EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

4-Nitrophenyl aminoethylurea

COLIPA n° B70

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

The SCCP adopted this opinion at its 12th plenary meeting on 19 June 2007

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.

Scientific Committee members

Claire Chambers, Gisela Degen, Ruta Dubakiene, Ramon Grimalt, Bozena Jazwiec-Kanyion, Vassilios Kapoulas, Jean Krutmann, Carola Lidén, Jean-Paul Marty, Thomas Platzek, Suresh Chandra Rastogi, Jean Revuz, Vera Rogiers, Tore Sanner, Günter Speit, Jacqueline Van Engelen, Ian White

Contact:
European Commission
Health & Consumer Protection DG
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-Sc6-Secretariat@ec.europa.eu

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http://ec.europa.eu/health/ph_risk/risk_en.htm

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Dr. C. Chambers
Prof. V. Kapoulas
Prof. C. Lidén
Prof. J.-P. Marty
Prof. T. Platzek (chairman)
Dr. S.C. Rastogi
Prof. T. Sanner
Dr. J. van Engelen
Dr. I.R. White (rapporteur)

External experts

Dr. M.-L. Binderup	National Food Institute, Technical University of Denmark
Dr. H. Norppa	Finnish Institute of Occupational Health, Finland
Prof. K. Peltonen	Finnish Food Safety Authority, EVIRA, Finland
Dr. J. van Benthem	RIVM, the Netherlands

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1. BACKGROUND

Submission I for 4-Nitrophenyl aminoethylurea was submitted in March 1990 by COLIPA[1,2].

The Scientific Committee on Cosmetology (SCC) adopted at its 50th plenary meeting on 2 June 1992 an opinion (SPC/106/92) with the final conclusion that:

"A proper evaluation of skin and eye irritating properties of the test compound is not possible because of the fact that the guinea pig is used instead of the rabbit and no good motivation is given for this choice. Furthermore no material is supplied to compare the results of the used test species with the conventional test using the rabbit. However, only minor effects were seen and further animal usage for irritancy testing was considered to be not justified. The test compound showed no signs of sensitization. The dermal absorption was 0.103 % for the applied solution and 0.0132 % or 0.0171 % for the formulation with or without hydrogen peroxide, respectively. The compound appeared to be not teratogenic or foetotoxic after administration of 10 mg/kg bw No mutagenic activities were observed when the test substance was tested in various test systems. Based on the effects found in the 90-day oral study, especially those found in the spleens of females, no no-effect level can be established. (In male rats no effects were seen at 5 mg/kg bw) For normal use of Ureidogelb the following calculation can be made: 500 mg of Ureidogelb comes in contact with the human skin in permanent hair dye condition and 175 mg in semipermanent hair dye condition (based on a maximum usage volume of 100 ml and 35 ml hair dye containing 0.5 % Ureidogelb, respectively). With a maximum dermal penetration of 0.0132 % this results in a dermal absorption of 0.066 mg per treatment for permanent hair dye and 0.023 mg per treatment for semipermanent hair dye, which is 0.0011 mg/kg bw and 0.0004 mg/kg bw, respectively (assuming a body weight of 60 kg). Because 5 mg/kg bw was the lowest effect level the safety margin is calculated on 0.5 mg/kg bw. So a safety margin of 455 can be calculated for the permanent hair dye. For the semipermanent hair dye a safety margin of 1250 can be calculated. It should be noted that the lowest effect level is based on daily exposure for 90 days, while human exposure to permanent hair dye is unlikely to be more than once a month and human exposure to semipermanent hair dye is unlikely to be more than once a week. No additional data are needed and the safety margins for both permanent and semipermanent hair dye are considered to be acceptable."

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 49 on the List of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission II was submitted in July 2005 by COLIPA. According to this submission 4-Nitrophenyl aminoethylurea is used as:

a) a non-reactive hair colouring agent ("direct dye") in non-oxidative hair dye formulations at a maximum on-head concentration of 0.5%. It is common practice to apply 35 to 50 g of the product over a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at weekly intervals.

b) a non-reactive hair colouring agent ("direct dye") in oxidative hair dye formulations at a maximum on-head concentration of 0.25%. If used in oxidative hair dye formulations, the dye component and developer (hydrogen peroxide) are mixed in ratios between 1:1 to 1:3. It is common practice to apply up to 100 g of the finished mixed product for a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at monthly intervals.

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

[2] According to records of COLIPA

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 4-Nitrophenyl aminoethylurea safe for use as a non-oxidative hair dye with an on-head concentration of maximum 0.5% taken into account the scientific data provided?*

2. *Does the SCCP consider 4-Nitrophenyl aminoethylurea safe for use in oxidative hair dye formulations with an on-head concentration of maximum 0.25% taken into account the scientific data provided?*

3. *Does the SCCP recommend any further restrictions with regard to the use of 4-Nitrophenyl aminoethylurea in any non-oxidative or oxidative hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

The name mentioned in the opinion of 2 June 1992 (SPC/106/92) was 1-(2'-ureidoethyl)-amino-4-nitro-benzene. Ureidogelb is one of the trade names.

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

4-Nitrophenyl aminoethylurea (INCI)

3.1.1.2. Chemical names

Urea, 2-[(4-nitrophenyl)amino]ethyl-, (CA Index name, 9CI)
N-[2-(4-nitroanilino)ethyl]-urea (IUPAC)

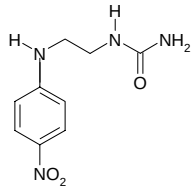
3.1.1.3. Trade names and abbreviations

Ureidogelb
Nitrogelb (OSL)
COLIPA n° B070

3.1.1.4. CAS / EINECS number

CAS: 27080-42-8
ELINCS: 410-700-1

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_9H_{12}N_4O_3$

3.1.2. Physical form

Yellow powder

3.1.3. Molecular weight

Molecular weight: 224.22 g/mol

3.1.4. Purity, composition and substance codes

[a] Purity and impurities of various batches of 4-Nitrophenyl aminoethylurea

Description	**Batch number**					
	Ch. B1/89	**Ch. B3/89**	**Ch. L4/130**	**LEH 5/2**	**L 4/113**	**Ch. 110889 (R96003805)**
Identification /characterisation	NMR, HPLC	NMR, MS, HPLC, IR, UV_Vis	NMR, HPLC, Elemental analysis	NMR, MS, HPLC, IR, UV_Vis,	NMR, HPLC	NMR, MS, IR, UV_Vis, HPLC, Elemental analysis
NMR content (g/100 g)	99.5	96.5	92.7	96.8	98.0	99.5
HPLC content* (g/100g)	97.5	98.1	91.6	95.2	99.2	Reference material
Impurities** A(ppm) B (g/100 g)	<100[1] 0.113	<50[1] 0.05	<50[1] 0.12	<50[1] 1.06	<50[1] 0.18	<50[1] 0.22
Water content (g/100g)	0.02	0.01	6.7	0.07	0.04	0.02
Loss on drying	0.03	0.02	6.6	0.10	0.01	0.01
Residue on ignition (g/100 g)	0.05	0.07	0.09	2.37	0.05	0.15
Element screening, ppm Levels above 25 ppm are reported		Si 91 K 236 Fe 18	Na 40 Mg 39 Si 241 K 98 Ca 171 Fe 46	Na 52 Si 117 K 26300 Fe 20	Si 34	Si 34 K 527

