



Scientific Committee on Consumer Safety

SCCS

OPINION ON

Acid Black 1

COLIPA n° B15

The SCCS adopted this opinion at its 18th plenary meeting
of 26 February 2013

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 Safety (SCCS), 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.

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SCCS

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

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ISSN 1831-4767

Doi:10.2772/56529

ISBN 978-92-79-31187-1

ND-AQ -13-022-EN-N

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged. Revised opinions carry the date of revision.

Keywords: SCCS, scientific opinion, hair dye, B15, Acid Black 1, directive 76/768/ECC, CAS 1064-48-8 (disodium salt), EC 231-903-1 (disodium salt)

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Acid Black 1, 26 February 2013, revision of 12 December 2013

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1. BACKGROUND

Submission I of hair dye Acid Black 1 was delivered to the Scientific Committee on Cosmetology in March 1984 by COLIPA¹. No opinion could be given at that time due to lack of data.

Acid Black 1 is identical with CI 20470 also used as a colouring agent allowed for use in cosmetic products intended to come into contact only briefly with the skin.

Submission II of Acid Black 1 (B015) was submitted by COLIPA in July 2005.

The Scientific Committee on Consumer Safety (SCCS) adopted at its 6th plenary meeting the 23 of March 2010 the opinion (SCCS/1226/09) with the following conclusion:

Based on the low margin of safety for the use as a direct hair colouring agent in non-oxidative hair dye formulations, the SCCS is of the opinion that Acid Black 1 at a maximum on-head concentration of 0.5% poses a risk to the health of the consumer.

Acid Black 1 (CI 20470) is listed in Annex IV, part 1 – List of colouring agents allowed for use in cosmetic products, field of application: 4 – colouring agents allowed exclusively in cosmetic products intended to come into contact only briefly with the skin.

The use of Acid Black 1 (CI 20470) as a cosmetic colorant should be evaluated.

This Submission III contains the results and conclusions obtained in a new dermal penetration study and the requested clarification related to the analytical work that was part of the previous Submission II.

2. TERMS OF REFERENCE

1. *Does the SCCS consider Acid Black 1 safe for use in non-oxidative hair dye formulations with a concentration of maximum 0.5% in the finish product taken into account the scientific data provided?*
2. *And/or does the SCCS recommend any restrictions with regard to the use of Acid Black 1 in non-oxidative hair dye formulations (e.g. maximum concentration in the finish cosmetic product, warning)?*

¹ COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Acid Black 1 (INCI name)

3.1.1.2. Chemical names

1-Naphthol-3,6-disulfonic acid, 8-amino-7-(p-nitrophenylazo)-2-phenylazo- disodium salt
2,7-Naphthalenedisulfonic acid, 4-amino-5-hydroxy-3-[(4-nitrophenyl)azo]-6-(phenylazo)-, disodium salt

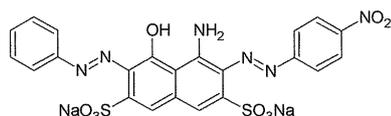
3.1.1.3 Trade names and abbreviations

Black n° 401
Naphthalene Black 10B
Amido Black 10B
CI 20 470
COLIPA n° B15

3.1.1.4. CAS / EC number

CAS: 1064-48-8 (disodium salt)
EC: 213-903-1 (disodium salt)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{22}H_{14}N_6Na_2O_9S_2$

3.1.2. Physical form

Dark red to black powder

3.1.3. Molecular weight

Molecular weight: 616.49 g/mol

3.1.4. Purity, composition and substance codes

Chemical characterisation by NMR, IR and LC-MS. UV spectrum 200-800 nm and a LC chromatogram was provided.

Ref.: 12

Comparison of batches and specification of commercial raw material

Batch	9405	M90114	J00927	Commercial quality
Purity	94.8%	97.1%	94.2%	99,5% by HPLC
Benzene *	360 ppm	400 ppm	60 ppm	< 400 ppm
p-nitroaniline **	14.74 ppm	45.89 ppm	7.98 ppm	< 8 ppm
4-Aminoazobenzene **	8.18 ppm	0.88 ppm	0.13 ppm	< 0.13 ppm
4-Aminobiphenyl **	0.31 ppm	0.068 ppm	0.062 ppm	< 100 ppb
Aniline **	9.39 ppm	10.6 ppm	3.0 ppm	< 5 ppm
Heavy metals	-	-	-	Arsenic * < 5 ppm Antimony < 5 ppm Lead * < 20 ppm Cadmium < 10 ppm Mercury < 5 ppm

* Method given in Japanese Standard of Cosmetic Ingredients (JSCI) and authorised by the Ministry of Health, Labor and Welfare

** FDA Method

Comments

1. CMR classification of the impurities in EU:

Benzene: carcinogenic, category 1 (CLP: Carc. 1A); mutagenic, category 2 (CLP: Muta. 1B)

4-Aminoazobenzene: carcinogenic, category 2 (CLP: Carc. 1B)

4-Aminobiphenyl: carcinogenic, category 1 (CLP: Carc. 1A)

Aniline: carcinogenic, category 3 (CLP: Carc. 2); mutagenic, category 3 (CLP: Muta. 2)

(CLP: Regulation (EC) 1272/2008 on classification, labelling and packaging of substances and mixtures)

p-nitroaniline: MAK commission; Carcinogenic, category 3A (Germany)

These CMR substances will not pose any relevant cancer risk at the levels given in the table above.

- Approximately 3-6% of the impurity(ies) in the various batches of Acid Black 1 have not been characterised. In addition no documentation is provided for the identification and quantification of the reported impurities.
- In one batch, the amount of 4-aminobiphenyl is relatively high (0.31 ppm), higher than that reported for the specification (<100 ppb) of commercial raw material.
- The amount of 4-Aminoazobenzene in two of the batches of Acid Black 1 is higher than that reported for the specification of commercial raw material
- The amount of aniline in two batches of Acid Black 1 is higher than that reported for the specification of commercial raw material.
- The amount of p-nitroaniline in two batches of Acid Black 1 is higher than that reported for the specification of commercial raw material..
- No documentation is provided for higher purity, and thus fewer impurities, of commercial Acid Black 1 than the three batches of Acid Black 1 used for toxicity testing.

3.1.5. Impurities / accompanying contaminants

See point 3.1.4. Purity, composition and substance codes

3.1.6. Solubility

Water: > 3%
DMSO: > 10%
Ethanol: < 0.2%

Comment

The water solubility was not determined by the EC method.

3.1.7. Partition coefficient (Log Pow)

Log P_{ow} : - 4.53 (*dianionic form*) (*calculated*)
Log P_{ow} : 1.2 (determined by EEC method)

Ref. 10

Comment

The calculated Log P_{ow} value is significantly different from the value experimentally determined.

3.1.8. Additional physical and chemical specifications

Melting point:	> 350 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	Absorption at 323 nm and 620 nm (λ_{max}) – similar in acidic, basic and neutral solution

3.1.9. Homogeneity and Stability

Acid Black 1 is stable under normal laboratory conditions.

0.5 mg/ml, 3.0 mg/ml and 18.0 mg/ml solutions of Acid Black 1 in distilled water containing 1% carboxymethylcellulose (used in 13 week oral toxicity study), stored at 2 – 8 °C for 7 days were stable (variation up to \pm 12% of the nominal concentration). These solutions were also homogeneous.

The UV/vis spectrum of a solution of Acid Black I in DMSO (approximately 0.33 mg/ml) does not change significantly over the storage period of 7 hours (storage conditions not given), conforming that Acid Black I is stable in DMSO for at least 7 hours.

General Comments on physico-chemical characterisation

- All batches Acid Black 1 contain 3-6% unidentified impurities.
- Acid Black 1 contains several carcinogenic and/or mutagenic impurities. The concentration of these impurities should be kept at a minimum.
- No documentation is provided for higher purity of commercial Acid Black 1 than the three batches of Acid Black 1 used for toxicity testing.
- The stability of Acid Black 1 in typical hair dye formulations is not reported.

3.2 Function and uses

Acid Black 1 is used as a direct hair colouring agent up to an on-head concentration of 0.5% in non-oxidative hair dye formulations.

Acid Black 1 is listed in Annex IV – List of colouring agents allowed for use in cosmetic products – to Directive 76/768/EEC on cosmetic products, column 4: colouring agents allowed exclusively in cosmetic products intended to come into contact only briefly with the skin.

