Scientific Committee on Consumer Products

SCCP

OPINION ON

2-Hydroxyethyl picramic acid

COLIPA n° B72

The SCCP adopted this opinion at its 18th plenary of 16 December 2008

About the Scientific Committees

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

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

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

SCCP

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

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Opinion to be cited as: Opinion of the SCCP on 2-hydroxyethyl picramic acid, 16 December 2008

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1. BACKGROUND

Submission I for 2-Hydroxyethyl picramic acid was submitted in September 1994 by COLIPA[1, 2].

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) has at its 8th meeting on 23 June 1999 expressed its opinion (SCCNFP/0182/99) with the conclusion that:

"The SCCNFP is of the opinion that 1-Hydroxy-2-(ß-hydroxyethyl)-amino-4,6-dinitro-benzene can be used safely in permanent hair dye formulations at a maximum concentration of 3.0%. Since permanent hair dyes are mixed with hydrogen peroxide before application, the in-use concentration is 1.5%. In hair tinting products and colouring setting lotions the concentration of 1-Hydroxy-2-(ß-hydroxyethyl)-amino-4,6-dinitro-benzene should not exceed 2.0%."

Submission II for 2-Hydroxyethyl picramic acid was submitted by COLIPA in July 2005. According to this submission the substance is used:

a) as a non-reactive hair colouring agent ("direct dye") in oxidative hair dye formulations at a maximum on-head concentration of 1.5%. The colorant component and a developer (hydrogen peroxide) are mixed at ratios between 1:1 to 1:3. It is common practice to apply 100 g of the product over a period of 30 to 45 minutes followed by rinse off with water and shampoo.

b) as a non-reactive hair colouring agent ("direct dye") in non-oxidative hair dye formulations at a maximum on-head concentration of 2%. It is common practice to apply 35 to 50 g of the product over a period of about 30 minutes followed by rinse off with water and shampoo. The application may be repeated at weekly intervals.

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

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider 2-Hydroxyethyl picramic acid safe for use as non-oxidative hair dye with an on-head concentration of maximum 2.0% taken into account the scientific data provided?*

2. *Does the SCCP consider 2-Hydroxyethyl picramic acid safe for use in oxidative hair dye formulations with an on-head concentration of maximum 1.5% taken into account the scientific data provided?*

3. *Does the SCCP recommend any further restrictions with regard to the use of 2-Hydroxyethyl picramic acid in any non-oxidative or oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

3. OPINION

In its opinion SCCP/1058/06, adopted on 19 June 2007, the SCCP concluded that: "The SCCP is of the opinion that the information submitted is insufficient to allow a final risk assessment to be carried out. Before any further consideration, the possible genotoxic potential must be excluded".

Following this opinion, submission III was received from the applicant in March 2008, providing the requested clarifications. Section 3.3.6.2. has been revised according to this.

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

2-Hydroxyethyl picramic acid (INCI)

3.1.1.2. Chemical names

Phenol, 2-[(2-Hydroxyethyl)amino]-4,6-dinitro- (CA INDEX NAME 9CI)
2-[(2-Hydroxyethyl)amino]-4,6-dinitrophenol (IUPAC)
2-(2-hydroxy-3,5-dinitroanilino) ethanol (ECB)
1-Hydroxy-2-(β-hydroxyethyl)amino-4,6-dinitrobenzene
N-(2-Hydroxyethyl) picramic acid

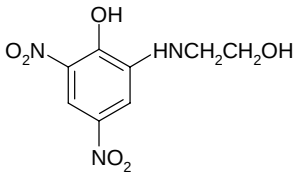
3.1.1.3. Trade names and abbreviations

Picraminrot
COLIPA: B72

3.1.1.4. CAS / EINECS number

CAS: 99610-72-7
ELINCS: 412-520-9 (picramic rot)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_8H_9N_3O_6$

3.1.2. Physical form

Red Powder, wetted (SCCNFP/0182/99: *Orange coloured fine crystalline powder*)

3.1.3. Molecular weight

Molecular weight: 243.17

3.1.4. Purity, composition and substance codes

The reported data on purity and impurities of 2-hydroxyethyl picramic acid in various batches of test substance is described in Table 1.

Comments:
- Supportive documents for the reported data on purity/impurities of all batches of 2-hydroxyethyl picramic acid are not submitted.
- In general, the contents of impurities A, B and C in different batches varied in several orders of magnitude.
- 2-hydroxyethyl picramic acid is a secondary amine. Nitrosamine content in 2-hydroxyethyl picramic acid is not reported.

Declaration by the Applicant
Batch 21989/8 is of lower purity (86%) than the other batches used for toxicological studies. It not only contains more than 0.1% of picric acid, but also other unknown impurities. However, batch 2189/8 was only used in a single *in vitro* mutagenicity test (Ames) performed in 1992 and the results of this assay were not relevant for the evaluation of the overall genotoxic potential. The latter was based on *in vivo* genotoxicity studies performed with batches JFB I 95, 2/99, and DO 2007, each with a dye content of more than 95% by NMR.

3.1.5. Impurities / accompanying contaminants

See 3.1.4

3.1.6. Solubility

Water:	157 mg/l (20°C)
Acetone/water (1:1):	<10 g/l
DMSO:	>100 g/l

3.1.7. Partition coefficient (Log P_{ow})

Partition coefficient of 2-hydroxyethyl picramic acid depends on the concentration of the test substance in the solution used.

Log P_{ow}: 1.28 (pH 3.9, 23±1°C), P_{ow} 19.23 at concentration 1248 mg/L solution in octanol
Log P_{ow}: 1.16 (pH 3.9, 23±1°C), P_{ow} 14.55 at concentration 624 mg/L solution in octanol
Log P_{ow}: 1.0 (pH 3.9, 23±1°C), P_{ow} 9.95 at concentration 312 mg/L solution in octanol

3.1.8. Additional physical and chemical specifications

Melting point:	134.6 – 137.1°C
Boiling point:	141°C (decomposition starting)
Flash point:	/
Vapour pressure:	2.4 x 10^{-8} hPa
Density:	1.58
Viscosity:	/
pKa:	/
Refractive index:	/

pH:	3.6 (saturated aqueous solution, 20°C)
UV_Vis spectrum	/

3.1.9. Homogeneity and Stability

No data

General Comments to physico-chemical characterisation

- Supportive documents for the reported data on purity/impurities of only 2 batches of 2-hydroxyethyl picramic acid were submitted.
- In general, the contents of impurities A, B and C in different batches varied in several orders of magnitude.
- No data on stability of 2-hydroxyethyl picramic acid in test solutions and in marketed products is reported.
- 2-hydroxyethyl picramic acid is a secondary amine. Nitrosamine content in the compound is not reported

3.2. Function and uses

2-Hydroxyethyl picramic acid is used as a hair colouring agent in oxidative hair dye formulations at a maximum on-head concentration of 1.5%, after mixing with hydrogen peroxide.
2-Hydroxyethyl picramic acid is used as a hair colouring agent in semi-permanent hair dye formulations at a maximum on-head concentration of 2%.