[a] Supportive analytical data for only some batches was provided
* Reference material Ch. 110889 (R96003805)
** A: 1-chloro-4-nitrobenzene
B: N-(2-aminoethyl)-4-nitroaniline
Several unidentified impurities (other than A and B) with HPLC peak area 0.01-0.3% were observed in the attached chromatograms.
[1] Detection limit, not detected

3.1.5. Impurities / accompanying contaminants

See 3.1.4

3.1.6. Solubility

Water:	0.0787 g/l, at 21°C, pH 8.1(saturated solution)
Ethanol:	< 10 g/l
DMSO:	>100 g/l
Acetone/water 1:1:	<10 g/l

3.1.7. Partition coefficient (Log P_{ow})

Log K_{ow}: 0.88 (pH 7.7, 22°C)

3.1.8. Additional physical and chemical specifications

Melting point:	180.9 °C –decomposition during melting
Boiling point:	not applicable
Flash point:	/
Vapour pressure:	17.4 hPa (20°C)
Density:	0.636 (20°C)
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum:	381 and 230 nm

3.1.9. Stability

4-Nitrophenyl aminoethylurea on storage in dryness and protected from light is considered to be stable for 7 years
The stability of test solution was tested over a period of 7 days. The test solutions were stored at room temperature and protected from light.

DMSO solution (approx. 10% w/v):	Stable, 99.5-100.5% of initial concentration
Acetone/water 1:1 solution (approx. 0.25%, w/v):	Stable, 98.5-99.5% of initial concentration
Water solution (approx. 0.01% w/v):	Stable, 99.7-101.0% of initial concentration

General comment to Physico-chemical characterisation

- 4-Nitrophenyl aminoethylurea is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in 4-nitrophenyl aminoethylurea is not reported.
- Several unidentified impurities were revealed in the HPLC chromatograms of the test compound.
- > 1% N-(2-aminoethyl)-4 nitroaniline was detected in batch LEH/52. This substance is a secondary amine. No further information was given.
- Stability of 4-nitrophenyl aminoethylurea in the presence of hydrogen peroxide and in marketed products is not reported.

3.2. Function and uses

4-Nitrophenyl aminoethylurea is used as direct dye in semi-permanent hair formulations at a maximum on-head concentration of 0.5%. 35-50 g of the product is applied for a period of 30 min, followed by rinse off with water and shampoo

4-Nitrophenyl aminoethylurea is used as a hair colouring agent (direct dye) in oxidative hair dye formulations at a maximum on-head concentration of 0.25%. The dye component and the developer are mixed at ratios 1+1 to 1+3. About 100 g of the finished mixed product is applied for a period of 30 min.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	Not indicated but in line with OECD 401(1981)
Species/strain:	Rat, Wistar strain Mice, CF1
Test substance:	1-(2-ureidoethyl)-amino-4-nitrobenzene
Batch:	Ch. L4/130
Purity:	91.6% (HPLC)
Dose:	Rats: 6000 and 8000 mg/kg bw Mice: 4200, 6600, 9000 and 11400 mg/kg bw
GLP:	Not in compliance

The test substance suspended in a 10% gummi arabicum solution was administered at 4 dose levels from 4200 to 11400 mg/kg bw to 10 female CF1 mice per dose and at 6000 and 8000 mg/kg bw to 6 Wistar rats per sex and dose once by oral gavage. Mortality and clinical signs were checked daily for the 14-day observation period. Body weights were recorded weekly and all animals were submitted to a gross necropsy at the end of the observation period.

Results
In the main experiment clinical signs such as reduced activity and clonic-tonic spasms were noted in the mice at doses of 9000 and 11400 mg/kg bw within the first four hours after substance administration. In the rats, no clinical signs were observed. Animals died within 2-24 hours (CF1 mice) and between 3 and 7 days (Wistar rats) after substance administration. At necropsy, no macroscopic organ changes/damages were noted.
Based on the observed mortality rates, the following LD50's were calculated using the method of Spearman-Kärber:

LD_{50} rat (female): 8 g/kg bw
LD_{50} rat (male): > 8 g/kg bw
LD_{50} mouse (female): 7.32 g/kg bw

Ref.: 16

Comment
Although the study was not performed in compliance with GLP, the results are considered valuable for the evaluation of the acute oral toxicity of the test substance.

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402 (1981) (limit test)
Species/strain:	Rat, strain Sprague Dawley (Him:OFA, SPF), 5/sex
Test substance:	Nitrogelb
Batch:	B1/89
Purity:	99.0 area% (HPLC, 254 nm)
Dose level:	2000 mg/kg bw
Observation period:	14 days
GLP:	In compliance

The test substance was moistened with distilled water immediately before testing, and was administered to the dorsal skin of 5 male and 5 female young-adult Sprague Dawley rats (Him:OFA, SPF), clipped free of hair the day before. The treated area of 30 cm² (corresponding to at least 10% of the body surface) was covered by a cellulose patch and kept in place by a tape. The area was covered additionally by a Fixomull dressing. After 24 hours exposure, the dressing, tape and patch were removed and residual test substance was wiped off. Clinical signs were noted at 10, 30 min, 1, 2, 4 and 6 hours after administration and at least once daily for the remaining 2 week observation period. Body weight was determined before administration and on days 7 and 14 after administration of the test substance.

Results
All animals survived until the end of the observation period. The body weight gain was reduced in 2 females during the second week as compared to untreated animals.
Slight and temporary clinical signs, such as chromodacryorrhoea, were noted in 2 males and 1 female within 1 day after administration. Yellow stained fur was observed in 1 male and all females post mortem.

Conclusion
The acute dermal toxicity study with 4-nitrophenylaminoethylurea in 5 male and 5 female rats revealed a dermal LD50 of > 2000 mg/kg bw.