3.3 Toxicological Evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Taken from SCCS/1226/09

Guideline: /
 Species/strain: rat, Wistar II
 Group size: 50 females, 10 per group
 Test substance: Acid Black 10B
 Batch: /
 Purity: /
 Vehicle: water
 Dose levels: 3.1, 10, 15, 16 and 18 g/kg bw, 30% concentration
 Route: stomach tube
 GLP statement: /
 Study period: September 1974

This study was provided as a 2004 certified translation with the comment that '*the raw data documentation did not correspond to current standards*'.

The doses started at higher concentration than the current guideline. Clinical signs noted 1 h after dosing, were piloerection, diarrhoea, prone/lateral position.

Ref.: 1

Comment

The results as presented are valueless as the table provided has transcriptional errors in it.

Guideline: /
 Species/strain: mouse, CF1 (Winkelmann)
 Group size: 10 males
 Test substance: Acid Black n° 1
 Batch: /
 Purity: /
 Vehicle: bi-distilled water
 Dose levels: 5000 mg/kg bw
 Dose volume: 20 ml/kg bw
 Route: stomach tube
 Administration: single application
 GLP statement: /
 Study period: February 1983

This study was provided as a 2005 certified translation. The lethal dose (LD₅₀) was > 5,000 mg/kg body weight. There were no symptoms of intoxication.

Ref.: 2

3.3.1.2 Acute dermal toxicity

No data submitted

3.3.1.3 Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

Taken from SCCS/1226/09

Guideline: /
 Species/strain: albino rabbit, New Zealand
 Group size: 6 males
 Test substance: Acid Black 1
 Purity: /
 Batch: /
 Vehicle: tap water
 Dose level: 10% (w/v) Acid Black 1 in tap water
 Dose volume: 0.5 ml
 GLP: /
 Study period: July 1982

The rabbits were clipped on the back 24 hours prior to the test. 10% aqueous solution/suspension (pH adjusted with ammonia to pH 8-10) of the dye was applied on the skin and covered with occlusive patches, which were secured to the skin. At the end of a 2 hours exposure, the patches were removed and skin reactions were examined immediately and after 24, 48 and 72 hours of patch removal.

Results

None of the 6 animals showed signs of irritation, neither after immediate reading nor after 72 hours.

Conclusion

Under the conditions in this experiment, the test substance (10% Acid Black 1) is not irritant to rabbit skin.

Ref.: 4

Comments

It is not reported if any staining of the skin affected the evaluation. No explanation was given for the pH adjustment. The batch and the purity of the test material were not identified, but it was mentioned that the sponsor of the study supplied the test material as 100% pure. The study was not performed according to GLP and OECD guideline.

3.3.2.2 Mucous membrane irritation

Taken from SCCS/1226/09

Guideline: /

Species/strain: albino rabbit, New Zealand
 Group size: 6 males
 Test substance: Acid Black 1
 Purity: /
 Batch: /
 Vehicle: tap water
 Dose level: 5% (w/v) Acid Black 1 in tap water
 Dosing volume: 0.1 ml
 GLP: /
 Study period: July 1982

On test day 1, an aliquot of 0.1ml of 5% aqueous solution/suspension (pH adjusted with ammonia to pH 8 - 10) was applied in the conjunctival sac of the right eye of each animal. The left eye remained untreated and served as reference control. Rinsing of the eyes was not performed.

Scoring of irritation based on the Draize method was performed 1, 24, 48 and 72 hours after single application.

Results

No irritation reactions of cornea, iris and conjunctiva were observed.

Conclusion

Acid Black 1 at 5% (w/v) concentration is not irritating to eye.

Ref.: 3

Comment

No explanation was given for the pH adjustment. The batch and the purity of the test material were not identified, but it was mentioned that the sponsor of the study supplied the test material as 100% pure. The study was not performed according to GLP and OECD guideline.

3.3.3 Skin sensitisation

Taken from SCCS/1226/09

Local Lymph Node Assay (LLNA)

Guideline: OECD 429 (2002)
 Species/strain: mouse, CBA/CaOlaHsd (nulliparous and non-pregnant)
 Group size: 20 females (4 animals per group)
 Test substance: Acid Black 1
 Batch: J00927
 Purity: 94.2%
 Vehicle: DMSO
 Concentration: 1, 5, 10 and 20% (w/v) in DMSO
 Positive control: α -hexylcinnamaldehyde in acetone:olive oil, 4:1 (v/v) (October 2004)
 GLP: in compliance
 Study period: 19 – 25 January 2005

In a non-GLP pre-test in two mice, 2.5, 5.0, 10.0 and 20.0% (w/v) suspension of Acid Black 1 in DMSO were tested on one ear each. Due to the intense black colour of the test material, local irritation reactions could not be detected. No swelling of the ears was observed. The results of the pre-test indicated that the final test can be performed at the highest test concentration (20%).

In the final test, four groups each of four female mice were treated by 25 µl topical application of 1% (w/v), 5% (w/v), 10% (w/v) and 20% (w/v) Acid Black 1 in DMSO at the dorsum of each ear lobe (both left and right) on three consecutive days. In addition a control group of four female mice was similarly treated with 25 µl vehicle. Five days after the first application, ³H-methyl thymidine was intravenously injected into a tail vein. 5 hours later mice were sacrificed by intraperitoneal injection of Na-thiopental and the draining auricular lymph nodes taken and pooled for each experimental group. Single cell suspensions of pooled lymph nodes were prepared. Cells were washed with PBS and precipitated with 5% trichloro-acetic acid (TCA). 18 hours later the pellets were resuspended in TCA and transferred into the scintillation cocktail. The proliferation capacity of lymph node cells was determined by the incorporation of ³H-methyl thymidine.

Data were provided from a positive control study, performed in October 2004, with α-hexylcinnamaldehyde in acetone:olive oil 4:1 (v/v) using CBA/CaOlaHsd mice.

Results

No signs of local toxicity at the ears of animals and no systemic toxicity findings were observed during the study period. Due to the intense black colour of the test material, local irritation reactions such as ear redness could not be detected. However, no swelling of the ears was observed.

The stimulation indices of 2.4, 4.6, 5.9 and 7.4 were determined with the Acid Black 1 concentrations of 1, 5, 10 and 20% (w/v) in DMSO, respectively.

Concentration	Stimulation Index
Test item	
1%	2.4
5%	4.6
10%	5.9
20%	7.4
α-hexylcinnamaldehyde	
5%	2.0
10%	3.0
25%	4.9

Conclusion

Acid Black 1 was found to be a moderate skin sensitiser. An EC3 value of 2.1% (w/v) was derived.

Ref.: 19

Comment

According to the grading scheme used by the SCCS (SCCP/0919/05), Acid Black 1 should be considered as a moderate sensitiser.

3.3.4 Dermal / percutaneous absorption

New study, submission III, 2012

Guideline:	OECD 428 (2004)
Tissue:	split-thickness human skin (1 abdomen, 1 "abdomen/breast", 1 "abdomen, arms and back" and 2 breast) from donors aged 23 to 42 years old; 200-400 µm
Group size:	12 samples from 5 different donors per experiment
Diffusion cells:	flow-through diffusion cell, 0.64 cm ²
Skin integrity:	tritiated water barrier integrity test
Test substance:	Acid Black 1 [nitroaniline-ring- ¹⁴ C] Acid Black 1

Batch:	J81204F1, lot n° 100400111 135153/A (radiolabelled), 71.5 µCi/mg
Purity:	99.0% 99.31% (radiochemical purity)
Test item:	[¹⁴ C]-Acid Black 1 in Test Preparation 1 (ca 0.5%, w/w) [¹⁴ C]-Acid Black 1 in Test Preparation 2 (ca 0.2%, w/w)
Doses:	20 mg/cm ²
Receptor fluid:	Physiological saline containing streptomycin (0.1 mg/mL) and penicillin G (100 units/mL)
Solubility receptor fluid:	85.3 mg/L
Stability:	/
Method of Analysis:	liquid scintillation counting
GLP:	in compliance
Study period:	9 May – 30 May 2011

The dermal absorption of 1-Naphthol-3,6-disulfonic acid, 8-amino-7-(p-nitrophenylazo)-2-phenylazodisodium salt (Acid Black 1, Colipa No. B015) was studied following topical application of the hair dye formulation to excised human skin under in-use conditions. Radiolabelled Acid Black 1 was incorporated into a typical hair dye formulation at two concentrations, 0.5% (w/w) and 0.2% (w/w), for testing under non-oxidative conditions.