Table 1: Purity and impurities of 2-hydroxyethyl picramic acid in various batches of test substance

Description	**Batch number**									
	DO 2007	**21989/5**	**RK156**	**JFB I 195**	**21989/8**	**PO2**	**BR 624**	**2/93**	**PO1**	**2/99**
NMR content, % w/w	99.7	97.0	97.2	97.9	86.7	96.5	98.6	97.0	96.1	95.1
HPLC purity, area % 210 nm 254 nm 410 nm	 99.8 99.9 99.8	 98.8 99.2 98.6	 99.4 99.6 99.6	 99.4 99.6 99.6	 92.3 94.3 86.7	 97.4 98.4 97.1	 99.2 99.4 99.1	 99.7 99.9 99.8	 97.6 98.1 96.0	 98.4 98.7
HPLC content*, % w/w	99.5	98.7	98.5	100.1	90.8	96.8	97.1	99.2	99.3	
Impurities**, % w/w A B C	 <0.0017[a] 0.098 0.033	 0.1340 0.0059 <0.0025[a]	 <0.0025[a] 0.291 0.815	 <0.0025[a] 0.310 0.296	 0.1390 0.079 Ca.0.0025	 0.0070 1.38 0.881	 <0.0025[a] 0.294 0.349	 <0.0025[a] 0.127 <0.0025[a]	 <0.0025[a] 0.330 2.43	 <0.01 0.99 0.0061
Water content, % w/w	<0.01	0.52	0.01	0.01	0.78	0.32	0.01	0.03	0.17	0.29
Loss on drying, % w/w	0.01	0.19	0.10	0.02	0.63	0.09	0.04	0.10	0.03	0.41
Sulfated Ash, % w/w	0.02	0.39	0.03	***	0.63	0.08	0.01	0.20	0.02	0.20
Screening for chemical elements[b]	63ppm Sn 12ppm Na	40ppm Fe 27ppm Mn 1790ppm Na	11ppm Fe	39ppm Br	36ppm Fe 27ppm Mo 1700ppm Na	389ppm I		127ppm Fe 57ppm Br		57ppm Fe 16ppm Br

*batch 2/99 was used as reference material for the determination of 2-hydroxyethyl picramic acid. The UV_Visible spectrum of test material is not submitted, and thus the correct wavelength for the quantification is not known.
**Impurities
A: Picric acid (banned according to Directive 76/768/EEC, annex II, n° 268; MAK classification carcinogenic class 3B)
B: 2-Amino-4,6-dinitrophenol
C: 2-[Di(2-hydroxyethyl)amino]-4,6-dinitrophenol
*** not done due to lack of substance
[a] not detected, values given are detection limits
[b] In all batches no significant impurities detected, with exception of mentioned ones

3.3. Toxicological Evaluation

2-Hydroxyethyl picramic acid is classified as toxic to reproduction category 3 (R62: possible risk of impaired fertility): the assessment (BgVV, 1993) was based on testes atrophy in the 450 mg/kg bw/d dose group of the 28-day study.

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from the opinion n° SCCNFP/0182/99

Guideline:	/
Species/strain:	Wistar rat CF1 mouse
Group size:	15 male and 15 female
Test substance:	2-hydroxyethylpicramic acid (HEP)
Batch:	/
Purity:	/
Dose:	rat females: 600, 800, 1000, 1200 mg/kg bw rat males: 800, 1000, 1200, 1400 mg/kg bw mice both sexes: 250, 500, 750, 1000 mg/kg bw
Route:	oral, by gavage
Exposure:	single administration and a 14 days observation period
GLP:	not in compliance

HEP, 5% suspension in 10% gum Arabic, was administered once via stomach tube to Wistar rats (6/sex) and CF1 mice (10/sex). The rats received doses of 800, 1000, 1200 mg/kg bw; female rats were additionally exposed to 600 and male rats with 1400 mg/kg bw. Mice received doses of 250, 500, 750 and 1000 mg/kg bw. During an observation period of 14 days, the mortality and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals. The test substance caused accelerated breathing, exitus and red colour of urine.

LD_{50} for male and female rats were 1134 and 900 mg/kg bw, respectively. LD_{50} for male and female mouse were 525 mg/kg bw

Although no analytical data of the test batch are available and although the study was not performed in compliance with GLP, the data can be considered sufficiently reliable to evaluate the acute oral toxicity of HEP.

Ref.: 13

3.3.1.2. Acute dermal toxicity

Taken from the opinion n° SCCNFP/0182/99

Guideline:	OECD 402 (1987)
Species/strain:	rat, Him:OFA
Group size:	5/sex
Test substance:	Pikraminrot (I)
Batch:	21989/5
Purity:	> 99%
Dose:	2000 mg/kg bw
Exposure:	dermal
GLP:	in compliance

Pikraminrot, moistened with distilled water, was administered once dermally to Sprague-Dawley rats (5/sex) at a concentration of 2000 mg/kg bw. After an exposure time of 24 hours, the patch was removed and the test substance residues were wiped off. During an observation period of 14 days, the mortalities and clinical toxicological findings were recorded. The body weights were recorded weekly. All animals survived till the end of the study. The test substance caused staining of the fur.
The test substance is slightly toxic.

Ref.: 14

Comment
The LD_{50} was > 2000 mg/kg bw.

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404
Species:	New Zealand white rabbits
Group:	3 females
Substance:	1-Hydroxy-2-(2'-hydroxyethyl)amino-4,6-dinitrobenzene
Batch:	21989/5
Purity:	> 99%
Dose:	0.5 g
Vehicle:	water
GLP:	in compliance

Approximately 6 cm^2 shaved skin (dorsal thoracal region) of the animals was exposed to 0.5 g of test substance soaked with 1.5 ml water under semi-occlusive condition for 4 hours. Animals were examined for erythema/eschar and oedema as well as for other local and systemic signs approximately 1 h, 24 h, 48 h and 72 h after exposure.

Results
No general toxic effect of the test substance was noted. No erythema or eschar formation was noted up to 72 hours after exposure with the test substance. No oedema was observed in 2/3 animals; and a score of "1" was noted 24 and 48 hours after the end of exposure in one animal (mean score 0.7). Only scores of "0" were observed at additional observation times up to 21 days after the end of exposure.

Conclusion
1-Hydroxy-2-(2'-hydroxyethyl)amino-4,6-dinitrobenzol is not irritant to rabbit skin.

Ref.: 15

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405
Species:	New Zealand white rabbits
Group:	3 females
Substance:	1-Hydroxy-2-(2'-hydroxyethyl)amino-4,6-dinitrobenzene
Batch:	0120081229
Purity:	> 99%
Dose:	39-50 mg (ca. 0.1 ml)

GLP: in compliance

Approximately 0.1 ml of the test substance, i.e. 39, 46 and 50 mg, was applied to the conjunctival sac of the right eye after gently pulling the lower lid away from the eyeball. The lids were then gently held together for about one second to prevent loss of the test substance. The left eye served as control.
Both eyes of the animals were examined within 24 hours before application and approximately 1 h, 24 h, 48 h and 72 h post application Additional examinations were performed up to 21 days post application. At all observation times, the animals were also examined for other than local changes.