Ref.: 17

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	/
Species:	Rabbit, New Zealand White
Group:	3 males
Substance:	Nitrogelb
Batch:	B1/89
Purity:	97.5%
Dose:	0.5g (moistened with water), single dermal application, 4h, semi-occlusive
GLP:	In compliance

0.5g of Nitrogelb moistened with distilled water was applied to an area of about 6.25cm^2 clipped dorsal skin of each of 3 male New Zealand White rabbits and kept in contact with the skin for 4 hours under semi-occlusive conditions. The treated area was covered with gauze and Micropore tape and then loosely bound with an elastic bandage.

The effects on the skin were evaluated at 1, 24, 48 and 72 h after patch removals.

Results
In one of the rabbits there was a slight erythema at 1 h after patch removal. No other reaction was observed at any other time point.

Conclusion
Under the conditions of the study, nitrogelb was not irritant to rabbit skin.

Ref.: 18

3.3.2.2. Mucous membrane irritation

Guideline: /
Species: Rabbit, New Zealand White
Group: 3 males
Substance: Nitrogelb
Batch: B1/89
Purity: 97.5%
Dose: 100mg (undiluted), instilled into the right conjunctival sac.
GLP: In compliance

100mg of Nitrogelb (undiluted) was instilled into the conjunctival sac of the right eye of 3 male New Zealand White rabbits without rinsing. The left eye served as the control. The effects on the eyes were evaluated at 1, 24, 48 and 72 h after application and at day 7.

Results
No corneal lesions were noted at any reading.
A slight iritis was noted in 2 animals 24 h after application, which had resolved at 48 h. Slight to moderate conjunctival erythema was noted in all animals at up to 48 h and up to 72 h in 2 animals. At 7 days, no abnormality was observed.

Conclusion
Under the conditions of the study, nitrogelb was irritant to rabbit eyes.

Ref.: 19

3.3.3. Skin sensitisation

Guideline: OECD 429
Species: Mouse, strain CBA/J
Group: 5 females per concentration
Substance: 4-nitrophenyl aminoethylurea (WR23348)
Batch: 110889
Purity: 99.5%
Dose: 0.5, 1.5, 5.0 and 10% (w/v) in DMSO
Control: negative – DMSO alone; positive - p-phenylenediamine 1% in DMSO
GLP: In compliance

The skin sensitising potential of 4-nitrophenylaminoethylurea was investigated in 5 female CBA/J mice per concentration by measuring the cell proliferation in the draining lymph nodes after topical application to the ear.

25 µl of 0 (vehicle only), 0.5, 1.5, 5 and 10% of 4-nitrophenylaminoethylurea in DMSO were applied to the surface of the ear of five female CBA/J mice per group for three consecutive days. After application, the ears were dried by means of a hair dryer for about 5 minutes. As positive control, p-phenylenediamine (PPD) at 1% in DMSO was investigated in parallel under identical test conditions.

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

At day 5, mice received an intravenous injection of 250µl phosphate buffered saline containing 23.8 µCi of [H^3] methyl thymidine. Approximately five hours later, mice were killed by CO_2-inhalation, and the draining auricular lymph nodes were removed and weighed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the 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 per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
Based on the data provided in the certificate of analysis a sufficient solubility and stability of the test item in the solvent used (DMSO) can be assumed.

The mean stimulation indices revealed no differences to the concurrent vehicle control group at any test concentration. Mean stimulation indices of 1.0, 0.9, 1.0 and 0.9 were obtained for the 4 test concentrations of 0.5, 1.5, *5* and 10%*,* respectively. Thus, no EC3 value (equal to the concentration inducing a stimulation index of 3) was calculated since all stimulation indices were below 3.
The responses noted do not indicate a skin sensitising potential of 4-nitrophenylaminoethylurea.
The positive control (PPD, 1% in DMSO) caused a stimulation index of 7.0 which demonstrated the sensitivity of the test system used.

Conclusion
4-Nitrophenylaminoethylurea induced no immune response in local lymph nodes after dermal application to the mouse ear using DMSO as vehicle. Consequently, no EC3 value was calculated and 4-nitrophenyl aminoethylurea is evaluated to not be a skin sensitiser under the described test conditions.

Ref.: 20

Comment
Three Magnusson/Kligman tests have been undertaken with 4-nitrophenylaminoethylurea. These tests were part of a former submission and were considered as negative although there were limitations with regard to the test design and/or material used. They do not contribute to the hazard identification of 4-nitrophenyl aminoethylurea.

3.3.4. Dermal / percutaneous absorption

Percutaneous absorption in vitro (Study 1: Non-oxidative formulation)

Guideline:	OECD 428 (2004)
Tissue:	Porcine back and flank skin (thickness: 960 ± 70 µm)
Method:	Diffusion Teflon-chambers
Test substance:	Nitrogelb (WR 23348) tested at a concentration of 0.5 % in a typical non-oxidative hair dye formulation
Batch:	110889 (sample R96003805; lot 131004-100)
Purity:	99.5%
Dose:	0.5 mg/cm^2 tested as part of a non-oxidative formulation
No. of chambers:	6 (five for the formulation containing the test item and one for the blank formulation)
Duration/exposure:	Single application for 60 min
GLP:	In compliance

The skin absorption of Nitrogelb at the maximum concentration intended for non-oxidative hair colorants was investigated with pig skin (Schweizer Edelschwein, female, 110 kg) prepared from the back and the flanks (960 ± 70 µm thick). 2 mg of the dye was applied to the skin in a commercial hair dye formulation (400 mg aqueous cream formulation containing 0.5 % dye applied to 4 cm^2 skin).

The integrity of the skin was monitored at the beginning of the experiment using tritiated water. A diffusion Teflon-chamber was used. The receptor solution (physiological phosphate buffer containing NaCl and antibiotics) was pumped through the receptor chamber at a rate of 5 ml/h. Sixty minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, once with 4 ml washing solution (shampoo-formulation) and again twice with water. The washing solutions were combined and the amount of dye was determined by HPLC.

Fractions of the receptor fluid were collected after 16, 24, 40, 48, 64 and 72 hours and stored at -20°C until analysis. At termination of the experiment, the skin was heat-treated and the upper skin compartment (stratum corneum and upper stratum germinativum, referred to as epidermis) was mechanically separated from the lower skin compartment (lower stratum germinativum and upper dermis, referred to as upper dermis). The two skin compartments were extracted separately and the dye content quantified by means of HPLC analysis.

Results
The limit of detection was 0.2 ng/HPLC injection and the limit of quantification was 1 ng/HPLC injection.

Based on the data for solubility (28.7 mg/ml in receptor fluid) and stability (≥ 7 days in water- based systems) provided in the report, the substance is considered stable.

The integrity of each skin preparation was demonstrated by examination of penetration characteristics with tritiated water. The figures obtained (0.65 to 1.44% of the applied dose found in the receptor fluids) were all within the limit of acceptance (≤ 1.5%).