Split-thickness human skin membranes were mounted into flow-through diffusion cells. Receptor fluid, physiological saline containing streptomycin (0.1 mg/mL) and penicillin G (100 units/mL), was pumped underneath the skin at a flow rate of 1.5 mL/h ± 0.15 mL/h. The skin surface temperature was maintained at 32°C ± 1°C throughout the experiment. A tritiated water barrier integrity test was performed and any human skin sample exhibiting absorption greater than 0.6% of the applied dose was excluded from subsequent absorption measurements.

Percutaneous absorption was assessed by collecting receptor fluid in 30 min fractions from 0 to 1 h post application, hourly fractions from 1 to 6 h post application and then in 2-hourly fractions from 6 to 72 h post application. At 30 min post application, exposure was terminated by washing the skin surface with water followed by a dilute shampoo solution and rinsing the skin again with water.

Results

Only results of the 0.5% test concentration are reported in the following.

Distribution of [¹⁴C]-Acid Black 1 (µg equiv./cm²) at 72 h Post Dose Following Topical Application of [¹⁴C]-Acid Black 1 in Test Preparation 1 (0.5%, w/w) to Human Split-Thickness Skin

Revision of the opinion on Acid Black 1

	Cell Number and Donor Number												Mean	SD
	Cell 1 0300	Cell 2 0300	Cell 4 0285	Cell 5 0285	Cell 6 0285	Cell 7 0290	Cell 8 0290	Cell 10 0296	Cell 11 0296	Cell 12 0296	Cell 14 0275	Cell 15 0275		
Skin Wash 30 min	98.46	94.61	105.78	105.93	100.03	102.71	101.37	104.91	104.36	91.10	99.38	100.21	100.61	4.95
Tissue Swab 30 min	1.91	0.77	2.73	2.23	0.64	2.04	4.15	0.17	2.65	6.28	1.84	2.31	2.50	1.71
Pipette Tips 30 min	0.38	0.11	0.11	0.17	0.72	0.07	0.26	0.20	0.24	1.07	0.53	0.11	0.32	0.30
Dislodgeable Dose 30 min	100.76	95.49	108.62	108.34	101.60	104.82	105.78	105.29	107.25	98.45	101.76	102.63	103.44	4.38
Skin Wash 72 h	0.60	1.04	0.29	0.26	0.04	0.40	0.31	0.43	0.26	0.29	0.43	0.36	0.43	0.24
Tissue Swab 72 h	0.18	0.29	0.28	0.16	0.10	0.39	0.31	0.58	0.65	0.34	1.41	0.82	0.50	0.38
Pipette Tips 72 h	0.03	0.01	0.01	0.01	*0.00	0.04	0.14	0.01	0.04	0.03	0.01	0.04	*0.03	*0.04
Donor Chamber Wash	0.06	0.06	0.07	0.04	0.07	0.12	0.07	0.08	0.15	0.54	0.12	0.17	0.14	0.15
Dislodgeable dose 72 h	0.87	1.40	0.65	0.47	0.21	0.96	0.82	1.10	1.11	1.20	1.96	1.38	1.09	0.43
Total Dislodgeable Dose	101.63	96.89	109.26	108.80	101.81	105.77	106.60	106.38	108.36	99.65	103.71	104.02	104.53	4.15
Stratum Corneum 1-5	4.43	6.34	3.58	3.26	0.29	3.42	3.63	4.31	2.94	1.07	4.22	4.80	3.86	1.37
Stratum Corneum 6-10	0.54	1.25	0.33	0.57	0.26	0.53	0.26	0.36	0.36	0.25	0.94	0.75	0.56	0.33
Stratum Corneum 11-15	0.37	0.41	0.15	0.13	0.11	0.19	0.05	0.30	0.16	0.06	0.30	0.45	0.24	0.15
Stratum Corneum 16-20	0.22	0.21	0.08	0.08	0.12	0.17	0.04	0.09	0.10	0.07	0.39	0.26	0.15	0.11
Stratum Corneum	5.56	8.20	4.14	4.04	0.77	4.31	3.99	5.06	3.57	1.45	5.84	6.25	4.81	1.82
Unexposed Skin	*0.00	*0.00	0.00	0.00	*0.00	0.03	0.01	*0.00	*0.00	0.07	0.00	0.01	*0.01	*0.02
Total Unabsorbed	107.19	105.09	113.41	112.85	102.58	110.12	110.60	111.45	111.93	101.17	109.56	110.28	109.35	3.83
Epidermis	1.92	1.04	0.27	0.36	0.06	1.40	0.21	1.28	0.31	1.85	1.72	0.61	0.96	0.69
Dermis	0.01	0.05	0.01	0.02	0.09	0.03	0.01	0.04	0.05	0.03	0.05	0.02	0.03	0.02
Receptor Fluid	*0.06	*0.03	*0.05	*0.07	*0.09	*0.07	*0.05	*0.05	*0.05	*0.06	*0.04	*0.05	*0.05	*0.01
Receptor Chamber Wash	*0.01	*0.00	*0.02	*0.00	*0.03	0.04	*0.00	*0.00	*0.01	0.19	*0.00	0.11	*0.03	*0.06
Receptor Rinse	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00
Total Absorbed	0.07	0.04	0.07	0.07	0.12	0.11	0.06	0.05	0.05	0.25	0.04	0.16	0.09	0.07
Mass Balance	109.19	106.22	113.77	113.30	102.86	111.66	110.88	112.82	112.34	103.30	111.37	111.07	110.43	3.34

Cells 6 and 7 are rejected from mean and SD calculations due to incomplete receptor fluid collections

*=Results calculated from data less than 30 d.p.m. above background which corresponds to 0.000782% or 0.869 ng equiv./cm²

=Mean includes results calculated from data less than 30 d.p.m above background

Conclusion

In conclusion, following topical application of [¹⁴C]-Acid Black 1 in a non-oxidative hair dye formulation at a concentration of 0.5%, w/w (Test Preparation 1) to human skin, the total absorbed dose of [¹⁴C]-Acid Black 1 (the sum of the receptor fluid, receptor rinse and receptor chamber wash) was 0.08% (0.09 µg equiv./cm²).

Ref.: IV, 2 (subm III)

Comment

The mean + SD (0.09 + 0.07) = 0.16 µg equiv./cm² will be used to calculate the MoS.

Taken from SCCS/1226/09

Guideline: OECD 428 (draft, 2000)
Tissue: split-thickness pig skin, 200 µm
Group size: 7 membranes from 3 donors
Diffusion cells: flow-through diffusion cell, 0.64 cm², 31 ± 1 °C
Skin integrity: permeability coefficient (Kp) < 2.5 x 10⁻³ cm/h, using tritium water
Test substance: Acid Black 1
[Nitroaniline-ring-¹⁴C] Acid Black 1; 2646 kBq/mg (71.5 µCi//mg)
Batch: 9405 (Acid Black 1)
KL/141 (radio-labelled Acid Black 1)
Purity: 94.8% (Acid Black 1)
98.74% (radio-labelled Acid Black 1)
Test item: Commercial product Elumen Hair Color Clear formulation
containing 0.5% (w/w) [¹⁴C] Acid Black 1
Doses: 95 µl (0.70 mg Acid Black 1/cm²)
Receptor fluid: physiological saline, flow rate 3 ml/h
Solubility receptor fluid: solubility in water > 3%
Stability: stable in aqueous solution containing 1% carboxymethylcellulose
Method of Analysis: Liquid scintillation counting
GLP: in compliance
Study period: 20 - 27 October 2003

Revision of the opinion on Acid Black 1

Split thickness skin membranes (thickness 200 µm) originating from frozen pig ear skin were used. The integrity of the skin membranes was determined by the permeability testing using tritiated water. A 95 µl aliquot of the formulation Elumen Hair Color containing 0.5% (w/w) ¹⁴C-Acid Black was applied manually to each skin membrane. Thus a dose level of approximately 0.70 mg Acid Black 1/cm² was applied to each skin membrane. The formulation was applied to the skin membranes for 30 minutes and then removed from skin with shampoo solution (three times and once with tap water). Following the washing procedure the donor chambers were filled with 1ml of saline. The perfusates were collected at ambient temperature in time intervals as follows:

- 0-4 hours: 0.5 hour interval (8 intervals)
- 4-8 hours: 1 hour interval (4 intervals)
- 8-48 hours: 2 hours interval (20 intervals)

48 hours after application, the perfusate sampling was terminated. The epidermis was separated from the dermis by tape strip method (18 tapes), the amount of dye found in the upper skin is considered not to have passed the skin. The consecutive strippings were combined to 3 fractions, 6 tape strips each. The remaining skin membranes after stripping were digested in tissue solubilizer reactivity was determined by LSC and considered as penetrated.