Results
No general toxic effect of the test substance was noted. Examination at one hour post application, slight opacity of the cornea (score 1) in all animals, slight chemosis of the conjunctiva (score 1) in one animal and slight redness (score 1) in one animal were noted.
24-72 h p.a., slight redness in treated eyes of all animals, and slight chemosis in two animals was observed. The chemosis in the third animal persisted only up to 48 h. Slight corneal opacity in two animals persisted up to 72 h, including moderate effect (score 2) at 48 h in one animal. No effect on cornea of third animal was observed. Slight redness (score 1) in treated eyes of all animal was noted during 24-72 p.a.
No effect of the test substance on the iris of any animal was observed up to 72 h.
Slight opacity of the cornea in 2 animals was still observed 6 days p.a. In one animal minimal redness and chemosis (score 1 for each) was found 6 days p.a. All other scores were "0" at additional observation times.

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

Ref.: 16

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species:	Mouse, CBA/J (10 weeks)
Group:	25 females, 5 groups of 5 animals
Substance:	2-Hydroxyethyl picramic acid WR23233
Batch:	2/99
Purity:	98.4% (HPLC peak area)
Dose:	0.5%, 1.5%, 5% and 10%
Vehicle:	DMSO
Control:	Treatment by vehicle only
GLP:	in compliance

For positive control using para-phenylenediamine (PPD), data from study 762/40 is reported (but the study not submitted).
The chosen concentrations of the test item are based on maximum solubility in the vehicle.

25 µl of the test item formulations 0% (vehicle only), 0.5%, 1.5%, 5% and 10% were applied on the dorsal surface of each ear of five animals for three consecutive days. A hairdryer was used for approximately 5 min to dry the application sites. Animals were checked for morbidity/mortality at least once daily. Observation for clinical signs was done daily. Body weight was determined on day 1 and day 6.

On day 6, the mice received an intravenous injection of 250 µl of phosphate buffered saline containing 23.8 µCi of ^{3}H-methylthymidine. Approximately five hours later the mice were sacrificed by CO_2-inhalation and the draining auricular lymph nodes were removed and

weighed. After preparing a single cell suspension of lymph nodes of each mouse, cells were precipitated by trichloroacetic acid, and the radioactivity (incorporation of ^{3}H-methylthymidine in the pellets) was determined by means of liquid scintillation counting as dpm. The mean dpm per treated group was determined and stimulation index was calculated.

Results
Neither clinical signs nor mortality were observed throughout the study period. Body weight gain was not affected by the treatment. With 2-Hydroxyethyl picramic acid in DMSO, mean stimulation indices of 1.1, 1.1, 2.6 and 2.8 were obtained for the 4 test concentrations of 0.5%, 1.5%, 5% and 10% respectively.
The positive control (PPD, 1% in DMSO) caused a mean stimulation index of 7.0 and an increase in lymph node weight by a factor of 1.2.

Individual stimulation index:

Treatment	***PPD 1.0 % ****	***2-Hydroxyethyl picramic acid***			
		0.5 %	***1.5 %***	***5.0 %***	***10.0 %***
	4.9	0.9	0.9	1.4	2.7
	4.1	1.2	0.7	2.6	2.8
	4.7	1.3	1.1	4.3	2.6
	7.4	1.1	1.2	2.8	3.9
	14.0	1.1	1.3	2.2	1.8
Mean SD	7.0 4.1	1.1 0.2	1.1 0.2	2.6 1.1	2.8 0.8

*Data from Study 762/40

Conclusion
2-Hydroxyethyl picramic acid was not proven to be a skin sensitiser under the test conditions used.

Ref.: 17

Comments
The following sensitisation tests were reported in an earlier submission (SCCNFP/0182/99):

- Ref. 7: Sterner W., et al. Delayed contact hypersensitivity In guinea pigs, modified method of Magnusson-Kligman. IBR Forschung GmbH. Sudkampen; 29-10-1985. Result: Test not accepted

- Ref. 8: Cuthbert J.A., Jackson D. Pikraminrot (1): Magnusson-Kligman maximisation test in Guinea pigs. Inveresk Research International. Tranent; 23-4-1991.
  Result: Negative.
  The report mentions that the study was performed according to GLP. Batch n° 21989/5 (purity: 98.7%) was used.

3.3.4. Dermal / percutaneous absorption

Percutaneous absorption *in vitro*, without hydrogen peroxide

Guideline:	OECD 428
Tissue:	Pig dermatomed skin (fresh, outer ear). Pig ears obtained from a slaughter house
Group size:	For each experiment skin (thickness 300-400 µm) from different pigs was used. Three independent experiments were performed with 6 skins per experiment
Diffusion cells:	Flow through diffusion cells, receptor fluid volume 1 ml, exposed skin area 1cm^2

Skin integrity:	Monitored by conductivity
Test substance:	2-Hydroxyethyl picramic acid
Batch:	2-99 (R0050493)
Purity:	98.4% (HPLC peak area)
Test item:	A cream formulation (221004-100) containing 2% 2-Hydroxyethyl picramic acid
Doses:	20 µl/cm^2
Receptor fluid:	Phosphate buffered saline, pH 7, temperature 32±1°C
Solubility in receptor fluid:	Soluble in PBS (maximum tested concentration 2 µg/ml PBS)
Stability:	Stable in PBS up to 72 h (test period) at room temperature
Method of Analysis:	HPLC, method validation reported
GLP:	in compliance

20 µl of the formulation was applied to each skin sample for 60 min and then washed off using 2 x 1 ml deionised water, 2 x 1 ml 10% shampoo solutions followed by 2 x 1 ml deionised water. The penetration was monitored for 72 h under non-occluded conditions. The receptor fluid was fractionated 0.5, 1, 2, 4, 6, 8, 24, 48 and 72 h after the application of the test item. The stratum corneum was separated from the remaining skin by heat separation. The membranes were extracted with 2 ml of a 70:30 mixture of 0.06 ammonium formate (pH 6.7) and methanol. The content of 2-Hydroxyethyl picramic acid in receptor fluid samples, membrane extracts and other solutions was determined by HPLC.

Results
The amount of 2-Hydroxyethyl picramic acid penetrated through the skin during 72 h was as given in the following table (data from 3 cells was excluded due to >110% recovery).

Skin compartment	**Skin penetration of 2-Hydroxyethyl picramic acid**			
	µg/cm^2		**% of applied amount**	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**
Upper skin (*Stratum corneum*)	1.47±0.64	0.498-2.783	0.477±0.198	0.155-0.764
Lower skin (Epidermis + dermis)	0.25±0.19	0.002-0.546	0.077±0.062	0.001-0.186
Receptor fluid	4.24±3.39	0.308-13.640	1.308±1.138	0.118-3.853
Dermal absorption*	4.49±3.96	0.310-13.846	1.384±1.146	0.382-3.911
Recovery				91.2 – 110.9

*sum of amounts in lower skin + receptor fluid

Ref.: 18

Comment
The study was performed in March 2005 and the data of (latest) positive control with caffeine (March 2005), benzoic acid (March 2004) and testosterone (June 2004) is provided. The standard deviation of dermal penetration is extremely high. The maximum amount of 2-Hydroxyethyl picramic acid absorbed into the skin (lower skin + receptor fluid) i.e. 13.85 µg/cm^2 may be used for the calculation of margin of safety under non-oxidative conditions.