The total recovery of 96.1 ± 1.0% of the applied dose confirmed the validity of the test. The majority of *4*- Nitrogelb remained on the skin surface representing 96.2 ± 1.1% (480.9 ± 5.2 µg/cm^2) of the applied dose.

After 72h a cumulative amount of 0.02 ± 0.02 µg/cm^2 (range 0.00 to 0.06) was determined in the receptor fluid. The figures for the upper dermis and the epidermis were 0.03 ± 0.01 µg/cm^2 (range 0.02 to 0.03) and 0.19 ± 0.06 µg/cm^2 (range 0.11 to 0.26). The absorption kinetics indicate that the small amounts of Nitrogelb remaining on the skin after the washing steps tend to migrate to deeper layers, and thus might act as a reservoir. Therefore, the dye content in the epidermis was considered to become potentially bioavailable.

Conclusion
Under the described test conditions, a mean skin penetration of 0.24 ± 0.07 µg/cm^2 was obtained for Nitrogelb by summing up the amounts for receptor fluid, dermis and epidermis. The maximum skin penetration was 0.31 µg/cm^2.

Ref: 21

Percutaneous absorption in vitro (Study 2: oxidative formulation)

Guideline:	OECD 428 (2004)
Tissue:	Porcine back and flank skin (thickness: 900 ± 100 µm)
Method:	Diffusion Teflon-chambers

Test substance:	Nitrogelb (WR 23348) tested at a concentration of 0.25 % in an oxidative hair dye formulation (3 % hydrogen peroxide)
Batch:	110889 (sample R96003805; lot 131004-100)
Purity:	99.5%
Concentration:	0.25 mg/cm^2 tested as part of an oxidative formulation
No. of chambers:	6 (five for the formulation containing the dye stuff and one for the blank formulation) number of animals not mentioned
Duration/exposure:	Single application for 60 min
GLP:	In compliance

The skin absorption of Nitrogelb at the maximum concentration intended for oxidative hair colorants, was investigated with pig skin (Schweizer Edelschwein, male, 110 kg) prepared from the back and the flanks (900 ± 100 µm thick).

1 mg of the dye was applied to the skin in a commercial hair dye formulation (400 mg formulation containing 0.25% dye and 3% hydrogen peroxide applied to 4 cm^2 skin surface).

The integrity of the skin was monitored at the beginning of the experiment using tritiated water. A diffusion Teflon-chamber was used. The receptor solution (physiological phosphate buffer containing NaCl and antibiotics) was pumped through the receptor chamber at a rate of 5 ml/h.

Sixty minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, once with 4 ml washing solution (shampoo-formulation) and again twice with water. The washing solutions were combined and the amount of dye was determined by HPLC.

Fractions of the receptor fluid were collected after 16, 24, 40, 48, 64 and 72 hours and stored at -20°C until analysis. At termination of the experiment, the skin was heat-treated and the upper skin compartment (stratum corneum and upper stratum germinativum, referred to as epidermis) was mechanically separated from the lower skin compartment (lower stratum germinativum and upper dermis, referred to as upper dermis). The two skin compartments were extracted separately and the dye content quantified by means of HPLC analysis.

Results
The limit of detection was 0.2 ng/HPLC injection and the limit of quantification was 1 ng/HPLC injection. Based on the data for solubility (28.7 mg/ml in receptor fluid) and stability the substance is considered stable for the given test procedure.

The integrity of each skin preparation was demonstrated by examination of penetration characteristics with tritiated water. Figures of 1.3 to 2.1% of the applied dose were obtained. All *5* skin samples treated with the dye formulation were within the limit of acceptance (≤ 1.5%). The single skin with a value of 2.1% was chosen as background control.

The mean recovery of the test item was 96.84%. The vast majority of Nitrogelb remained on the skin surface representing 96.79 ± 4.87% (0.242 ± 0.012 mg/cm^2) of the applied dose.
After 72 hours, the cumulative content of the test item in the receptor fluid was 0.01 ± 0.01 µg /cm^2 (range 0.00 to 0.02). In the upper dermis and in the epidermis, 0.02 ± 0.01 µg/cm^2 (range 0.01 to 0.03) and 0.07 ± 0.07 µg/cm^2 (range 0.02 to 0.19) were measured, respectively. The absorption kinetics indicate that the small amounts of Nitrogelb remaining on the skin after the washing steps tend to migrate to deeper layers and might act as a reservoir. Therefore, the dye content in both the upper dermis and the epidermis was considered as potentially bioavailable.

Conclusion
Under the described test conditions, a skin penetration of 0.1 ± 0.07 µg/cm^2 was obtained for Nitrogelb by summing up the amounts for receptor fluid, upper dermis and epidermis. The maximum skin penetration was 0.22 µg/cm^2.

Ref: 22

Comment on percutaneous absorption studies *in vitro*
At 0.5% in a non-oxidative formulation, the maximum penetration of Nitrogelb through porcine skin was 0.31 µg/cm^2. At 0.25% in an oxidative formulation it was 0.22 µg/cm^2. However, the experiment was not performed according to the SCCP Notes of Guidance (only five replicates from one donor; 100 mg formulation/cm² applied instead of 20 mg/cm²).

Percutaneous absorption in vivo

Guideline:	/
Species/strain:	Rat, strain Sprague Dawley Him:OFA, SPF
Group size:	3 per sex and treatment group
Test substance:	(Ureidogelb); ^{14}C-4-nitrophenylaminoethyl urea (ring-labelled)
Radioactive purity:	>97%
Batch:	/
Dose levels:	Application area: 9 cm^2 A) 0.25% commercial formulation *without* hydrogen peroxide: equal to 0.28 mg/cm^2 B) 0.25% commercial formulation *with* hydrogen peroxide: equal to 0.28 mg/cm^2 C) 0.83% solution in DMSO/water (1:0.69) equal to 0.29 mg/cm^2
Dosing schedule:	Single cutaneous application for 0.5 h; total study period 72 h
GLP:	In compliance

^{14}C-4-Nitrophenylaminoethyl urea was applied dermally to groups of three male and three female rats (body weight about 200 g at the day of application). The application area was 9 cm^2 and the test substance was applied at concentrations of 0.83% in DMSO (equal to 0.29 mg/cm^2). Commercial hair dye formulations containing 0.25% of the test item (equal to 0.28 mg/cm^2) were applied either in the absence or in the presence of hydrogen peroxide. The contact time was 30min. Application was performed under anaesthesia.

The test substance was scraped off (formulation only) and the skin was rinsed with a shampoo solution (about 100 ml) and warm water. After rinsing, the area was covered with gauze and an air permeable plastic cone to further prevent licking of the treated area during the 72 h observation period.