Results

93.8 ± 3.3% of the applied dose could be washed off from the skin membranes. Only 4% of the dose remained on the skin membrane after the washing procedure. A significant part of this radioactivity was found in physiological saline (2.3 ± 0.5%), which was applied to the skin after washing. The rest of the radioactivity (1.8%) was found in the stratum corneum of the skin membrane: tape strip I = 1.53 ± 0.56%, tape strip II = 0.28 ± 0.29%, tape strip III = 0.04 ± 0.04%. In the lower levels of the skin, a maximum absorption of 0.03% (range <0.01- 0.03% = 0.081-0.243 µg/cm²) of the dose was found. Most of the determined values in the lower skin were below the limit of determination (LQ), i.e. 0.01µg/cm². Thus, the study authors considered the LQ as the amount present in the lower skin. The amount of Acid Black 1 penetrated through the skin as measured in the receptor fluid was 0.173 ± 0.117 µg/cm². The amounts of Acid Black 1 measured in the receptor fluid and in the lower skin were considered to be absorbed by the skin. This amount was considered bioavailable.

Conclusion

According to the study authors, the worst case consideration of penetrated test item was 0.173 µg/cm² (receptor fluid) + 0.081 µg/cm² (lower skin) = 0.254 µg/cm². The mean recovery of the test item was 98.12 %.

Cell number	1	2	4	6	10	11	12	mean	SD
Penetrated (µg/cm ²)	0.390	0.288	0.110	0.108	0.105	0.105	0.105	0.173	0.117
Penetrated (%)	0.048	0.032	0.010	0.009	0.006	0.006	0.006	0.017	0.017
Amount present in lower skin (%)	<0.01	0.03	<0.01	0.01	<0.01	<0.01	0.01	0.03 (max) = 0.24 µg/cm²	/
Recovery (%)	105	99	95	97	99	95	97	98	3

Ref.: 11

Comment

Too few chambers were used for the study. The volume of the test material applied on the skin, 95 µl/cm² (approximately 95 mg/cm²) is too high compared to the recommended dose of 20 mg/cm².

Due to these shortcomings, an absorption of mean + 2SD will be used in the calculation of MoS. For the amount present in lower skin, a SD could not be determined, since in 4/7 samples the amount of Acid Black 1 was below the LQ. For this compartment, the A_{\max} (0.03%, corresponding to $0.24 \mu\text{g}/\text{cm}^2$) will be used in the calculation. The worst case estimation for dermal absorption of Acid Black 1 in non-oxidative hair dye formulation is therefore $0.17 + 2 \times 0.12$ (mean + 2SD, receptor fluid) + $0.24 \mu\text{g}/\text{cm}^2$ (A_{\max} lower skin) = $0.65 \mu\text{g}/\text{cm}^2$.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (14 days) oral toxicity

Guideline: /
 Species/strain: rat, Wistar Hanlbm: WIST (SPF)
 Group size: 40 (5 per sex and per dose)
 Test substance: Acid Black 1
 Batch: 9405
 Purity: 94.8%
 Vehicle: bi-distilled water containing 1% carboxymethylcellulose
 Dose levels: 0, 100, 300 and 1000 mg/kg bw/day (0, 95, 285, and 950 mg active dye/kg bw/day)
 Dose volume: 10 ml/kg bw
 Route: oral gavage
 Administration: daily for 14 days
 GLP: /
 Study period: 10 – 30 December 1999

This was a range finding study for the 90 day study. Doses were prepared daily. Homogeneity and stability of Acid Black 1 in bi-distilled water containing 1% CMC was performed.

The animals were observed for mortality twice daily and for clinical signs once daily. Food consumption and body weights were recorded once prior to treatment then weekly.

On Day 15, animals were anesthetized, weighed, and killed. Macroscopic observations were recorded, selected organs weighed (adrenals, brain, heart, liver, kidneys, spleen, testes), and selected tissues collected and preserved (adrenals, heart, kidneys, liver, spleen, gross lesions).

Results

At the high dose, two males (Days 5 and 9) and four females (Days 2, 5 12 and 15) were found dead. Clinical observations included dark faeces at all dose levels; orange urine and sedation at the high and mid dose levels; and ataxia, piloerection, salivation, half closed eye lids and emaciation at the high dose level. Body weight, body weight gain and food consumption was decreased only in the high dose group.

The relative spleen weight was significantly increased in all animals from both the mid dose and high dose groups (dose related). In the high dose males there was a significant increase of the relative liver weight. These finding were considered to be treatment-related.

At necropsy several animals in the high dose groups showed black discolouration of the intestines, liver, kidney, spleen, stomach, urinary bladder and uterus. Enlarged spleens were observed in mid- and high dose animals.

Conclusion

Based on these results, the doses for the 90-day study were proposed to be 0, 5, 30 and 180 mg/kg bw/day.

Ref.: 5

3.3.5.2 Sub-chronic (90 days) toxicity (oral, dermal)

Study 1

Guideline:	OECD 408 (1998)
Species/strain:	Wistar rat, Hanlbn:WIST (SPF)
Group size:	80 (10 per sex and dose)
Test substance:	Acid Black 1
Batch:	9405
Purity:	94.8%
Stability:	> 72h (in water)
Vehicle:	bi-distilled water containing 1% carboxymethylcellulose
Dose levels:	0, 5, 30 and 180 mg/kg bw/day (0, 4.7, 28, and 171 mg active dye/kg bw/day)
Dose volume:	10 ml/kg bw
Route:	oral gavage
Administration:	daily for at least 91 days
GLP:	in compliance
Study period:	7 February – 17 November 2000

The doses were prepared weekly. Concentration, homogeneity and stability (after 2 hours and 7 days) of doses were determined.

The following parameters were evaluated: Mortality twice daily; clinical signs daily; functional observational battery in week 13; grip strength and locomotor activity; body weight and food consumption weekly; ophthalmoscopy at pre-test and in week 13; clinical laboratory investigations and macroscopy at termination; organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thyroid, thymus) and histopathology (kidneys, liver and spleen as well as gross lesions in all animals; in control and high-dose group for other organs and tissues). There were no recovery groups.

Results

There were no deaths. Body weight, body weight gain and food consumption, functional observational battery, locomotor activity, grip strength and ophthalmoscopy were comparable with the controls.

Clinical signs were dark faeces in mid and high dose groups, and green urine and bedding stained orange in the high dose group.

Statistically significant haematological changes included decreased red blood cell count (mid and high dose males, high dose females), decreased haemoglobin (high dose males/females), decreased haematocrit (high dose males/females), increased mean corpuscular volume (MCV) (mid and high dose males, high dose females), increased mean corpuscular haemoglobin (MCH) (mid and high dose males/females), decreased mean corpuscular haemoglobin concentration (MCHC) (high dose males/females), increased platelets count (high dose males), reticulocytosis (all male dose groups, high dose females), Heinz bodies (high dose males/females), increased methaemoglobin levels (mid and high dose males/females), polychromatophilia (high dose males/females). All these changes indicated haemolytic anaemia with compensatory reticulocytosis.

Statistically significant changes in clinical biochemistry included increased urea (mid and high dose females), increased uric acid (high dose males/females), increased bilirubin (mid and high dose males, high dose females). In high dose animals, crystals with a general resemblance to those of indigotine were noted in the urinalysis, as well as increased protein level and higher pH in high dose females. These findings were considered by the study authors as being test article related.