Percutaneous absorption *in vitro* under oxidative conditions

Guideline:	OECD 428
Tissue:	Pig dermatomed skin (fresh, outer ear). Pig ears were obtained from a slaughter house
Group size:	For each experiment skin (thickness 300-400 µm) from different pigs was used. Three independent experiments were performed with 6 skins per experiment
Diffusion cells:	Flow through diffusion cells, receptor fluid volume 1 ml, exposed skin area 1cm^2
Skin integrity:	Monitored by conductivity
Test substance:	2-Hydroxyethyl picramic acid
Batch:	2-99 (R0050493)
Purity:	98.4% (HPLC peak area), 95.1% (w/w) by NMR
Test item:	A cream formulation (221004-100) containing 3% 2-Hydroxyethyl picramic acid. Peroxide solution Welloxon Perfect 12% 40 Vol. (Batch No. F290855). Cream formulation was mixed 1:1 with peroxide solution before application on the skin.
Doses:	20 µl/cm^2
Receptor fluid:	Phosphate buffered saline, pH 7, temperature 32±1°C
Solubility in receptor fluid:	Soluble in PBS (maximum tested concentration 2 µg/ml PBS)
Stability:	Stable in PBS up to 72 h (test period) at room temperature.
Method of Analysis:	HPLC, method validation reported
GLP:	in compliance

20 µl of the formulation was applied to each skin sample for 60 min and then washed off using 2 x 1 ml deionised water, 2 x 1 ml 10% shampoo solutions followed by 2 x 1 ml deionised water. The penetration was monitored for 72 h under non-occluded conditions. The receptor fluid was fractionated 0.5, 1, 2, 4, 6, 8, 24, 48 and 72 h after the application of the test item. The stratum corneum was separated from the remaining skin by heat separation. The membranes were extracted with 2 ml of a 70:30 mixture of 0.06 ammonium formate (pH 6.7) and methanol. The content of 2-Hydroxyethyl picramic acid in receptor fluid samples, membrane extracts and other solutions was determined by HPLC.

Results
The amount of 2-Hydroxyethyl picramic acid penetrated through the skin after during 72 h was as given in the following table (data from 1 cell was excluded due to non-integrity of the membrane).

Skin compartment	**Skin penetration of 2-Hydroxyethyl picramic acid**			
	µg/cm^2		**% of applied amount**	
	Mean ± SD	**Range**	**Mean ± SD**	**Range**
Upper skin (Stratum corneum)	0.813±0.878	0.002-2.719	0.344±0.375	0.001-0.883
Lower skin (Epidermis + dermis)	0.562±0.577	0.253-0.951	0.239±0.243	0.113-1.121
Receptor fluid	2.679±3.041	0.467-11.084	1.311±1.271	0.141-4.560
Dermal absorption*	3.241±3.183	0.586-11.382	1.384±1.332	0.249-4.683
Recovery				96.5 – 106.7

*sum of amounts in lower skin + receptor fluid

Ref.: 19

Comment
The study was performed in March 2005 and the data of (latest) positive control with caffeine (March 2005), benzoic acid (March 2004) and testosterone (June 2004) is provided. The standard deviation of dermal penetration is extremely high. The maximum amount of 2-Hydroxyethyl picramic acid absorbed into the skin (lower skin + receptor fluid) i.e. 11.38 µg/cm^2 may be used for the calculation of margin of safety under oxidative conditions.
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3.3.5. Repeated dose toxicity

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

Taken from opinion n° SCCNFP/0182/99

Guideline:	OECD 407 (1981)
Species/strain:	Wistar
Group size:	40 animals
Test substance:	2-hydroxyethylpicramic acid (HEP)
Batch:	21989/8
Purity:	99% (HPLC)
Dose:	50, 150 and 450 mg/kg bw day
Route:	Oral, in 0.5% carboxymethylcellulose
Exposure:	28 days
GLP:	in compliance

HEP was administered by gavage once daily to 3 groups of Wistar rats (5/sex, bw males 212 g, bw females 164 g) for 28 days. The test substance was administered at dosage levels of 50, 150 and 450 mg/kg bw/day. The control group (5/sex) received the vehicle (aqueous solution of 0.5% carboxymethylcellulose) only. In addition, two recovery groups (high and control, 5/sex) were kept for further 15 days without test substance administration. All animals were sacrificed at the end of the study (day 29 and 44).
Animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Opthalmological examinations were performed on all animals, in pre-test and in week 4. Blood samples were taken from all animals of all groups for haematological (10 parameters) and clinical chemistry (13 parameters).
Investigations were carried out on day 0, 28 and 43. Urine samples (8 parameters) were taken from all rats of all test groups on day 0, 28 and 43. Organ weights (4 organs) were recorded. Macroscopy and histopathology (35 organs/tissues) was performed on all animals.

Results
Three animals died during the study (two of the recovery control and one of the 150 mg/kg group, due to the sampling). The uric acid level was significantly increased and the glucose level was significantly decreased in the 450 mg/kg bw/day group (after the recovery period, these effects were disappeared). A dose related staining of the urine was noted in all test groups indicating systemic exposure. At necropsy (day 29 and 44), in all animals of the test groups fur and skin were stained medium brick-red. Absolute and relative adrenal weights were significantly increased in the males of the high group. All animals of the 450 mg/kg bw/day group showed increased relative and absolute kidney weights. Testicular weights were decreased in 3/5 animals in 450 mg/kg bw/day group and 2/5 animals of the 450 mg/kg bw/day recovery group. Histopathological examination revealed testicular atrophy in males of the 450 mg/kg bw/day group and hepatic bile duct proliferation in the 150 and 450 mg/kg bw/day groups.

Conclusion

The NOAEL was defined at 50 mg/kg bw per day.

Ref.: 13 (submission I)

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

Guideline:	OECD 408
Species/strain:	Wistar
Group size:	15 per sex and dose group 15 animals per sex high dose satellite group
Test substance:	2-hydroxyethylpicramic acid (HEP)
Batch:	BR 624
Purity:	>99.4%
Dose:	20, 60 and 180/120 mg/kg bw/day
Route:	Oral in deionized water
Exposure:	91 days, 5 days/week
GLP:	in compliance

Doses of 20, 60 and 180 mg/kg bw/day (120 mg/kg bw/day from week 2 onward due to solubility problems) of HEP were administered daily to groups of 15 male and 15 female Wistar rats. The mean bodyweight of the animals were 139.96 +/-6 and 161.45 +/-5 g for females and males, respectively. For recovery, satellite groups of additional 15 males and 15 females for the high dose and 5 females and 5 males for the control were studied after 4 weeks.
Mortality and clinical signs were recorded once daily and individual body weight, food and water intake were recorded. Ophthalmological investigation was done for high, mid and control groups before exposure and the end of the exposure.
Haematology and blood chemistry for 10 animals per sex and dose group (5 at 16 weeks for the recovery and control group) were performed after 6 and 13 weeks in all dose groups as well as after 16 weeks in the recovery group. Urine analysis was done after 5, 11 and 15 weeks.
Animals were subjected to a detailed necropsy and a number of organs (adrenals, brain, heart, kidneys, liver, spleen, testes and uterus) of 10 animals per sex and dose were weighed and archived. All gross lesions were examined and histopathological investigations for various tissues were carried out.