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

Results
Satisfactory recovery rates (98 to 107.9%) were obtained. The majority of the applied dose (97.7 to 107.6% of the applied amount) was recovered in the washing solutions for all groups.

For the DMSO solution, the amount of radioactivity remaining at the application site (skin) was 0.381 µg/cm^2 (equal to 0.131 ± 0.0393% of the applied dose). The respective figures for formulations without and with hydrogen peroxide were 0.89 µg/cm^2 (equal to 0.317 ± 0.150% of the applied dose) and 1.06 µg/cm^2 (equal to 0.377 ± 0.221% of the applied dose).

The mean amounts absorbed (sum of amounts detected in urine, faeces, and residual carcass) were low for the hair dye formulations: 0.048 µg/cm^2 (equal to 0.017 ± 0.007% of the applied dose) in the absence of hydrogen peroxide, and 0.037 µg/cm^2 (equal to 0.013 ± 0.006% of the applied dose) in the presence of hydrogen peroxide. The respective figure for the DMSO solution was 0.298 µg/cm^2 (equal to 0.103 ± 0.100% of the applied dose).

Elimination of the absorbed test substance occurred mainly via urine (78 to 79%). The elimination via urine was fast, as 58-91% of the absorbed amount were excreted within the first 24 hours. Excretion via faeces was generally of less importance (0.002 to 0.032*%).*

After 72 h, the remaining amount of ^{14}C-4-nitrophenylaminoethyl urea detected in the carcass was close to or below the detection limit of 0.003% of the applied dose for all three groups. The concentrations in the organs were generally low (below or close to the detection limit). Taking the mean amounts in urine, faeces, residual carcass and the total content of the dye remaining in the skin as potentially bioavailable, cutaneous absorption rates of 0.938 µg/cm^2 (equal to 0.334%) and 1.097 µg/cm^2 (equal to 0.390%) were found for commercial formulations without and with hydrogen peroxide, respectively, applied under typical use conditions. The respective figures for the DMSO solution were 0.679 µg/cm^2 (equal to 0.234%) of the applied dose. The higher amounts noted for formulations compared to DMSO may be due to less efficient washing steps following application of the formulations, leading to higher residues in the skin of the application site.

Conclusion
^{14}C-4-Nitrophenylaminoethyl urea applied dermally to rats in a semipermanent and in an oxidative hair dye formulation as well as in DMSO/water is mainly excreted via urine and to a minor extent via faeces. Excretion via urine was fast, with approximately 75% of the absorbed dose being excreted within the first 24 hours. Low tissue residue levels distributed through the general carcass were found independent of the vehicle used, suggesting that bio-accumulation is not to be expected following dermal exposure. Based on the conservative assumption that the total amount found in the skin will become completely bioavailable, mean absorption rates of 1.097 µg/cm^2 and 0.938 µg/cm^2 were obtained for ^{14}C-4- nitrophenylaminoethyl urea when applied in a commercial formulation under typical use conditions in the presence or absence of hydrogen peroxide, respectively. In DMSO, the respective value was 0.679 µg/cm^2.

Ref: 24

Comment
In the absence of valid *in vitro* studies, the above values of 1.097 µg/cm^2 and 0.938 µg/cm² respectively in the presence and absence of hydrogen peroxide may be used for the calculation of the Margin of Safety.

3.3.5. Repeated dose toxicity

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

No data submitted

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

Guideline:	OECD 408 (1998)
Species/strain:	Rat, strain Sprague Dawley CFY
Group size:	10 per sex and dose
Test substance:	4-nitrophenylaminoethylurea
Batch:	110889
Purity:	98.9% (HPLC, 254 nm)

Dose levels:	1, 5, 25 and 125 mg/kg bw
Vehicle:	Bi-distilled water (containing 1% CMC)
Route:	Oral, gavage
Exposure period:	13 weeks, 7 days/week
GLP:	In compliance

The objective of this study was to determine the toxicity of the test item following daily oral administration (by gavage) to 10 Sprague Dawley rats per sex and dose for 13 consecutive weeks. A control group consisting of 10 Sprague Dawley rats per sex was treated with the vehicle alone. The four dose groups received 1, 5, 25 and 125 mg/kg bw of 4-nitrophenylaminoethylurea.
Morbidity/mortality checks were performed at least twice daily and clinical signs were recorded daily. Additional clinical examinations were performed weekly. Individual body weights and food consumption were recorded weekly.
Behavioural and functional tests as well as laboratory investigations were performed in week 13. Ophthalmological examinations were carried out before the start of the treatment and repeated in week 13.
At the end of the treatment period, all animals were killed and selected organs were weighed. Tissue samples were fixed and preserved at necropsy for all animals. Selected tissues from all animals were examined histopathologically.

Results
Homogeneity and concentrations of the suspensions of *4-nitrophenylaminoethylurea* in the vehicle were analytically confirmed. The mean concentrations of the test samples (analysed three to four times during the treatment period) were 102.5%, 101.6%, 94.8% and 93% of the nominal values of the 4 dose groups, confirming the proper dosing.
Orally administered 4-nitrophenylaminoethylurea in the dose range of 1 to 125 mg/kg bw was well tolerated by the rat. There were no unscheduled deaths in any dose group. Treatment-related changes, i.e. discolouration of the fur at dose levels of 5 mg/kg bw and above and coloured urine at all dose levels were noted. These findings were not considered as adverse effects, but a common finding for dyes. Body weight gain and food consumption were unaffected by treatment at all dose levels.
There were no effects of treatment at any dose level on the behavioural and functional tests performed. No treatment-related ocular lesions were noted at any dose level.
Treatment-related effects on the haematological profile occurred in both sexes at 125 and 25 mg/kg bw after 13 weeks. The effect at 125 mg/kg bw was considered a mild anaemia characterised by small reductions in RBC count, haemoglobin concentration and packed cell volume (males only), with increased MCV and reticulocyte count. Similar but weaker effects were evident in both sexes at 25 mg/kg bw. Erythrocyte-related parameters were unaffected by treatment at 1 mg/kg bw and 5 mg/kg bw. Furthermore, no treatment-related effect was noted on total and different WBC counts at any dose level.
Apart from colouration, no treatment-related effects were noted with regard to urinalysis or serum clinical chemistry.
No treatment-related gross abnormalities were found at necropsy at any dose level, apart from staining of the fur. There was a treatment-related increase in the spleen weights of both sexes at 125 mg/kg bw, but not at the lower dose levels. The mean kidney weight of females at 125 mg/kg bw was increased, mainly due to two animals with pronounced mineralisation or pyelonephritis. No other treatment-related effects on organ weights were observed at any dose level.
Treatment-related histopathological alterations occurred in the spleen and the bone marrow in association with the above described reductions in red blood cell parameters. In the spleen, a slightly increased incidence and severity of extramedullary haemopoiesis was evident in both sexes at 25 or 125 mg/kg bw, associated with increased haemosiderosis in both sexes at 125 mg/kg bw and in females at 25 mg/kg bw. The increased extramedullary haemopoiesis and haemosiderosis were considered to represent a response to a haemolytic anaemia. A slight decrease in the amount of fatty tissue seen in the femoral bone marrow of

a few animals at 125 mg/kg bw also suggested a regenerative response of the bone marrow.