Increased relative spleen weight and relative kidney weight were observed in the high dose group. These correlated with the histology (extramedullary erythropoiesis in the spleen at

all doses; increased lipofuscin in kidneys of mid and high dose females and high dose males). In addition, in the kidneys, a brownish pigment was recorded in tubular cells of all treated groups but mostly in high dose animals, and minor morphological alterations like increased incidence of lymphoid foci and tubular mineralization in high dose males, and of pelvic mineralization in high dose females were observed.

In the liver, increased incidence and/or severity of haemosiderin deposits mainly stored in macrophages was observed. In addition haemopoietic cell foci increased in incidence of the high dose females; this was considered by the study authors to be adaptive in nature.

Conclusion

Due to the changes in haematological parameters and the correlated histological findings in the spleen, the NOEL of Acid Black 1 could not be established.

Ref.: 6

Comment

Regarding the changes in haematological parameters, the SCCS noted that only reticulocytosis was statistically significantly changed in the low dose group. The SCCS agrees with the conclusion of the study authors

Study 2

Guideline:	OECD 408 (1998)
Species/strain:	rat, HanBrl:WIST (SPF)
Group size:	100 (group 1 and 4: 15 per sex and per dose; group 2 and 3: 10 per sex and per dose)
Recovery group:	5 per sex and per dose (control and high dose group)
Test substance:	Acid Black 1
Batch:	9405
Purity:	94.8%
Stability:	> 72h (in vehicle)
Vehicle:	bi-distilled water containing 1% carboxymethylcellulose sodium salt
Dose levels:	0, 0.3, 1.5 and 5 mg/kg bw/day (0, 0.28, 1.4, and 4.7 mg active dye/kg bw/day)
Dose volume:	10 ml/kg bw
Route:	oral gavage
Administration:	daily for 91 days
GLP:	in compliance
Study period:	11 December 2003 – September 2004

The doses were prepared weekly. Concentration, homogeneity and stability (after 2 hours and 7 days) of doses were determined.

The recovery groups were kept for a further treatment-free 28 days.

The following parameters were evaluated: Mortality twice daily; clinical signs daily; body weight and food consumption weekly; ophthalmoscopy at pre-test, in week 13 and in week 17 (recovery group); clinical laboratory investigations and macroscopy at termination (week 13 and 17); organ weights (adrenals, brain, heart, kidneys, liver, uterus, ovaries, spleen, testes, epididymides, thyroid, thymus) and histopathology of selected tissues.

Results

There were no effects on mortality, absolute and relative food consumption, mean body weight and mean body weight gain, and ophthalmology. Dark faeces were seen from week 2 up to the first week of recovery in the high dose group.

Statistically significantly haematological changes included decreased mean haemoglobin level in high dose males, decreased mean corpuscular haemoglobin in low and mid dose males, and decreased mean corpuscular haemoglobin concentration at all doses in males

and in high dose females. Red cell distribution width was significantly reduced in mid and high dose males and high dose females. The haemoglobin distribution width was significantly reduced in high dose females. The reticulocyte maturity indices in the high dose males were shifted significantly towards the high fluorescence reticulocytes by the end of the treatment phase but were within the historical control range. The relative reticulocyte count was significantly elevated in the high dose males at the end of the 4-week recovery period, but this was, according to the study authors, due to a slightly lower control value. After the recovery period, a compensatory shift towards low fluorescence reticulocytes occurred, but the study control values were considered by the study authors to have abnormally low values skewing the clinical picture. Mid and high dose females had significantly increased eosinophil counts during the treatment period but returned to control levels during the recovery period. The haematological changes were thought to be adaptive by the study authors as they were reversed in the recovery period, were within the historical control range, and were considered to be indicative of metabolic reactions rather than adverse changes.

Biochemical and urine parameters changes were seen in high dose females, but were minor and within the historical control values except triglycerides. The mean triglycerides were significantly lower and outside the lower historic control range. The levels rose during the recovery phase but not to levels comparable to the controls. The study authors suggested that this was due to metabolic changes in the liver. Alpha-1-globulin levels were also significantly reduced at the high dose.

Absolute or relative organ weights were comparable with the controls. No substance related macroscopic or adverse microscopic findings were noted.

Conclusion

The study authors considered the NOAEL for Acid Black 1 to be 5 mg/kg/day (4.7 mg active dye/kg bw/day) on the basis of the minimal reduction of haemoglobin accompanied by "left-shifting" of reticulocyte maturity indices without compensatory reticulocytosis.

Ref.: 7

SCCS comments to the two 90-day studies:

The SCCS has revealed some inconsistencies between the results of various haematological parameters in male animals in the low-dose (study 2) and high-dose (study 1) 90-day studies in general, e.g. RBC decreased in high-dose study, but increased or constant in low-dose study; MCV increased in high-dose study, but decreased in low-dose study; reticulocyte count consistently increased in high-dose study, but only increased at the highest dose in the low-dose study. In addition, there are considerable differences in several haematological parameters between the two control groups and between the 5 mg/kg bw/day groups in the low-dose and high-dose 90-day studies. The results of some of the haematological parameters in the low-dose study control group have been claimed by the study report authors to be abnormal. Based on the available data it cannot be excluded that the results of the haematological parameters in the treated groups also are abnormal. On this background, the SCCS considers that the results of the haematological parameters in the low-dose study presumably are not fully reliable although supporting some of the findings in the high-dose study. The safety assessment will therefore be based on the high-dose 90-day study with the lowest dose level of 5 mg/kg bw/day (4.7 mg active dye/kg bw/day) as a LOAEL.

3.3.5.3 Chronic (> 12 months) toxicity

No data submitted

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity in vitro

Bacterial Reverse Mutation Assay, study 1

Guideline:	OECD 471 (1997)
Species/Strain:	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537 and <i>Escherichia coli</i> uvrA
Replicates:	triplicates in three independent experiments
Test substance:	Acid Black 1
Batch:	M90114
Purity:	> 85%
Vehicle:	de-ionised water
Concentration:	Experiment I and II: 42, 125, 417, 1250, 2500 and 5000 µg/plate, without and with S9-mix Experiment III: 666, 1000, 1666, 2500, 3333 and 5000 µg/plate, without S9-mix; TA98 and TA1537 only
Treatment:	pre-incubation method with 30 minutes pre-incubation and at least 48 h incubation, both without and with S9-mix
GLP:	in compliance
Study period:	31 May – 19 July 1999

Acid Black 1 was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9 fraction from male Syrian golden hamsters was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment with all strains; eight concentrations up to the prescribed maximum concentration of 5 mg/plate were tested for toxicity and mutation induction. The pre-experiment was reported as experiment I since criteria for a proper experiment were met. Toxicity was evaluated on the basis of a reduction in the number of spontaneous revertant colonies and/or a clearing of the bacterial background lawn. All experiments were performed with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results

In experiment II a slight toxic effect, evident as a reduction in the revertant colony numbers, was seen in strain TA1537 at 5000 µg/plate in the presence of S9-mix. A clearing of the background lawn was not observed in any of the experiments.

In experiment I without S9-mix a dose dependent increase in the number of revertants was observed in TA1537. However, this result could not be reproduced in experiments II and III. In all three experiments Acid Black 1 induced a dose-dependent increase in the number of revertants in strain TA98 without S9-mix.

Conclusion

The study authors concluded that under the experimental conditions used Acid Black 1 was mutagenic in the gene mutation tests in bacteria in TA98 in the absence of S9 metabolic activation.

Ref.: 13

Comment

The SCCS noted that, although the increases in TA98 were dose-dependent and more than two-fold, they were within the historical control range of the laboratory.

Bacterial Reverse Mutation Assay, study 2

Guideline:	OECD 471 (1997)
Species/Strain:	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537 and <i>Escherichia coli</i> WP2 uvrA
Replicates:	triplicates in two independent experiments
Test substance:	Acid Black 1
Batch:	9405
Purity:	94.8%
Vehicle:	de-ionised water
Concentration:	Experiment I and II: 33, 100, 333, 1000, 2500 and 5000 µg/plate, without and with S9-mix
Treatment:	pre-incubation method with 30 minutes pre-incubation and at least 48 h incubation, both without and with S9 mix
GLP:	in compliance
Study period:	12 - 21 January 2000

Acid Black 1 was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9 fraction from male Syrian golden hamsters was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment with all strains; eight concentrations up to the prescribed maximum concentration of 5 mg/plate were tested for toxicity and mutation induction. The pre-experiment was reported as experiment I since criteria for a proper experiment were met. Toxicity was evaluated on the basis of a reduction in the number of spontaneous revertant colonies and/or a clearing of the bacterial background lawn. All experiments were performed with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results

Neither relevant toxic effects nor a clearing of the background lawn were observed in any of the experiments. A biologically relevant increase in the number of revertants was not found in any of the tester strains following treatment with Acid Black 1 at any dose level neither in the presence or absence of metabolic activation.