Results
No HEP related deaths occurred and besides staining of the urine indicating systemic exposure from week 3 of treatment onwards, no clinical signs were reported. Ophthalmology did not reveal any difference between the exposure groups and control animals. Food and water consumption and body weight gain was not affected by the HEP exposure. All figures in haematology, blood chemistry and urine analysis were with in the normal variation of this particular strain of rats.
The absolute and the relative organ weights were not affected by the exposure. At histopathology an activation of the thyroid epithelial cells in males and protein cylinders in the kidney in both sexes were noted at 60 mg/kg bw/day and above. They were interpreted as treatment related effects.

Conclusion
The NOAEL was defined at 20 mg/kg bw per day.

Ref.: 23

Guideline:	/
Species/strain:	Wistar
Group size:	15 per sex 15 animals per sex high dose satellite group
Test substance:	2-hydroxyethylpicramic acid (HEP)

Batch:	Po2
Purity:	>98.4%
Dose:	15 mg/kg bw/day
Route:	Oral in 1% aqueous carboxymethylcellulose
Exposure:	91 days, 5 days /week
GLP:	in compliance

Wistar rats (15 male, mean bw 103.5 g and 15 female mean bw 100.0 g) were exposed to 15 mg/kg bw/day of HEP by gavage.
Mortality and clinical signs were recorded once daily and individual body weight, food and water consumptions were recorded. Opthalmologic investigations were carried out before exposure and at the day of 85.
Haematology, blood chemistry and urine analysis for 10 animals per sex were done at days of 0, 41 and 83.
All animals were subjected to a detailed necropsy and a number of organs of all animals were weighed and the tissues and organs archived for the possible future use. All gross lesions were examined and histopathological investigations for various tissues were carried out.

Results
No HEP related deaths occurred. Urine was stained to orange red on day 5 onwards which was the only clinical sign reported. Ophthalmology did not reveal any difference between the exposure groups and control animals. Food and water consumption and body weight gain was not affected by the HEP exposure. All figures in haematology, blood chemistry and urine analysis were with in the normal variation of this particular stain of rats.
At necropsy no macroscopical findings were noted and no biologically relevant HEP related effects were reported for the absolute or relative organ weights. HEP related findings in histopathology were not reported

Conclusion
The NOAEL was defined at 15 mg/kg bw per day.

Ref.: 24

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 (1983)
Species/strain:	*Salmonella typhimurium*, TA1535, TA1537, TA98, TA100 and TA1538
Replicates:	Three plates per concentration in two independent experiments
Assay conditions:	Direct plate incorporation method, both in the presence and absence of Aroclor 1254 induced rat liver S9-mix.
Test substance:	2-hydroxyethyl picramic acid
Batch:	21989/8
Purity:	94.3 %
Concentrations:	3.2, 16, 80, 400, 2000 µg/plate
Solvent:	DMSO
GLP:	In compliance

2-hydroxyethyl picramic acid was tested in a preliminary toxicity test in TA100 at 9 concentrations from 19.5 to 5000 µg/plate. Based on this toxicity test the highest concentration tested in the two main tests was 2000 µg/plate both in the absence and presence of metabolic activation. Negative (solvent) and positive controls were included in all experiments in accordance with OECD guidelines.

Results
Toxicity was observed at the highest concentration tested (2000 µg/plate) in both assays both with and without metabolic activation. 2-hydroxyethyl picramic acid induced a substantial concentration related increase in revertants in all tester strains both with and without metabolic activation at concentrations $\geq$ 16 µg/plate. All positive controls used gave a distinct increase of induced revertant colonies.

Conclusion
Under the experimental conditions used in this study, 2-hydroxyethyl picramic acid was mutagenic in the bacterial reverse mutation test.

Ref.: 25

Remarks
No laboratory historical control data was submitted. An older bacterial mutation test is also available but not submitted. This limited study was also positive.

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

Guideline:	OECD 476 (1983)	
Species/strain:	Mouse lymphoma cell line L5178Y (*tk* locus)	
Replicates:	Duplicate cultures in two independent experiments	
Metabolic act.:	Aroclor 1254 induced rat liver S9-mix	
Test substance:	2-hydroxyethyl picramic acid	
Batch:	do/2007	
Purity:	99.9%	
Concentrations:	First experiment:	62.5, 125, 250 and 500 µg/ml (without S9-mix) 2.5, 5, 7.5 and 10 µg/ml (with S9-mix)
	Second experiment:	200, 300, 400 and 500 µg/ml (without S9-mix) 6, 8, 10 and 12 µg/ml (with S9-mix)
Treatment:	Pulse (3h) treatment both in the absence and presence of S9-mix and 72 hours expression period.	
Solvent:	DMSO	
GLP:	In compliance	

2-hydroxyethyl picramic acid was evaluated for its genotoxic activity at the *tk* locus in the mouse lymphoma cell line L5178Y. Two independent experiments using duplicate cultures each were performed. Both experiments used a pulse (3-hour) treatment and were conducted in the absence and presence of metabolic activation (S9-mix prepared from the liver of rats given Aroclor 1254). In the first study 6 and 7 concentrations were tested with and without metabolic activation respectively. The concentrations chosen for evaluation were based on toxicity of the test compound.
In the first experiment the following concentration were evaluated:

62.5, 125, 250 and 500 µg/ml (without S9-mix)
2.5, 5, 7.5 and 10 µg/ml (with S9-mix)

In the second experiment the following concentrations were evaluated:

200, 300, 400 and 500 µg/ml (without S9-mix)
6, 8, 10 and 12 µg/ml (with S9-mix)

Dimethylsulphoxide (DMSO) was used as solvent control, while methylmethanesulphonate (MMS µg/ml) without and benzo[a]pyrene (BP) with metabolic activation system were used as positive controls. Mutant frequency, colony size and cell survival (measured as total suspension growth) were determined.

Results
Toxicity was noted at 500 µg/ml and above in the absence of S9-mix (experiment I: relative cell growth of 38.4 % at 500 µg/ml and < 0.31 % at 750 µg/ml; Experiment II: 13.5 % at 500 µ/ml and 2.3 % at 600 µg/ml) and in the presence of S9-mix at 10.0 µg/ml in experiment I with a relative cell growth of 40.4 %.
In the presence of S9-mix a statistically significant increase in the number of mutant colonies as compared to the concurrent controls and the historical control range were noted in all concentrations tested in the first experiment (at 2.5, 5.0, 7.5 and 10.0 µg/ml, 2.25, 2.83, 3.31 and 4.94 times the control value, respectively) and the second experiment (at 8, 10, 12 µg/ml, 4.92, 5.52 and 6.22 times the control value, respectively). The observed mutation frequency in the absence of S9-mix was also significantly higher than those of the solvent control at 500 µg/ml in the first (induction factor of 6.49) and in the second experiment (400 µg/ml and 500 µg/ml, induction factor 2.29 and 4.28, respectively.
Differentiation between small and large colonies did not reveal a clear pattern. Whereas a statistically significant increase of the small colonies was noted in the first experiment in the presence and absence of S9-mix and in the second experiment with S9-mix, such an effect was noted only at one concentration (8 µg/ml) in the second experiment without S9-mix. However, this finding indicates that 2-hydroxyethyl picramic acid might cause both gene mutations and chromosomal aberrations in mammalian cells *in vitro*.
The concurrent positive controls induced a distinct increase in mutation frequency.