Conclusion
In conclusion, the haemopoietic system was identified as a target organ for 4-nitrophenylaminoethylurea based on the occurrence of anaemia and adaptive responses in the spleen and in the bone marrow at dose levels of 25 or 125 mg/kg bw. The "No observed adverse effect level" (NOAEL) was 5 mg/kg bw.

Ref.: 25

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

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:	LGH 110583/2 (4-nitrophenylaminoethylurea)
Batch:	LEH 5/2
Purity:	96.5 area% (HPLC, 254 nm)
Concentrations:	Exp. 1: 1, 10, 100, 300, 1000, 2000, 3000, 4000 and 5000 µg/plate Exp. 2: 100, 300, 1000, 3000 and 5000 µg/plate Exp. 3: 300, 1000, 2000, 3000, 4000 and 5000 µg/plate (TA100 and TA1538 only)
Solvent:	DMSO
GLP:	In compliance

4-nitrophenylaminoethylurea was tested for mutagenicity in the *Salmonella*/microsome assay both with and without Aroclor 1254 induced rat liver S9-mix. Negative (solvent) and positive controls were included in all experiments in accordance with OECD guidelines.

Results
From 3000 µg/plate and above precipitation as fine crystals in the aqueous agar-medium was observed. Toxicity measured as reduced background growth was observed at all concentrations in the presence and absence of S9-mix in strains TA1535, TA1537 and TA98. For strains TA1538 and TA100 reduced background growth was noted only at low test concentrations (1 to 1000 µg/plate) with and without metabolic activation system. A concentration related and reproducible increase in revertant colony numbers was observed in TA100 and TA1538 in three independent experiments. No mutagenic effects was observed in the strains TA1535, TA1537 and TA98 up to a concentration of 5000 µg/plate, either with or without S9-mix. All positive controls used gave a distinct increase of induced revertant colonies.

Conclusion
Under the experimental conditions used in this study, 4-nitrophenylaminoethylurea was genotoxic (mutagenic) in the bacterial reverse mutation test.

Ref.: 26

Remarks
A further bacterial gene mutation assay was part of a former submission. As this test reveals several limitations with regard to technical performance and as unspecified test

material was used, the study is not presented here in detail. Result: No mutagenic activity was seen either with or without metabolic activation.

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

Guideline:	OECD 476 (1997)
Species/strain:	Mouse lymphoma cell line L5178Y (*tk* locus)
Replicates:	Duplicate cultures in two independent experiments
Metabolic activation:	phenobarbital/ß-naphthoflavone rat liver S9-mix
Test substance:	4-nitrophenylaminoethylurea
Batch:	110889
Purity:	98.9 area% (HPLC, 254 nm)
Concentrations:	Experiment I: 43.8, 87.5, 175.0 and 350.0 µg/ml with and without metabolic activation Experiment II: 21.9, 43.8, 87.5, 175.0 and 350.0 µg/ml with and without metabolic activation
Treatment:	Incubation time was 4 hours in the presence and 24 hours in the absence of S9-mix. The expression period was 72 hours after 4 h treatment and 48 h after 24 h treatment
Solvent:	DMSO
GLP:	In compliance

4-nitrophenylaminoethylurea was evaluated for its mutagenic/genotoxic activity at the *tk* locus in the mouse lymphoma cell line L5178Y. In a range-finding toxicity test the highest applied concentration (700 µg/ml) was based on the maximum solubility in the culture medium.
The main test was performed as two independent experiments using duplicate cultures. Incubation time was 4 hours in the presence and 24 hours in the absence of S9-mix.
DMSO was used as solvent control, while methylmethanesulphonate (MMS, 13 µg/ml) and cyclophosphamide (CPA, 3 µg/ml) were used as positive controls without and with metabolic activation system, respectively.
Mutant frequency, colony size and cell survival (measured as relative suspension growth (RSG) and relative total growth (RGT) were determined

Results
In the initial range-finding study, concentrations up to 700 µg/ml with and without S9-mix were evaluated for toxicity. No relevant toxic effects were observed up to the maximum concentration at both treatment intervals. Due to heavy precipitation at 700 µg/ml at the end of the incubation period, 350µg/ml was considered the maximum analysable concentration in the main experiments.
In the main experiment, precipitation occurred at 175.0 µg/ml and above in the absence and presence of metabolic activation. No marked cytotoxicity was observed in the main study in the presence of S9-mix, but higher concentrations could not be tested due to precipitation of the test item. In experiment II (24 hours) without metabolic activation a concentration dependant toxic effect was observed. RTG was 20.8 and 29.5 at the highest concentration tested in the two cultures respectively.
In the first experiment, the mutation frequency exceeded the laboratory negative control range at 350 µg/ml. However, this effect was only observed in one culture, and the induction factor was only 1.3 and therefore not considered biological relevant. In the second experiment without S9-mix and 24 hours treatment, there was a more than twofold increase in mutation frequency at 87.5 and 350 µg/ml. However, the mutation frequency remained within the historical control range, it was not concentration related and was only observed in one culture and therefore considered biological irrelevant. It was concluded that no biologically relevant or dose-dependent increase in the number of mutant colonies was noted in the presence or absence of S9-mix up to the maximum test concentration

compared to the concurrent controls and the historical control range. The concurrent positive controls induced a distinct increase in mutation frequency.

Conclusion
Under the test conditions used in this study 4-nitrophenylaminoethylurea was not considered to be genotoxic (mutagenic and/or clastogenic) in mammalian cells, either in the absence or in the presence of metabolic activation.

Ref: 26

Remarks
In a former submission, a further **mouse lymphoma assay** (1983) was included. This test is of limited validity as e.g. non-standard test conditions were applied and unspecified test material was used and is therefore not considered relevant for the hazard assessment of 4-nitrophenylaminoethylurea.
Result: No mutagenic effects were observed.