Conclusion

Under the experimental conditions used Acid Black 1 was not mutagenic in this gene mutation tests in bacteria both in the absence and the presence of metabolic activation.

Ref.: 15

***In vitro* Mammalian Cell Gene Mutation Test, study 1**

Guideline:	OECD 476 (1997)
Species/strain:	L5178Y <i>tk</i> ^{+/-} mouse lymphoma cells
Replicates:	two parallel cultures in two independent experiments
Test substance:	Acid Black 1
Batch:	M90114
Purity:	> 85%
Vehicle:	de-ionised water
Concentrations:	Experiment I: 75, 150, 300, 600 and 1200 µg/ml without and with S9-mix Experiment II: 156.3, 312.5, 625, 1250 and 2500 µg/ml without and with S9-mix
Treatment	Experiment I: 4 h both without and with S9 mix, expression period 72 h, selection growth 10-15 days. Experiment II: 24 h without S9 mix, expression period 72 h, selection growth 10-15 days

GLP: in compliance
 Study period: 28 June – 24 August 1999

Acid Black 1 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring relative survival growth. In the main test, cells were treated for 4 h (experiment I: both without and with S9-mix) or 24 h (experiment II: without S9-mix), followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation and a selection growth 10-15 days. Liver S9-mix fraction from male Syrian golden hamsters was used as exogenous metabolic activation system. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results

In experiment I precipitation of Acid Black 1 occurred at 1200 µg/ml and above and in experiment II at 1250 and 2500 µg/ml both without and with S9-mix. The appropriate level of toxicity (10-20% survival after the highest dose) was not reached in both experiments without or with S9-mix pointing to insufficient exposure of the cells. A rather weak but reproducible increase in mutant frequency was observed at the highest dose tested in experiment I. The ratio of small *versus* large colonies was shifted towards the small colonies at concentrations producing a mutagenic effect, indicating a clastogenic rather than a mutagenic potential of Acid Black 1.

The authors stated that under the experimental conditions reported a weak mutagenic potential of Acid Black 1 can not be entirely excluded both without and with S9-mix at concentrations at or above the limit of solubility. On the other hand, they stated that there was also no indication of a clear mutagenic response leading to an equivocal outcome of the study.

Conclusion

According to the study authors and under the experimental conditions used, the results obtained with Acid Black 1 in this mouse lymphoma assay at the *tk* locus have to be considered as inconclusive.

Ref.: 14

Comment

The appropriate level of toxicity (10-20% survival after the highest dose) was not reached in both experiments without and with S9-mix which may point to insufficient exposure of the cells.

***In vitro* Mammalian Cell Gene Mutation Test, study 2**

Guideline: OECD 476 (1997)
 Species/strain: L5178Y *tk*^{+/-} mouse lymphoma cells
 Replicates: two parallel cultures in two independent experiments
 Test substance: Acid Black 1
 Batch: 9405
 Purity: 94.8%
 Vehicle: de-ionised water
 Concentrations: Experiment I: 156.3, 312.5, 625, 1250 and 2000 µg/ml without S9-mix
 156.3, 312.5, 625, 1250 and 2500 µg/ml with S9-mix
 Experiment II: 312.5, 625, 1250, 2500 and 5000 µg/ml without S9-mix
 156.3, 312.5, 625, 1250 and 2500 µg/ml with S9-mix

Revision of the opinion on Acid Black 1

Treatment	Experiment I: 4 h both without and with S9-mix, expression period 72 h, selection growth 10-15 days. Experiment II: 4 h without S9-mix, expression period 72 h, selection growth 10-15 days 24 h both without and with S9-mix, expression period 72 h, selection growth 10-15 days.
GLP:	in compliance
Study period:	14 December 1999 – 21 February 2000

Acid Black 1 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring relative survival growth. In the main test, cells were treated for 4 h (experiment I: both without and with S9-mix; experiment II with S9-mix) or 24 h (experiment II: without S9-mix only), followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation and a selection growth 10-15 days. Liver S9-mix fraction from male Syrian golden hamsters was used as exogenous metabolic activation system. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results

Precipitation of Acid Black 1 was only reported in the pre-experiment; at 5000 µg/ml after 4 h treatment both without and with S9-mix and at 2500 µg/ml and above after 24 h treatment without S9-mix only. The appropriate level of toxicity (10-20% survival after the highest dose) was mostly reached in experiments without S9-mix but not with S9-mix, pointing to insufficient exposure of the cells in the latter case. An increase in mutant frequency was observed at 2000 µg/ml without S9-mix and at 2500 µg/ml with S9-mix in culture 1 of experiment I. Since these results were not reproducible, they were considered not biologically relevant. In the other cultures of both experiments I and II a biologically relevant and dose dependent increase in the number mutant colonies was not observed independent of the test concentration or the presence or absence of S9-mix.

Conclusion

Under the experimental conditions used, Acid Black 1 was not mutagenic in this mouse lymphoma assay using the *tk* locus as reporter gene.

Ref.: 16

Comment

The appropriate level of toxicity (10-20% survival after the highest dose) was not reached in cultures treated in the presence of S9-mix which may point to insufficient exposure of the cells.

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

***In vivo* Mammalian Erythrocytes Micronucleus Test**

Guideline:	OECD 474 (1997)
Species/strain:	mouse, NMRI
Group size:	5 mice/sex/group
Test substance:	Acid Black 1
Batch:	M90114
Purity:	> 85%
Vehicle:	deionised water
Dose level:	500, 1000 and 2000 mg/kg bw
Route:	oral

Sacrifice times: 24 h after treatment for all concentrations, 48 h for the highest dose only.
GLP: in compliance
Study period: 11 April – 24 May 2000

Acid Black 1 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based in a pre-experiment on acute toxicity at 1, 6, 24 and 48 h after start of treatment. In the main experiment mice were exposed orally to 0, 500, 1000 and 2000 mg Acid Black 1/kg bw. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/NCE). Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results

In the a pre-experiment on acute toxicity with exposure up to 2000 mg Acid Black 1/kg bw, reduction of spontaneous activity and coloured urine was found up to 24 h after administration. On the basis of these data the oral application of 2000 mg/kg bw was estimated to be suitable. The mean number of NCEs was not relevantly increased after treatment with Acid Black 1 as compared to the mean value in untreated concurrent control animals indicating that Acid Black 1 was not cytotoxic for bone marrow cells. Biologically availability of Acid Black 1 is certain since coloured urine of treated animals confirms systemic distribution. A biologically relevant and/or statistically significant increase in the number of cells with micronuclei was not found following treatment with Acid Black 1 at any dose or time point.

Conclusion

Under the experimental conditions used, Acid Black 1 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 17

In vivo unscheduled DNA synthesis (UDS) test

Guideline: OECD 486 (1997)
Species/strain: rat, Wistar Hanlbm: WIST (SPF)
Group size: 4 male rats/group
Test substance: Acid Black 1
Batch: M90114
Purity: > 85%
Vehicle: carboxymethylcellulose 1% w/v
Dose level: 500 and 2000 mg/kg bw
Route: oral (gavage)
Sacrifice times: 2 h and 16 h after dosing
GLP: in compliance
Study period: 3 May – 18 July 2000

Acid Black 1 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test concentrations were based on results from a pre-experiment on acute toxicity evaluated at 1 and 48 h after start of treatment. In the main experiment mice were exposed orally to 0, 500 and 2000 mg Acid Black 1/kg bw. Hepatocytes for UDS analysis were collected at 2 h and 16 h after administration of Acid Black 1. All animals from each group were perfused with collagenase for the collection of hepatocytes and establishment of cultures. After attachment of the cultures they were labelled for 4 h with 5 µCi/ml ³H-thymidine (specific activity 20 Ci/mmol). Evaluation of autoradiography was done after 12 days exposure.