Conclusion
Under the test conditions used in this study, 2-hydroxyethyl picramic acid was considered to be mutagenic and/or clastogenic in mammalian cells, both in the absence and in the presence of metabolic activation.

Ref.: 26

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	Not indicated but in line with OECD 474
Species/strain:	Mouse, strain NMRI
Group size:	5 males, 5 females per dose group and per sacrifice time
Test substance:	2-hydroxyethyl picramic acid
Batch:	JFB I 95
Purity:	99.6 %
Dose level:	0, 30, 100 and 300 mg/kg bw administered as single doses
Route:	Oral, gavage
Vehicle:	DMSO
Sacrifice times:	24 hours after dosing and 48 and 72 hours (high dose and control only)
GLP:	In compliance

The clastogenic/aneugenic potential of 2-hydroxyethyl picramic acid was investigated in bone marrow cells of mice. The animals were dosed once by gavage. For the vehicle and high dose, three groups were treated to allow sampling after 24, 48 and 72 hours.
Bone marrow cells were sampled from sacrificed mice 24 hours after dosing in all dose groups and positive control animals and additionally after 48 and 72 h in the high dose group and vehicle control. 1000 PCEs per animal were scored for the incidence of micronuclei. Toxicity on the bone marrow was measured as the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE ratio) for each animal.
Positive (cyclophosphamide (CPA)) and negative (vehicle) controls were included.

Results
Signs of toxicity after dosing were abdominal position, reduced spontaneous activity and incomplete eye-closure in the animals of the group treated with the highest dose. Four animals (3 males and 1 female) of the highest dosage died within 24 or 48 hours after administration. The remaining animals recovered within 24 hours after treatment and survived until sacrifice.
2-hydroxyethyl picramic acid showed no clear cytotoxic effect in the bone marrow as the PCE/NCE ratio did not substantially change after treatment with the test item. However, the observed systemic toxicity and the available kinetic data indicate that 2-hydroxyethyl picramic acid becomes bio-available and systemically distributed after oral administration.
There was no statistically significant or biologically relevant increase in the number of micronuclei in mice of any 2-hydroxyethyl picramic acid treated group compared to the respective vehicle control group.
The positive control (CPA) induced a distinct and significant increase in micronucleated PCEs.

Conclusion
Under the test conditions used in this study 2-hydroxyethyl picramic acid was not genotoxic (clastogenic and/or aneugenic) in the *in vivo* micronucleus test in mice.

Ref.: 27

Comet assay *in vivo* in male rats

Guideline:	Performed according to an internationally accepted protocol for the comet assay *in vivo (*Ref.: 28)
Species/strain:	Rat, Wistar CRL:(WI) BR
Group size:	5 males at doses of 0, 500 and 1000 mg/kg bw 2 males at the dose of 2000 mg/kg bw
Test substance:	2-hydroxyethyl picramic acid
Batch:	2/99
Purity:	99.8%
Dose levels:	0, 500, 1000 and 2000 mg/kg bw
Route:	Twice orally by gavage (20 hours apart)
Sacrifice time:	3 hours after second treatment
Organs studied:	liver, stomach and urinary bladder
Vehicle:	Deionised water
GLP:	In compliance

The potential genotoxic effect of 2-hydroxyethyl picramic acid was assessed in the comet assay in liver, stomach and urinary bladder epithelium cells after *in vivo* treatment of male rats. A range finder test was conducted with 3 male rats, administered doses of 750 and 1500 mg/kg bw to identify the maximum tolerated dose (MTD). The doses used in the main study were based on the observed clinical signs and on the histopathological findings in this pre-test. Intact cells of the different organs were isolated by in situ perfusion of the liver and enzymatic digestion of stomach epithelium and urinary bladder epithelium of the same animal. The comet assay was performed under alkaline conditions. Fifty cells per slide and two slides per animal (100 cells total) were evaluated for the occurrence of DNA damage (tail length, Olive tail moment and % tail DNA). The tail length, defined as the distance between the middle of the head and the end of the tail, was used as the assessment parameter because the historical data of the performing laboratory are based on this parameter (and upon sponsor request). The viability of the cells from the different organs was determined directly after the cell isolation by using the trypan blue exclusion method. Ethylmethane sulfonate in deionised water was used as positive control (400 mg/kg bw once via gavage 3 h before sacrifice).

Results
At 2000 mg/kg bw, the liver cell yield was too low in one rat and mortality associated with marked hepatocellular degeneration indicated this dose to exceed the MTD. No further animals were exposed to this dose. At the other doses tested, both in the pre-test and in the main study, rats showed clinical signs of toxicity such as piloerection, discoloured urine, accelerated breathing and discoloration of hairless skin areas at the selected doses of 2-hydroxyethyl picramic acid. These findings indicate adequate systemic exposure to the test agent. The DNA tail length of liver, stomach and urinary bladder cells of rats treated 500 and 1000 mg/kg bw 2-hydroxyethyl picramic acid were comparable to concurrent control values. In this study data on % DNA in tail and tail moment was also recorded. Using these parameters for evaluation there was a positive and dose related effect of 2-hydroxyethyl picramic acid in the bladder. This effect was statistically significant when DNA damage was measured as tail moment. The tail length in the positive control group was markedly increased compared to the negative control animals for liver (66 %), stomach (49 %) and urinary bladder epithelium (74 %) cells, demonstrating the sensitivity of the test system.

Conclusion
Under the conditions of the comet assay *in vivo* described in this study, 2-hydroxyethyl picramic acid is considered to be non-genotoxic to liver, stomach and urinary bladder epithelium cells of male Wistar rats after the oral administration of two sequential doses which clearly exhibited systemic toxic doses.

Ref.: 29

Comment
DNA damage was measured as tail length, % DNA in tail and tail moment. Upon sponsor request only data on tail length was used in the evaluation of the data. However, it has been recommended by several authors to use either % DNA in tail or tail moment, which gives the best dose response relationship, while tail length tends to reach a maximum at a low level of damage.
In this study data on % DNA in tail and tail moment was also recorded. Using these parameters for evaluation there was a positive and dose related effect of 2-hydroxyethyl picramic acid in the bladder. This effect was statistically significant when DNA damage was measured as tail moment. Laboratory historical control data was not submitted.
2-Hydroxyethyl picramic acid was genotoxic in urinary bladder cells when tail moment was measured.
An explanation for the inconclusive results is required.

Submission III, March 2008
In this submission, the applicant provided evidence that the observed increases in tail moment and % tail DNA were inside the range of negative control values in the test laboratory. Therefore, the SCCP endorses that HC Blue n° 12 is not genotoxic in the Comet assay with rats under the conditions used.

