



EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL
Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

Basic Brown 17

COLIPA N° B7

Adopted by the SCCP
during the 2nd plenary meeting of 7 December 2004

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1. BACKGROUND

Submission II for Basic Brown 17 (COLIPA¹ No. B 7) was submitted in line with the second step of the strategy on the evaluation of hair dyes [<http://pharmacos.eudra.org/F3/cosmetic/doc/HairDyeStrategyInternet.pdf>] and provides new data on mutagenicity, sensitization and a new in-vitro percutaneous absorption study.

2. TERMS OF REFERENCE

1. *On the basis of currently available information, the SCCP is asked to assess the risk to consumers of Basic Brown 17 when used in hair dye formulations.*
2. *Does the SCCP recommend any further restrictions with regard to the use of Basic Brown 17 in hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name
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Basic Brown 17 (INCI name)

3.1.1.2. Chemical names

8-[(4-Amino-3-nitrophenyl)azo]-7-hydroxy-N,N,N-trimethyl-2-naphthalenaminium chloride
 2-Naphthalenaminium, 8-[(4-amino-3-nitrophenyl)azo]-7-hydroxy-N,N,N-trimethyl-, chloride
 [8-[(4-amino-3-nitrophenyl)azo]-7-hydroxy-2-naphthyl]trimethylammonium chloride.
 1-[(3-Nitro-4-aminophenyl)azo]-2-hydroxy-7-trimethylammoniumchloride naphthalene
 1-(3'-Nitro-4'-amino)-phenyl-azo-2-hydroxy-7-trimethylammonium chloride naphthalene

3.1.1.3. Trade names and abbreviations
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Trade name	:	Arianor Sienna Brown, Jaracol Sienna, Brown SAT 000918 Sienna Brown
Colour Index	:	CI 12251

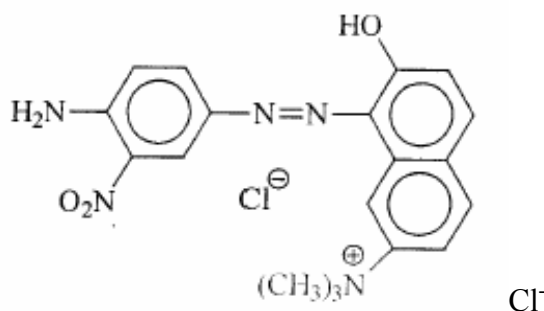
¹ COLIPA – European Cosmetics Toiletry and Perfumery Association

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3.1.1.4. CAS / EINECS number

CAS : 68391-32-2
 EINECS : 269-944-0

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula : C₁₉H₂₀N₅O₃. Cl
 Chloride content : 8.82%

3.1.2. Physical form

Dark brown powder

3.1.3. Molecular weight

Molecular weight : 401.85 (as chloride)

3.1.4. Purity, composition and substance codes

The data provided on the composition and purity of the material tested is quite obscure:

- In six studies there is no information at all.
- In the 3.3.3. Skin sensitisation / Study 1 the Batch n° 1425K is given without any information about its purity.
- A Batch n°. KS 5156 (purity: dye content 68 % as chloride) is used in the following tests:
 - * 3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity
 - * 3.3.6.1. Mutagenicity / Genotoxicity *in vitro*
 Bacterial Reverse Mutation Test
 Mammalian Cell Gene Mutation Test
 Unscheduled DNA Synthesis (UDS) Test
 - * 3.3.6.2. Mutagenicity / Genotoxicity *in vivo*
 Mammalian Liver Cells *in vitro*
 Mammalian Erythrocyte Micronucleus Test

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- * 3.3.8.2. Teratogenicity
 - Another Batch n° NDKS 1944 >94% pure is used in the following tests:
 - * 3.3.3. Skin sensitisation / Study 2
 - * 3.3.4. Dermal / percutaneous absorption / Study 2 (NDKS 1944, SAT000918 and SAT010439)

The data provided for the last two batches above are:

Batch n°. KS 5156 (Submission I)

Dye content (as chloride)	:	68%
Sugar content	:	13%
volatile matter/water of crystallisation	:	14%
inorganic salts (chloride, sulphate, etc.)	:	to 100%

Remark:

Apparently, this batch is a commercial formulation marketed by one or more companies. It gives no information about the purity, composition and impurities of the primary dye used in the formulation. Furthermore, it is not clear whether the reported percentage of the dye in the tested solutions refers to % of the dye itself or to % of the above formulation.

Batch n°. NDKS 1944 (Submission II)

Purity: > 94.5% in relation to a reference material including its water and chloride content

Impurities

1-[(2'-Nitro-4'-aminophenyl)-azo]-2-hydroxy-7-trimethyl-ammoniumchloride naphthalene (isomer)* : 3 %

* This impurity is the 2'-nitro-isomer of Basic Brown 17, which itself is a hair dye named Basic Red 118; it is included in the cosmetics' inventory with the same CAS and EINECS numbers, but with the colour index number CI 12251/1.