UDS was measured by counting nuclear grains and subtracting the number of grains in a nuclear sized area adjacent to the nucleus; this value is referred to as net grain count. Unscheduled synthesis was determined on 2 slides and 50 randomly selected hepatocytes per animal. Negative and positive controls were in accordance with the OECD guideline.

Results

In the a pre-experiment on acute toxicity with exposure up to 2000 mg Acid Black 1/kg bw, reduction of spontaneous activity and black coloured urine was found up to 24 h after administration. On the basis of these data the oral application of 2000 mg/kg bw was estimated to be suitable. The viability of the hepatocytes was not substantially affected due to the *in vivo* treatment with Acid Black 1; variations were within the historical control data. No dose level of Acid Black 1 induced UDS in the hepatocytes of treated animals as compared to concurrent control values.

Conclusion

Under the experimental conditions used Acid Black 1 did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 18

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

Dose-range finding prenatal development toxicity study

Guideline:	/
Species/strain:	rat, Wist HanIbm: WIST (SPF-Quality)
Group size:	20 mated females, 5 per group
Test substance:	Acid Black 1
Batch:	9405
Purity:	94.8%
Vehicle:	bi-distilled water containing 1% carboxymethylcellulose sodium salt
Dose levels:	0, 80, 240 and 720 mg/kg bw/day (0, 76, 228, and 683 mg active dye/kg bw/day)
Dose volume:	10 ml/kg bw
Route:	oral, gavage
Administration:	once daily, 11 days (GD 6 - GD 17)
GLP statement:	/
Study period:	10 December 1999 - 13 January 2000

The doses were prepared daily. Concentration, homogeneity and stability (after preparation and 4 hours) of doses were determined.

Females were killed on GD 21 and the foetuses were removed by Caesarean section.

Maternal data recorded included deaths, clinical signs, food consumption, body weights, reproductive data and pathology findings and foetal data such as external examination, sex ratios and body weights.

Results

Maternal effects

There was one death in the high dose group on GD 17. Clinical signs in this high group started at GD7 and the symptoms were tremor, ruffled fur, dyspnea, swollen throat region, dark faeces and green urine. The dead animal felt cold to touch on GD 16. Dark faeces were also noted in the low and mid dose group throughout the study.

Food consumption was decreased in the mid and high dose groups with concomitant reduction in bodyweights. A body weight gain of 5% was noted in the mid dose group and a body weight loss of 13% was noted in the high dose group versus a 10% body weight gain in the control group. Dose related increases in absolute spleen weight were recorded (low dose 33%, mid dose 64% and high dose 166%).

Reproductive effects

One low dose animal was not pregnant. Live foetuses were found in 4 mid dose and 2 high dose dams at the end of the study period. Pre-implantation losses were noted in all groups but were highest in the high dose group, since implantation occurs on GD6, this could be treatment related. Increased post-implantation losses were seen in the high (50%) and mid (11%) dose resulting in decreased foetal numbers and increased foetal deaths. Foetal resorptions were seen in one mid and 2 high dose dams.

Foetal findings

At the high dose, 13/14 live foetuses were male. The high post-implantation losses were possibly of female foetuses. Mean foetal weight were significantly reduced (60%) compared with controls. 12/14 foetuses from the 2 litters had malformations such as hydrocephalus (11), micrognathia (9), oligodactylia (4) and syndactylia (3).

In the low and mid dose groups, no treatment related effects were seen. The foetal sex ratio was not affected.

Conclusion

Based on the results of this study, dose levels of 5, 40 and 320 mg/kg bw/day were proposed for the main study.

Ref.: 8

Embryo-foetal development study

Guideline:	OECD 414 (1981)
Species/strain:	rat, Wist HanIbm: WIST (SPF-Quality)
Group size:	88 mated females, 22 per group
Test substance:	Acid Black 1
Batch:	9405
Purity:	94.8%
Vehicle:	bi-distilled water containing 1% carboxymethylcellulose sodium salt
Dose levels:	0, 5, 40 and 320 mg/kg bw/day (0, 4.7, 38, and 303 mg active dye/kg bw/day)
Dose volume:	10 ml/kg bw
Route:	oral, gavage
Administration:	once daily, from day 6 through day 17 <i>post coitum</i>
GLP statement:	in compliance
Study period:	7 February – 8 March 2000

Based on the previous range finding study, doses were lowered. The doses were prepared daily. Concentration, homogeneity and stability (after preparation and 4 hours) of doses were determined.

Females were killed on GD 21 and the foetuses were removed by Caesarean section.

Maternal data recorded included deaths, clinical signs, food consumption, body weights, spleen weights, reproductive data and pathology findings, and foetal data such as external examination, sex ratios and body weights.

Results

Maternal effects

No deaths occurred during the study. Only in the high dose group were all animals pregnant. One non-pregnant female was found in the other groups.

At the high dose, clinical symptoms observed were dark faeces, green discoloured urine and ruffled fur, as well as reduced food consumption and body weight gain. Compared with the controls, increased relative spleen weights (50%) were observed.

In the low and mid dose groups, no treatment related effects were seen.

Reproductive effects

No treatment related effects were seen in any dose group.

Foetal findings

At the high dose, reduced foetal body weight (13%) was noted. Consequently, though statistically significant differences in skeletal ossification were seen, these were considered to be due to delayed maturation and not evidence of a teratogenic potential. The frequency of other foetal abnormalities noted during external examination, visceral examination or skeletal examination were comparable with the controls at all doses.

Conclusion

Based on this study, the maternal and foetal No-Observable-Effect-Level (NOEL) of Acid Black 1 was considered to be 40 mg/kg bw/day (38 mg active dye/kg bw/day).

Ref.: 9

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

40 hairdressers with a known relevant allergy to 4-phenylenediamine (PPD) and or 2,5-diaminotoluene sulphate (DTS) and/or 2-nitro-4-phenylenediamine (ONPPD) and a history of hand eczema were examined.

Study Phase 1

Patch test concentration range finding study was performed among 10 volunteers. Acid Black 1 (0.2%, 0.6%; 2%) and a representative formulation containing 0.5% Acid Black 1 (1%, 3%, 10%) were prepared in aqua or petrolatum.

Study Phase 2

40 hairdressers with known allergy (to PPD and/or DTS and/or ONPPD) were tested with the highest non-irritating concentration of Acid Black 1.

The patch tests were performed with Van der Bend square chambers. Readings were made 2 and 3 days after application and if necessary on day 4. The grading was performed according to standard guidelines as follows: -, +, ++, +++, IR (IR = irritant reaction).

Results

Study Phase 1

In 10 volunteers no irritant reactions were observed at any of the concentrations. Based on the result the highest test concentration was used for study phase II.

Study Phase 2

No positive reaction was observed on Acid Black 1 neither at 48 hours nor at 72 hours.

Conclusion

Acid Black 1 is not an irritant under the reported test conditions. In addition, there is no cross sensitisation to PPD, DTS and ONPPD.

Ref.: 21

3.3.12 Special investigations

No data submitted

3.3.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(Acid Black 1)

(non-oxidative conditions)

Absorption through the skin	A	=	0.16 µg/cm²
Skin Area surface	SAS	=	580 cm²
Dermal absorption per treatment	SAS x A x 0.001	=	0.09 mg
Typical body weight of human		=	60 kg
Systemic exposure dose (SED)	SAS x A x 0.001/60	=	0.0015 mg/kg bw
Lowest observed adverse effect level (90-day, oral, rat)	LOAEL	=	4.7 mg/kg bw/d
50% bioavailability *		=	2.35 mg/kg bw/d
Uncertainty factor for LOAEL (3)		=	0.78 mg/kg bw/d

Margin of Safety	NOAEL / SED	=	522
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* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14 Discussion

Acid Black 1 is used as a direct hair colouring agent up to an on-head concentration of 0.5% in non-oxidative hair dye formulations.

Acid Black 1 is also listed in Annex IV – List of colouring agents allowed for use in cosmetic products – to Directive 76/768/EEC on cosmetic products, column 4: colouring agents

allowed exclusively in cosmetic products intended to come into contact only briefly with the skin. Exposure of Acid Black 1 from such products is not considered in this risk assessment.