Unscheduled DNA Synthesis *in vivo*/*in vitro*

Guideline:	OECD 486 (draft 1991)
Species/strain:	Rat, Wistar HanIbm:WIST (SPF)
Group size:	5 males per dose group and per sacrifice time
Test substance:	2-hydroxyethyl picramic acid
Batch:	DO 2007
Purity:	99.9%
Dose levels:	0, 750 mg/kg bw (2 h treatment); 0, 75 and 750 mg/kg bw (16 h treatment)
Route:	Oral, gavage, single dose (10ml/kg bw)
Vehicle:	PEG 400
Preparation time:	2 and 16 hours

GLP: In compliance

2-hydroxyethyl picramic acid was assessed for its potential to induce DNA-damage and -repair in the *in vivo / in vitro* UDS test using rat hepatocytes. Pre-experiments with 2 male rats each were performed to determine the MTD. Doses of 1200, 1000, 750 and 500 mg/kg bw were administered in PEG 400. Based on the findings in the pre-test 750 mg/kg bw was chosen as the MTD.
From each animal at least three primary hepatocyte cultures were established and exposed for 4 h to ^{3}H-thymidine. Possible liver cell toxicity was examined by means of the dye-exclusion (trypan blue) method. Four animals per dose group were evaluated. At least two slides per animal were evaluated for UDS, covering 100 cells/animal in total. Vehicle (PEG 400) was used as negative control and 2-acetylaminofluorene (2-AAF) as positive control (100 mg/kg bw).

Results
In the pre-test both animals dosed with 1200 mg/kg bw and one animal dosed with 1000 mg/kg bw died within 24 h. The surviving animal at 1000 mg/kg bw showed clinical signs of toxicity (reduced spontaneous activity, eyelid closure, piloerection). Both animals treated with 750 mg/kg bw expressed similar but less pronounced effects, whereas no effects were noted at 500 mg/kg bw. On the basis of these results 750 mg/kg bw was estimated to be close to the maximum tolerated dose and chosen as the maximum dose for the main study.
In the main study at the high dose (750 mg/kg bw, 16 h preparation interval) the urine and the tissues of the peritoneal cavity were orange-red coloured. This finding, together with the observed signs of toxicity, demonstrates the systemic availability of 2-hydroxyethyl picramic acid after oral gavage. The determined viability (trypan blue exclusion) of the isolated hepatocytes was very similar for control and treatment groups (65 - 78% and 52 - 69%, respectively).
2-hydroxyethyl picramic acid did not induce UDS in the hepatocytes of the treated animals as compared to the vehicle control. Neither the number of nuclear grains nor the resulting net grains were increased due to the *in vivo* treatment of the animals with 2-hydroxyethyl picramic acid for 2 or 16 h, respectively. *In vivo* treatment with 2-AAF induced distinct increases in the number of nuclear and net grain counts.

Conclusion
Under the experimental conditions reported 2-hydroxyethyl picramic acid did not induce DNA-damage leading to increased repair synthesis in rat hepatocytes *in vivo*.

Ref.: 30

Comment on additional mutagenicity tests
A range of old, limited and non-guideline studies have been performed, but were not submitted for this evaluation:
Ames test, UDS in HeLa cells, Sister chromatid exchange in mouse bone marrow cells,
Sister chromatid exchange in rat bone marrow, UDS assay in rats. These studies were evaluated in 1999 by SCCNFP and all but the Ames test were negative.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Guideline: OECD 416
Species/strain: rat/Wistar
Group size: 25 per sex and dose

Test substance:	2-hydroxyethylpicramic acid (HEP)
Batch:	2/93
Purity:	>99.8%
Dose:	30, 60, 120 mg/kg bw/day
Route:	Oral in 4.0 % aqueous carboxymethylcellulose
Exposure:	P-generation about 140 days, F1 generation about 220 days in total
GLP:	in compliance

HEP in 4% aqueous carboxymethylcellulose was administered daily by oral gavage to rats (males bw 199-240 g and females 138-178 g). Parent animals (P generation) of both sexes were treated during a 70 days premating period and a subsequent mating period. Female rats were treated during the gestation and lactation periods. Following weaning on day 21 post partum, the parent animals of the first filial (F1) generation were selected from the pups of the P generation dams (25 males and 25 females). Excess pups were killed and subjected to macroscopic investigations. F1 animals were exposed from day 22 and continued to maturation and mating (120) days. F1 parent females were exposed during gestation and lactation periods.

In a preliminary dose finding study doses of 0, 50, 150 and 450 mg/kg bw/day were used and administered by gavage. The F2 generation was reared until 7 days after weaning, but they were not directly exposed.

Food consumption was decreased and the numbers of implantations were lower and the litters were smaller in the 150 mg/kg bw/day dose group. At 450 mg/kg bw/day systemic and reproductive toxic effects were reported. A prolonged precoital time and no implantations were noticed at this dose Also the food consumption and body weight gain was reduced in dams. Necropsy indicated macroscopically abnormal testes and prostate and testes weights were significantly reduced.

Because of the above mentioned finding, doses of 30, 60 and 120 were selected for the main study. Each group of the P and F1 generations consisted of 25 male and 25 female Wistar rats.

Mortality and clinical signs were checked twice and body weights were recorded once a day. Food consumption and body weight gain were recorded. Implantation sites in the uteri were determined in all females including those who failed to deliver or lost their litter. Gestation, pregnancy and delivery rate were calculated.

Post mortem examinations of treated animals selected for mating in the P and F1 generations covered several organs and all macroscopic abnormalities were recorded.

The relative food consumption was increased in males at 60 (P generation) and 120 (P and F1 generations) mg/kg bw/day groups and in females in 120 mg/kg bw/day group (P-generation). However the body weight gain was not affected. HEP exposure did not affect to the organ weights and macroscopically or microscopically findings were not reported.

Reproduction parameters, including the number of implantations and duration of gestation, fertility and gestation indices, the conception rates, births, viability and the weaning indices were unaffected in both the P and F1 generations.

The litter data of the F1 and F2 generations were unaffected, except from the discolouration of the fur noted in all pups of the F1 and F2 generations.

Neither the birth weight (day 0 post partum) nor the mean body weight of the F1 and F2 offspring during the lactation period were affected.

In the F1 generation the ratio of males: females were statistically significantly increased in the low and the mid dose groups compared to the control group, but not in the high exposure group. In the F2 group the sex ratio was normal in all groups.

HEP related macroscopic findings were not reported in the F1 and in the F2 generations.

Ref.: 31

Comment
The fertility and the reproduction parameters of the P and F1 parent animals, as well as their progeny were unaffected in any dose levels. The NOAEL for reproductive toxicity was set at 120 mg/kg bw/day.
In the dose range finding study, testes atrophy was observed at 450mg/kg bw, similar to the observations of the 28-day study. No such effects were noted in the main study at 120 mg/kg bw per day.

3.3.8.2. Teratogenicity

Guideline:	OECD 414
Species/strain:	rat/Sprague-Dawley (SD)
Group size:	24 female per dose
Test substance:	2-hydroxyethylpicramic acid (HEP)
Batch:	2/99
Purity:	>98.4%
Dose:	50, 150, 450 mg/kg bw/day
Route:	Oral in 0.5 % aqueous carboxymethylcellulose
Exposure:	Once daily from day 6 to day 19 of pregnancy
GLP:	in compliance

Pregnant SD (n=24) rats were exposed to 50, 150 and 450 mg/kg bw/day from day 6 to day 19 of gestation. Animals were observed on a daily basis during the entire exposure period for clinical signs. Body weight gain and food consumption data was collected.