In a former submission, 4-nitrophenylaminoethylurea of specified quality (batch L/4113) was investigated in a **chromosomal aberration test** in CHO cells at concentrations of 25, 50 and 250 µg/ml in the presence of metabolic activation and at 5, 25, and 50 µg/ml in the absence of metabolic activation. The test substance did not produce a statistically significant increase in chromosome aberrations either in the absence or presence of metabolic activation. Although this test is not in compliance with the current guideline (no repeat experiment was performed), it indicates the absence of clastogenic potential in mammalian cells *in vitro*.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474 (1997)
Species/strain:	Mouse, strain NMRI
Group size:	6 males per test group and sacrifice time (cells of 5 animals per test group were analysed)
Test substance:	4-nitrophenylaminoethylurea
Batch:	110889
Purity:	98.9 % (HPLC, 254 nm)
Dose level:	250, 500 and 1000 mg/kg bw administered as single doses
Route:	Intraperitoneal
Vehicle:	30 % DMSO/ 70 % corn oil
Sacrifice times:	24 after dosing and 48 hours (high dose and control only)
GLP:	In compliance

The clastogenic/aneugenic potential of 4-nitrophenylaminoethylurea was investigated in bone marrow cells of mice. Single doses were administered intraperitoneally in a total volume of 10 ml/kg bw. For the high dose, two groups were treated to allow sampling after 24 and 48 hours.
Dose selection was based on findings in the pre-experiment for toxicity, in which a dose range of 100 to 1500 mg/kg bw was administered.
Bone marrow cells were sampled from mice after sacrifice (24 h after dosing) for all dose groups and additionally after 48 h for the high dose group. At least 2000 PCEs per animal were analysed. Toxicity on the bone marrow was measured as the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE ratio) for each animal. Five animals per test group were evaluated. Negative control groups received 30 % DMSO / 70 % corn oil (10 ml/kg bw) and concurrent positive control groups received 40 mg/kg bw cyclophosphamide (CPA) dissolved in deionised water

Results
In the pre-experiment (2 animals per sex and dose), intraperitoneal administration of 1250 mg/kg bw and of 1500 mg/kg bw caused death within 24 hours in one male and two males, respectively. No deaths were noted in females at those doses. Signs of toxic reactions were abdominal position, eyelid closure, ruffled fur, apathy, convulsion and reduced spontaneous activity. Furthermore, the urine of all animals was yellow (the colour could be clearly distinguished from the normal urine colour) up to 48 hours after administration. At the 4 remaining doses, ranging from 100 to 1000 mg/kg bw, no deaths occurred after intraperitoneal administration. Apart from yellow discoloured urine, no further clinical signs were noted up to 48 hours.
Based on these findings, doses of 250, 500 and 1000 mg/kg bw were chosen for the main study. In the pre-test, there was no substantial difference in sensitivity/toxicity to the test item between sexes. Therefore, only males were used in the main test.

In the main study, toxic signs like those described in the pre-experiment were noted for the high dose group up to 24 hours after administration, but no cases of death occurred. Yellow discoloured urine was noted up to 24 and 48 hours (highest dose level only) after intraperitoneal administration.
4-nitrophenylaminoethylurea showed no clear cytotoxic effect in the bone marrow as the PCE/NCE ratio did not substantially change after treatment with the test item up to systemically toxic doses. However, the discoloured urine and the observed systemic toxicity and the available kinetic data indicate that 4-nitrophenylaminoethylurea becomes bio-available and systemically distributed after intraperitoneal administration.
There was no statistically significant or biologically relevant increase in the number of micronuclei per 2000 PCEs in the mice of any of the 4-nitrophenylaminoethylurea treated groups versus the respective vehicle control groups.
The positive control group (CPA) produced a statistically significant increase in micronucleated PCEs and the vehicle control was well within the range of historical control data of the performing laboratory.

Conclusion
4-nitrophenylaminoethylurea was not genotoxic (clastogenic and/or aneugenic) in the *in vivo* micronucleus test using NMRI mice after a single intraperitoneal administration up to the maximum tolerated dose of 1000 mg/kg bw.

Ref.: 28

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	OECD 414 (2001)
Species/strain:	Rat, strain Wistar Crl:Wi/Br (SPF)
Group size:	25 pregnant females per dose group
Test substance:	4-nitrophenylaminoethylurea
Batch:	110889
Purity:	98.9% (HPLC, 254 nm)
Dose levels:	50, 250 and 600 mg/kg bw
Route:	Oral, gavage

Vehicle: 1% carboxymethylcellulose in bi-distilled water
Dosing schedule: Days 6 to 19 of gestation
GLP: In compliance

Three groups of 25 mated females received 4-nitrophenylaminoethylurea at dose levels of 50, 250 and 600 mg/kg bw by gavage from day 6 to day 19 of gestation. An equally sized group receiving the vehicle (1% carboxymethylcellulose in bi-distilled water) alone served as control.
Clinical condition and reaction to treatment were recorded at least once daily. Body weights and food consumption were reported/calculated for days 0, 6, 9, 12, 15, 18 and 20 of gestation. All surviving females were sacrified on day 20 of gestation for examination of their uterine contents, including examination of the placenta. At necropsy, the dams were examined macroscopically and live foetuses were weighed, sexed and examined for external, visceral and skeletal abnormalities.
Approximately one half of the foetuses was examined for skeletal malformations. The remaining foetuses did undergo a visceral examination.

Results
One female at 600 mg/kg bw was found dead on day 12 of gestation. Three females in this dose group and one receiving 250 mg/kg bw were killed in a moribund condition between days 14 and 17 of gestation. All the decedents had severe weight loss. There was no mortality in the control and the low dose groups.
All rats in the treated groups had abnormally yellow-discoloured urine throughout the treatment period. Most of the females in all treated groups had a yellow-stained fur after several days of treatment. There were no other clinical signs amongst the surviving females of any dose group.
There was a marked decrease in body weight gain and reduced food consumption throughout the treatment period for females dosed with 600 mg/kg bw. A slight decrease was also evident in the 250 mg/kg bw group. The 50 mg/kg bw group was not affected.
There were no treatment-related macroscopic findings at the terminal necropsy examination of the adult females.
The percentage post-implantation loss and mean live litter size was comparable in the treated and control groups. The foetal sex ratios did not show any treatment-related trends.
Compared to the control group, foetal weight was significantly reduced at dose levels of 250 (-10 %) and 600 mg/kg bw (-20 %). Consistent with the reduced foetal weight, an increased incidence of foetuses with reduced ossification was observed. Since clear signs of systemic toxicity were noted in the dams, effects in foetuses are regarded as a secondary reaction to maternal toxicity.
Nine foetuses from a single high dose litter had anasarca and cleft palate and one foetus from a litter given 250 mg/kg bw had an anophthalmia. These malformations are common spontaneous findings and were within the historical background for the rat strain at the testing facility. Due to the isolated nature of these malformations, they were not considered to be test item related.
No adverse effects were observed in the low dose group (50 mg/kg bw).