Physico-chemical properties

Besides several carcinogenic and/or mutagenic impurities, all batches of Acid Black 1 contain 3-6% unidentified impurities. The concentration of carcinogenic/mutagenic impurities should be kept at a minimum. The stability of Acid Black 1 in typical hair dye formulations is not reported.

The purity of commercial Acid Black 1 (>99.5%) has been reported to be much higher than the purity (94-97%) of three batches of Acid Black 1 used for toxicity testing. However, no documentation is provided for the higher purity (and thus less impurities) of the commercial material. Documentation of high quality of commercial Acid Black 1 compared to the test batches of Acid Black 1 should be provided, for example by presenting HPLC chromatograms showing peaks of Acid Black 1 as well as all organic impurities of test batches of Acid Black 1 and commercial Acid Black 1.

Toxicity

Acid Black 1 is of low acute toxicity with an oral LD50 > 5000 mg/kg bw.

Erythropoietic effects of Acid Black 1 suggesting the development of haemolytic anaemia were seen in both sub-chronic toxicity studies in rats. Males seem to be more susceptible. The results of the haematological parameters in the low-dose study are presumably not fully reliable although supporting some of the findings in the high-dose study. Therefore, the safety assessment will be based on the high-dose study with the lowest dose level of 5 mg/kg bw/day as a LOAEL.

The maternal and foetal No Observable Effect Level (NOEL) of Acid Black 1 was considered to be 40 mg/kg bw/day (38 mg active dye/kg bw/day) in a developmental toxicity study in rats.

No two generation reproduction study was submitted.

Skin/eye irritation and sensitisation

10% (w/v) Acid Black 1 in tap water was shown to be non-irritant to rabbit skin. Acid Black 1 at 5% (w/v) concentration was not irritating to eye. In a LLNA study, Acid Black 1 was found to be a moderate skin sensitiser.

Percutaneous absorption

The dermal absorption of B15 at 1% final concentration in a non-oxidative hair dye formulation was $0.09 \pm 0.07 \mu\text{g}/\text{cm}^2$.

The mean value + SD = $(0.09 + 0.07)$ or $0.16 \mu\text{g}/\text{cm}^2$ is used for calculating the MOS.

Mutagenicity/genotoxicity

Overall, the genotoxicity of Acid Black 1 is sufficiently investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Acid Black 1 did induce gene mutations in the first gene mutation tests in bacteria (batch M90114, purity >85%) but not in the second (batch 9405, purity >94.8%), although both tests were performed identically. In the same way in the first mouse lymphoma assay the results obtained with Acid Black 1 were inconclusive (batch M90114, purity >85%) whereas in the second experiment (batch 9405, purity >94.8%) negative results were found. The positive *in vitro* results for gene mutations were not confirmed in an *in vivo* unscheduled DNA synthesis test in rats.

The induction of chromosome aberrations by Acid Black 1 was only studied in an *in vivo* micronucleus test with mice; Acid Black 1 exposure did not result in an increase of cells with micronuclei in bone marrow cells.

As the positive *in vitro* results were not confirmed in an *in vivo* test, Acid Black 1 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity

No data submitted

4. CONCLUSION

The assessment of Acid Black 1 was for its use in non-oxidative hair dye formulations only.

The SCCS is of the opinion that the use of Acid Black 1 as a non-oxidative hair dye with a concentration on head of maximum 0.5% does not pose a risk to the health of the consumer.

The use of Acid Black 1 (CI 20470) as a cosmetic colorant should be reassessed.

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submission II, 2005

1. Bombard E., acute oral toxicity of Acid Black 1. Bayer AG, Institut für Toxikologie Wupperthal-Elberfeld, Extract of Test Report, 23 September 1974
2. Gloxhuber Ch. Orientating test of acute oral toxicity in mice. Henkel KGaA, D-4000, Düsseldorf, Study No.: R_9501226, 1983
3. Potokar M. Test of Acid Black 1 for primary ocular mucosa irritation in rabbit eyes. Henkel KGaA, Düsseldorf/Germany, Study nr R9501227, 22 July 1982
4. Potokar M. Test of Acid Black 1 for primary skin irritation by occlusion method in rabbits. Henkel KGaA, Düsseldorf/Germany, Study nr R9501228, 1982
5. Rosner E. 14-Day Oral Toxicity (Gavage) Study in the Rat. RCC Ltd, Itingen/Switzerland, Study No.: RCC 756336, March 2000
6. Hamann H-J., Braun W.H., Weber K., Knappe C. 13-Week Oral Toxicity (Gavage) Study in the Rat. RCC Ltd, Itingen/Switzerland, Study No.: RCC 758362, May 2000
7. Braun W.H., Damme B., Flade D., Konrad D. 13-Week Oral Toxicity (Gavage) Study in the Rat. RCC Ltd, Study nr 851932, 29 September 2004
8. Becker H., Biedermann K. Acid Black 1 (C.I. 20470): Dose range-finding study for effects on embryo-fetal development in the rat. RCC Ltd, Itingen/Switzerland, Study nr 756718, 3 February 2000
9. Becker H., Biedermann K. Acid Black 1 (C.I. 20470): Study for effects on embryo-fetal development in the rat. RCC Ltd, CH-Itingen, RCC 756720, August 24, 2000
10. Determination of the Partition Coefficient (n-Octanol / Water) of Acid Black 1 RCC Ltd, A. Tognucci, Itingen/Switzerland, August 30, 2002, Study No.: RCC 833883
11. Hassler S. Percutaneous penetration of C14-Acid Black 1 formulated as Elumen Hair Color through pig split thickness skin membrane (in vitro) RCC Ltd, 2004, Study No.: RCC 850572, 26 March 2004
12. Determination of the NMR-, IR-, UV/VIS Absorption, Mass Spectra and a HPLC Chromatogram. RCC Ltd, A. Tognucci, Itingen/Switzerland, Study nr 852012, 24.2.2004
13. Wollny H-E., Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay For Azo Dyes with Acid Black 1 RCC-CCR, Roßdorf, Study nr 636101, 13 August 1999
14. Wollny H-E. Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells with Acid Black 1 (C.I. 20470) (Modified Test Design for Azo-Dyes) RCC-CCR, Roßdorf. Study No.: RCC-CCR 636102, 10 December 1999
15. Wollny H-E., Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay For Azo Dyes with Acid Black 1. RCC-CCR, Roßdorf, Study nr 655301, 4 April 2000

16. Wollny H-E., Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells with Acid Black 1 (C.I. 20470) (Modified Test Design for Azo-Dyes) RCC-CCR, Roßdorf, Study nr 655302, 7 August 2000
17. Honarvar N. Micronucleus Assay in Bone Marrow Cells of the Mouse with Acid Black 1 (C.I. 20470). RCC-CCR, Roßdorf, Study nr 665301, 9 June 2000
18. Schulz M. In vivo / in vitro Unscheduled DNA Synthesis in Rat Hepatocytes with Acid Black 1 (C.I. 20470). RCC-CCR, Roßdorf, Study nr 665302, 11 August 2000
19. Honarvar N. LLNA in mice with Acid Black 1. RCC-CCR, Roßdorf, Study nr 870100, 21 February 2005
20. Contact Sensitisation: Classification according to Potency. ECETOX, Technical Report No. 87, April 2003
21. Hair dye sensitized hairdressers: the cross reaction pattern with new generation hair dyes. Fautz R, Fuchs A, van der Walle H, Contact Dermatitis, 46, 2002: 319-324

Submission III, 2012

- Wolfgang Dekant, Professor of Toxicology; Würzburg: Derivation of a NOAEL for Acid Black 1 from the results of the two available 90-day oral toxicity studies; October 2010
- Charles River Report No. 32319; Craig Blackstock, January 2012; the in vitro percutaneous absorption of radiolabelled Acid Black 1 (B015) at two concentrations (0.5%, w/w and 0.2% w/w) in a Non-Oxidative Hair Dye Formulation Through Human Skin
- Fautz R., Fuchs A., van der Walle H., Hair dye sensitized hairdressers; cross reaction pattern with new generation of hair dyes; Contact Dermatitis, 46, 2002: 319-324

Additional references

SCCP/0919/05. Memorandum; classification and categorisation of skin sensitisers and grading of test reactions. Adopted by the SCCP during the 5th plenary meeting of 20 September 2005