On day 20 of gestation all dams were sacrificed and macroscopic examination of the organs was carried out. The ovaries and the intact uteri were removed and the presence of resorption sites and foetuses were examined. Each live foetus was weighted, sexed and examined for gross external malformations. A skeletal and a visceral examination of the foetuses were performed. Placenta and uterus weights were recorded.
No differences compared to controls were noted at 50 mg/kg bw/day, except orange staining of the fur of dams.
At dose groups of 150 and 450 mg/kg bw/day HEP caused some maternal toxicity as indicated by the reduction in body weight from day 12 to day 20 of gestation. A reduction in food consumption was also reported. Gravid uterus weight was lower in 450 mg/kg bw/day group compared to controls.
An increased incidence of abdominal haemorrhages in foetuses was noted at the mid and high dose group.
In the high dose group a delay in development of foetuses was noted (small foetuses and delayed ossification)

For 2-hydroxyethylpicarmic acid the NOAEL of maternal toxicity is 50 mg/kg bw/day and the NOAEL of foeto/embryotoxicity is 50 mg/kg bw/day.

Ref.: 32

3.3.9. Toxicokinetics

Guideline:	/
Species/strain:	Human intestinal epithelial cell line TC-7
Group size:	/

Test substance:	2-hydroxyethylpicramic acid (HEP)
Batch:	2/99
Purity:	>98.4%
Dose:	50 microM in HBSS buffer, 1% DMSO
Route:	/
Exposure:	60 minutes incubation
GLP:	/

The bioavailability of HEP across the intestinal barrier was investigated in human intestinal epithelial cell *in vitro*. Analysis of the donor and receiver samples was done by using HPLC/MS/MS instrument. The permeability coefficient (Papp) was calculated for two independent experiments. 14C-mannitol (about 4 µM) was used to demonstrate the integrity of the cell monolayer.

The total recovery of HEP ranged from 79 to 96%. By using the reference compounds (atenolol, propranolol, vinblastine and ranitidine) the validity of the assay was demonstrated.

With HEP a mean permeability in human intestinal epithelial cells of 10.7 x 10-6 cm/sec was obtained.

Conclusion
The test substance has moderate permeability across the intestinal barrier *in vitro*.

Ref.: 33

3.3.10. Photo-induced toxicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

2-hydroxyethyl picramic acid
(non-oxidative)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**13.85 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**9.70 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.16 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**20 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**124**

2-hydroxyethyl picramic acid
(oxidative)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**11.38 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**8.00 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.13 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**20 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**151**

3.3.14. Discussion

Physico-chemical properties
2-Hydroxyethyl picramic acid is used as a hair colouring agent in oxidative hair dye formulations at a maximum on-head concentration of 1.5%, after mixing with hydrogen peroxide and it is also used as a hair colouring agent in semi-permanent hair dye formulations at a maximum on-head concentration of 2%. Supportive document for the reported data on purity/impurities for only 2 batches of 2-hydroxyethyl picramic acid were submitted. In general, the contents of impurities A (Picric acid), B (2-Amino-4,6-dinitrophenol) and C (2-[Di(2-hydroxyethyl)amino]-4,6-dinitrophenol) in different batches varied in several orders of magnitude. No data on stability of 2-hydroxyethyl picramic acid in test solutions and in marketed products is reported. 2-hydroxyethyl picramic acid is a secondary amine. Nitrosamine content in the compound is not reported. 2-hydroxyethyl picramic acid should not be used together with nitrosating agents. The test material, batch 21989/8, used for Ames test was only 87% pure. However, the results of this study did not influence the overall conclusion.

General toxicity
In a non GLP study the LD_{50} for male and female rats were 1134 and 900 mg/kg bw, respectively. LD_{50} for male and female mice were 525 mg/kg bw. In a 90 days study at histopathology an activation of the thyroid epithelial cell in males and protein cylinders in the kidney in both sexes were noted at 60 mg/kg bw and the No Observed Adverse Effect Level (NOAEL) was set to 20 mg/kg bw/day. The NOAEL for maternal toxicity was 50 mg/kg bw/day. The NOAEL of foeto/embryotoxicity is 50 mg/kg bw/day.
2-Hydroxyethyl picramic acid is classified as toxic to reproduction category 3 (R62: possible risk of impaired fertility). The assessment (BgVV, 1993) was based on testes atrophy in the 450 mg/kg bw/d dose group of the 28-day study.
A 2-generation toxicity study was performed. In the dose range finding study, testes atrophy was observed at 450 mg/kg bw, similar to the observations of the 28-day study. No such effects were noted in the main study at 120 mg/kg bw per day.
The NOAEL for reproductive toxicity was set at 120 mg/kg bw per day.

Irritation / sensitisation
1-Hydroxy-2-(2'-hydroxyethyl)amino-4,6-dinitrobenzene is not irritant to rabbit skin, and it is slightly irritant to the rabbit eye under the conditions of the test.
Under the test conditions, 2-Hydroxyethyl picramic acid was not shown to be a skin sensitiser in LLNA.

Dermal absorption
The maximum amount of 2-Hydroxyethyl picramic acid absorbed into the skin (lower skin + receptor fluid) i.e. 13.65 µg/cm^2 may be used for the calculation of margin of safety under non-oxidative conditions.
The maximum amount of 2-Hydroxyethyl picramic acid absorbed into the skin (lower skin + receptor fluid) i.e. 11.38 µg/cm^2 may be used for the calculation of margin of safety under oxidative conditions.

Mutagenicity
2-Hydroxyethyl picramic acid induced gene mutations in bacteria both in the absence and the presence of metabolic activation. The test item also induced gene mutations and/or clastogenic effect in mammalian cells at the *tk* locus of mouse lymphoma cells. The clastogenic effect could not be confirmed in an *in vivo* micronuclus test in mice up to systemic toxic doses. In an *in vivo/in vitro* UDS assay no genotoxic effect was observed in liver cells after oral exposure to 2-Hydroxyethyl picramic acid. In addition 2-Hydroxyethyl picramic acid was non-genotoxic to liver, urinary bladder cells and stomach cells in an *in vivo* comet assay in rats.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCP is of the opinion that the use of 2-Hydroxyethylpicramic acid as an ingredient in non-oxidative hair dye formulations with a maximum on-head concentration of 2.0% and in oxidative hair dye formulations with a maximum on-head concentration of 1.5% does not pose a risk to the health of the consumer.

2-Hydroxyethylpicramic acid is a secondary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

2-Hydroxyethyl picramic acid is classified as toxic to reproduction category 3 (R62: possible risk of impaired fertility). The CMR classification was based on the assessment by BgVV (1993) on testes atrophy in the 450 mg/kg bw/day dose group of the 28-day study. However, more recent studies on reproduction toxicity do not support this classification.

5. MINORITY OPINION

Not applicable

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