Conclusion
Treatment of females with 4-nitrophenylaminoethylurea at 600 mg/kg bw resulted in severe maternal toxicity including deaths and in corresponding embryo-foetal toxicity, i.e. reduction in the foetal weight and reduction of foetal ossification, but with no influence on embryo-foetal survival. At 250 mg/kg bw, less severe maternal toxicity and only slight reductions in foetal weight (-10 %) and in ossification were noted.
The low dose level of 50 mg/kg bw of 4-nitrophenylaminoethylurea was the NOAEL for both maternal and embryo-foetal toxicity.

Ref: 29

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

Guideline:	/
Cells:	Human intestinal epithelial cell line TC-7
Test substance:	4-nitrophenylaminoethylurea
Batch:	110889
Purity:	98.9 % (HPLC, 254 nm)
Test concentration:	50 µM in HBSS buffer containing 1 % DMSO
Incubation time:	60 min
GLP:	Not in compliance, but QAU checked

The bioavailability of 4-nitrophenylaminoethylurea across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro*. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37°C in 96-well transwell plates with shaking for a 60 min contact time. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of HLPC-MS/MS and the apparent permeability coefficient (P_{app}) was calculated for two independent experiments. ^{14}C-mannitol (about 4 µM) was used to demonstrate the integrity of the cell monolayer. Only monolayers revealing a permeability of $< 2.5 \times 10^{-6}$ cm/sec were used. Propranolol and ranitidine were analysed concurrently to demonstrate the validity of the test system.
According to the laboratory's classification system, a low permeability is considered for test items revealing a $P_{app} < 2 \times 10^{-6}$ cm/sec. A P_{app} of 2 - 20 $\times 10^{-6}$ cm/sec and a $P_{app} \geq 20 \times 10^{-6}$ cm/sec classify a substance to have a moderate and a high permeability, respectively. Ranitidine, which has a 50 % absorption in humans was used as low permeability reference compound, as recommended by FDA.

Results
The figures for the reference substances propranolol ($P_{app} = 25.9 \times 10^{-6}$ cm/sec), a high permeability reference compound with 90 % absorption in humans, and ranitidine ($P_{app} = 0.2 \times 10^{-6}$ cm/sec) revealing an absorption rate of about 50 % in humans were well within the acceptance range of 20 – 45 $\times 10^{-6}$ cm/sec and 0.2 – 2 $\times 10^{-6}$ cm/sec, respectively, and demonstrated the validity of the assay.
4-nitrophenylaminoethylurea revealed a P_{app} of 17.0×10^{-6} cm/sec and thus was classified to be of medium permeability, indicating a substantial absorption from the gastro-intestinal tract.

Ref.: 30

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Not applicable

3.3.14. Discussion

Physico-chemical specifications
4-Nitrophenyl aminoethylurea is used as direct dye in semi-permanent hair formulations at a maximum on-head concentration of 0.5%, and as a hair colouring agent (direct dye) in oxidative hair dye formulations at a maximum on-head concentration of 0.25%.
4-Nitrophenyl aminoethylurea is a secondary amine, and thus, it is prone to nitrosation. The nitrosamine content in 4-nitrophenyl aminoethylurea is not reported. 4-Nitrophenyl aminoethylurea should not be used in combination with nitrosating agents. Stability of 4-nitrophenyl aminoethylurea in the presence of hydrogen peroxide and in marketed products is not reported. Several unidentified small peaks were seen in HPLC. N-(2-aminoethyl)-4 nitroanilinine was present at more than 1% in batch LEH/52. This substance is a secondary amine. No further information was given.
Supportive analytical data for only some batches was provided

General toxicity
In the rat Sub-chronic (90 days) oral study the haemopoietic system was identified as a target organ for 4-nitrophenyl aminoethylurea based on the occurrence of anaemia and adaptive responses in the spleen and in the bone marrow at dose levels of 25 or 125 mg/kg bw. The "No observed adverse effect level" (NOAEL) was 5 mg/kg bw.
In the rat teratogenic study, treatment of females with 4-nitrophenyl aminoethylurea at 600 mg/kg bw resulted in severe maternal toxicity including deaths and in corresponding embryo-foetal toxicity. At 250 mg/kg bw, less severe maternal toxicity and only slight reductions in foetal weight (-10 %) and in ossification were noted. A dose level of 50 mg/kg bw of 4-nitrophenylaminoethylurea was the NOAEL for both maternal and embryo-foetal toxicity.

Irritation / sensitisation
Nitrogelb applied 'as is' was not irritant to rabbit skin but was irritant to rabbit eyes.
4-Nitrophenyl aminoethylurea induced no immune response in local lymph nodes after dermal application to the mouse ear using DMSO as vehicle. Consequently, no EC3 value was calculated and 4-nitrophenyl aminoethylurea was evaluated as not being a skin sensitiser.

Dermal penetration
In the absence of valid *in vitro* studies, the values of 1.097 µg/cm^2 and 0.938 µg/cm² derived from the *in vivo* studies in rats, respectively in the presence and absence of hydrogen peroxide, may be used for the calculation of the Margin of Safety.

Mutagenicity / Genotoxicity
4-nitrophenylaminoethylurea did reveal a potential to cause gene mutations in bacteria. In mammalian cells (mouse lymphoma assay), no potential to induce gene mutations or chromosomal aberration was found. The absence of a clastogenic potential of 4-nitrophenylaminoethylurea in mammalian cells *in vitro* was further supported by the results of a chromosome aberration test in CHO cells in the presence and absence of a metabolic

activation system, although the test was not performed according to the current guideline (and not submitted for this evaluation). In addition, no clastogenic/aneugenic effects were noted *in vivo*: In a micronucleus test in mice, performed with intraperitoneal administration of 4-nitrophenylaminoethylurea at dose levels up to the MTD (1000 mg/kg bw), no indication for a mutagenic effect was observed. In this study, the occurrence of discoloured urine and the observed signs of systemic toxicity indicate the systemic availability of 4-nitrophenylaminoethylurea.
Since 4-nitrophenylaminoethylurea induced gene mutations in bacteria, a proper genotoxicity test covering gene mutations *in vivo* is essential to definitively conclude on the genotoxicity of 4-nitrophenylaminoethylurea.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCP is of the opinion that the information submitted is insufficient to allow a final risk assessment to be carried out. An additional mutagenicity / genotoxicity test should be performed following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance in order to exclude its gene mutation potential.

4-Nitrophenyl aminoethylurea is a secondary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.
Its stability in the presence of hydrogen peroxide should be demonstrated.

5. MINORITY OPINION

Not applicable

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