1-[(3'-Nitro-4'-aminophenyl)-azo]-2-hydroxy-7-trimethyl-ammoniumchloride naphthalene : / (detected)

Chloride content : 3.6 %
Water content : 6 %

Remark:

The purity is reported as a percentage of a reference material of unknown purity, including water and chloride (apparently, including also the two impurities mentioned above). Thus, the difference of (100 - 94.5 =) 5.5 % is totally unknown; it cannot be either water (because the reference material is reported to contain water), or chloride (because it was quantitated). In addition, the reported chloride content = 3.6 % is much lower than the (94% x 8.82% =) ~8.3 % which is the theoretical chloride content of the dye itself. This means that the samples used in the respective toxicological tests were a mixture of the free dye and its chloride salt and not in the chloride form of the dye, as given in the formula. Especially in the percutaneous absorption

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study 2, this composition is expected to affect the experimental results in comparison with the real use conditions.

3.1.5. Impurities / accompanying contaminants

See point 3.1.4.

3.1.6. Solubility

Soluble in water and ethanol.

3.1.7. Partition coefficient (Log P _{ow})

Log P_{ow} : /

3.1.8. Additional physical and chemical specifications
--

Organoleptic properties	:	slight odour
Melting point	:	200-202 °C
Boiling point	:	/
Flash point	:	/
Vapour pressure	:	/
Density	:	/
Viscosity	:	/
pKa	:	/
Refractive index	:	

General comments on analytical and physico-chemical characterisation

The following properties do not or poorly comply with the basic requirements for proper characterisation:

- * the purity of the substance (only one batch mentioned) is completely unknown (see remarks above);
- * Information on impurities is inadequate; data is needed on presence of 2-nitro-p-phenylene diamine and on 2-Hydroxy-7-trimethylamino naphthaleneamine
- * no data on solvent residues, if any;
- * Log P_{ow} is not given;
- * No quantitative data are given for solubility;
- * no experimental data are given on stability provided.

3.2. Function and uses

Basic Brown 17 is used in direct hair dye formulations in concentrations up to 2.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline	:	/
Species	:	CFY rat
Group size	:	4 male + 4 female
Substance	:	Basic Brown 17 in 1% aqueous methylcellulose
Batch no	:	not stated
Dose	:	0, 0.1, 1.0, 4.0, 8.0 and 16.0 g/kg bw in a volume of 1 to 40 ml/kg
Observation period	:	14 days
GLP	:	not in compliance

The rats (CFY strain) were treated with Arianor Sienna Brown with a range of dose levels from 0.1 to 16 g/kg body weight. The test compound was prepared as 10 and 40 % (w/v) suspensions in 1% aqueous methylcellulose. Rats dosed with the vehicle alone served as controls. All animals were observed for a period of 14 days.

Signs or reaction to treatment, observed shortly after dosing, included lethargy, piloerection, decreased respiratory rate and abnormal body carriage (hunched posture). Two male rats from the highest dose group died and one female rat from the highest dose group died.

After 14 days observation, the LD50 was reported to be between 8 and 16 g/kg bw.

Ref.: 1

Guideline	:	pre-dated OECD Guideline 401
Species	:	CF1 mouse
Group size	:	3, respectively 10 males per dose
Substance	:	Basic brown 17 in aqueous solution
Batch no	:	not stated
Dose	:	1.0, 2.51 and 5.01 g/kg bw in a volume of 0.2 ml/10g
Observation period	:	7 days
GLP	:	not in compliance

The test material was applied to CF1 mice once by gavage at three dose levels between 1.00 and 5.01 g/kg body weight and administered at a volume of 0.2 ml/10 g body weight. All animals were observed for a period of 7 days.

Results

During the observation period no mortalities were recorded. The LD50 was reported to be greater than 5 g/kg bw.

Ref.: 2

3.3.1.2. Acute dermal toxicity

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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

Guideline	:	OECD n° 404
Species	:	rabbit (New Zealand White)
Route	:	skin (intact and scarified)
Group size	:	6 male and 6 female
Substance	:	Basic Brown 17
Batch no	:	not stated
Dose	:	0.5 g
Concentration	:	100%
GLP	:	not in compliance

The test material was applied undiluted at the level of 0.5 g per square inch either to the shorn intact or to scarified skin on the back of six albino rabbits of each sex. The sample was covered by a linen cloth and plastic foil which were fixed by an elastic bandage and left in place for 24 hours. Readings were made according to Draize upon removal of the test material and then daily for the following 14 days.

Results

There were no observable irritation reactions to the dye by this test procedure.

