3.7. Carcinogenicity

No carcinogenicity data was provided.
A paper giving only a summary of the results evaluating toxicity and carcinogenicity by skin painting with formulations of hair dyes was provided. 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 can be drawn from this study.

Ref.: 26
(submission 1, ref 15)

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data

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 specifed in study but given as BRA 1/315, Submission III*)*
Purity:	- (not specifed 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:	/

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

Ref.: 27
(submission I, ref 14)

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: /

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 37^0C 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 P_{app} 93.6 x 10^{-6} cm/sec that would indicate very good intestinal absorption.

Ref.: 28

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data

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 additional data

3.3.13. Safety evaluation (including calculation of the MoS)

Not applicable

3.3.14. Discussion

The toxicity studies were conducted mainly in the 1980s. Thus all these studies should have been made available with the previous submissions.

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.

In the earlier Opinion (SCCNFP/0183/99), a formulation (fC) describes the use of test substance in combination with dialkanolamine. The formulation also contains HC Blue 1, which is classified by IARC as Carcinogenic 2B.
0.18% aqueous solution of the dye, for which stability data is reported, is not in conformity with the water solubility of the test material (0.351 g/L) determined by OECD method. The stability in aqueous solutions was poor, after 6 h only 50% was still seen. There was no information on the degradation products.
No data is provided for the stability of the dye in marketed products. However, the applicant declares that hydroxyethyl-2-nitrotoluidine is stable under the conditions used in formulation.

Hydroxyethyl-2-nitrotoluidine has a low order of acute oral and dermal toxicity. Repeated dose studies with relatively low oral doses revealed few systemic effects.

Hydroxyethyl-2-nitrotoluidine is not an eye irritant or skin sensitiser.

Hydroxyethyl-2-nitro-p-toluidine is not mutagenic *in vitro*. It did not induce gene mutation in bacteria, in cultured mammalian cells or chromosome aberrations in mammalian cells *in vitro*. The test substance did not induce damage to chromosomes or the mitotic apparatus in the *in vivo* micronucleus test.

The percutaneous absorption studies are considered inadequate. In the *in vitro* study, the concentration of hydroxyethyl-2-nitro-p-toluidine, 0.63 %, was not at the maximum concentration intended for hair colorants (1 %) and the experimental design was not pertinent. The variations in the results were such that the data could not be interpreted.

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 can reasonably be set at 45 mg/kg bw/day.

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.

4. CONCLUSION

Hydroxyethyl-2-nitro-p-toluidine has been evaluated as a hair dye.

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

Before any further consideration, the following information is required:

- the stability of Hydroxyethyl-2-nitro-p-toluidine in the various solvents used (e.g. percutaneous absorption study) has to be clarified.

- an *in vitro* percutaneous absorption study according to the SCCNFP Notes of Guidance

5. MINORITY OPINION

Not applicable

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