Ref.: 4

3.3.2.2. Mucous membrane irritation

Guideline	:	OECD n° 405
Species	:	rabbit (New Zealand White)
Route	:	eye
Group size	:	3 male and 3 female
Substance	:	0.5% Basic Brown 17 0.5% (w/v) physiological saline
Batch no	:	not stated
Dose	:	0.1 ml
GLP	:	not in compliance

0.1 ml of a 0.5 % (w/v) solution in 0.9% saline solution was instilled into the conjunctival sac of the left eye of each of three rabbits. The right eye was treated with 0.1 ml of the vehicle and served as a control. The test material was not rinsed out.

The eye irritation reactions were read 30 and 60 minutes and after one and two days following instillation of the test material and were evaluated by the Draize method.

Results

Apart from a discoloration of the conjunctivae, the above treatment provoked no effects on the cornea and iris in any of the animals.

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Remark

The study was conducted before publication of OECD guideline 405, but appears to conform to its requirements. The material was tested at a concentration below the in-use level. In addition, a mild response could have been masked by the discoloration due to the dye.

Ref.: 3

3.3.3. Skin sensitisation

Study 1

Guideline	:	OECD (pre-dated)												
Species	:	guinea pig (Hartley/Dunkin)												
Group size	:	10 female												
Substance	:	Basic Brown 17 in aqueous solutions												
Batch no	:	9-1425K (purity not stated)												
Concentrations used	:	<table> <tr> <td>intradermal induction</td> <td>:</td> <td>0.1%</td> </tr> <tr> <td>topical induction</td> <td>:</td> <td>75%</td> </tr> <tr> <td>first challenge</td> <td>:</td> <td>25%</td> </tr> <tr> <td>second challenge</td> <td>:</td> <td>5%</td> </tr> </table>	intradermal induction	:	0.1%	topical induction	:	75%	first challenge	:	25%	second challenge	:	5%
intradermal induction	:	0.1%												
topical induction	:	75%												
first challenge	:	25%												
second challenge	:	5%												
GLP	:	not in compliance												

The material was prepared as a 0.1% w/v solution in water (injection 1). Freund's complete adjuvant was diluted with an equal volume of water (injection 2). A 1:1 mixture of the material solution and Freund's complete adjuvant solution was prepared (injection 3). The solutions (0.1 ml) were administered intradermally. One week after the injection, 0.4 ml of a solution of 75% w/v of the test material in distilled water was topically applied and occluded for a period of 48 hours. The animals were challenged topically two weeks after this second induction period with 0.1 ml of Basic Brown 17 at a concentration of 25% w/v in distilled water. Reactions were observed on the skin of 7 animals. The reactions consisted of erythema with slight oedema. To further evaluate the reactions, a second topical application was made one week later using 0.1 ml of Basic Brown 17 at a concentration of 5% in distilled water. Erythema was observed on the skin of 2 animals at 24 hours alone and at 48 hours alone in a third animal. The test material was by the authors not considered to show evidence of sensitisation despite the observed reactions.

Comment

Too low an intradermal induction concentration was used. The study is considered to be inadequate.

Ref.: 5

Study 2

Method	:	Local Lymph Node Assay (LLNA)
Guideline	:	OECD n° 429
Species	:	mice
Group size	:	3 test groups of 4 female mice and a control group of 4 mice
Substance	:	Basic Brown 17; >94% pure
Batch no	:	NDKS 1944
Concentrations used	:	1%, 5% and 25%

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GLP : in compliance

A homogenous aqueous dilution of the test substance was made shortly before each dosing. The highest non-irritating concentration was found in a pre-test with two mice. Related to these results, 1%, 5% and 25% dilutions were chosen for the main study.

Each test group of mice was treated by topical application to the dorsal surface of each ear lobe with different test item concentrations. The application volume, 25µl, was spread over the entire dorsal surface of each ear lobe once daily for 3 consecutive days. The control group was treated with water alone. Five days after the first topical application, all mice were administered with radio-labelled thymidine (³HTdR) by intravenous injection via a tail vein.

The draining lymph nodes were excised and pooled for each group (8 nodes per group). After preparation of the lymph nodes, disaggregation and overnight precipitation of macromolecules, these precipitations were re-suspended and transferred to scintillation vials.

The level of ³HTdR incorporation was measured by scintillation counting. The proliferation response of lymph node cells was expressed as the ration of ³HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index). An appropriate reference was used as a positive control.

The proliferation capacity of pooled lymph node cells was determined by the measurement of the incorporation of ³H-methyl thymidine. A test substance is regarded as a sensitiser if the exposure to at least one concentration results in incorporation of ³HTdR at least 3-fold than that recorded in control mice, as indicated by the stimulation index.

	Conc. Basic Brown 17	Stimulation index
Group 2	1%	0.6
Group 3	5%	0.7
Group 3	25%	1.0

Based on the above criteria, Basic Brown 17 was regarded as a non-sensitiser.

Ref.: 15

3.3.4. Dermal / percutaneous absorption

Study 1

Guideline : /
 Method : Human volunteer study
 Group size : 10 males
 Substance : 1 mM dye content in 40% aqueous isopropanol
 Batch no : not stated
 Application levels: 20 µl on 5.3 cm² skin of the inner forearm
 GLP : study not in compliance

Ten male volunteers participated in this investigation.

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20 µl of a 10⁻³ molar solution of the test material in 40% aqueous isopropanol were applied to five separate skin areas (5.3 cm²) of the inner forearm (equivalent to about 1.5 µg/cm²). After 10 minutes and 24, 48 and 72 hours, the dye stains of one treatment area after the other were removed by ten repeated strippings with Tesafilm-Spezial^R -tape. During the intervals between sampling, the skin areas were protected by a special non-occlusive mould. The stripping-tapes were glued on to a white cardboard and kept in the dark until they were evaluated by densitometry. From the recovery rates the amount of the dye that could possibly have penetrated the skin was estimated.

Results

It was reported that the dye did not diffuse into the horny layer. It was therefore concluded that Basic Brown 17 was not absorbed through the skin.

Ref.: 13, 14

Study 2

Guideline	:	OECD 428 (2000)
Test substance	:	Basic Brown 17
Batch no	:	NDKS 1944, SAT000918 and SAT010439
Tissue	:	pig (6-8 weeks old) dermatomed skin (trunk)
Skin integrity	:	skin electrical resistance measurement
Method	:	Static diffusion cell 2.54 cm ²
Receptor fluid	:	physiological saline 75 / ethanol 25
Formulation tested	:	aqueous-methanol solution (50/50) and standard commercial type formulation
Dose formulation applied	:	10 µl/cm ² and 10 mg/cm ²
Concentration ingredient	:	2 % w/w
Replicate cells	:	6 cells mounted and interpreted for each formulation
Duration of the contact	:	30 minutes non occluded
Duration of the diffusion	:	48 hours (sampling time: 0.5, 2, 6, 12, 24, 30 and 48 hours)
Analytical method	:	HPLC with visible detection
Validation	:	limit of quantitation (0.1 µg/ml)
GLP	:	in compliance

The skin penetration of Basic Brown 17 was evaluated in a static diffusion cell system. Fresh pig skin removed from the trunk was dermatomed to a constant thickness 400 µm, it was then stored frozen until use. The integrity of the skin was evaluated by the measurement of its electrical resistance. The skin surface temperature was monitored (32 ± 1 °C). Because of the low solubility of Basic Brown 17, the receptor fluid was saline / ethanol 75/25. This alcohol concentration is acceptable according to OECD (Guidance Document for the conduct of skin absorption studies, 1999) for which the amount of ethanol can reach 50 %. The test substance was prepared at a concentration of 2 % in a “reference vehicle” (solution in methanol) and in a “commercial type” formulation. Approximately 10 µl or mg/cm² of the formulation (exactly measured) was applied to 2.54 cm² for 30 minutes. The excess from the skin surface removed with a cotton swab soaked in water/ethanol, 50/50. The substance was measured using HPLC in the receptor fluid during the 48 hours of the diffusion. At the end, Basic Brown 17 was assayed by HPLC, in the horny layer collected by tape stripping (up to 21 strips), in the epidermis and dermis altogether. After assay of Basic Brown 17 in the washing material (skin excess) the mass

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balance of the study was calculated (88.0 ± 4.53 % of the applied dose for the alcoholic solution, 91.3 ± 5.22 % of the applied dose for the standard formulation)

Results

For the two formulations tested, most of the hair dye applied was recovered at the skin surface in the washing liquids (84 % for the aqueous-alcoholic formulation and 78 % for the standard formulation). The quantity of test substance penetrating, from both excipients, through the skin to the receptor fluid was lower than the quantitation limit (< 0.094 % of the dose for each product). No material was recovered in the horny layer whatever the formulation. The total amount absorbed calculated from the epidermis, the dermis and the receptor fluid contents was 0.106 ± 0.106 % of the applied dose for the aqueous-alcoholic solution, and 0.066 ± 0.024 % of the applied dose for the standard formulation at the end of 48 hours of diffusion after a contact with the skin of 30 minutes.

Ref.: 17

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 n° 408
Species	:	rat (Mu Ra Han 67)
Group sizes	:	10 male and 10 female
Substance	:	Basic Brown 17 in aqueous solution
Batch no	:	KS 5156
Dose levels	:	0, 50, 150 and 450 mg/kg bw in a volume of 10 ml/kg
Exposure	:	15 weeks
GLP	:	in compliance

The test substance, dissolved in water, was administered by oral gavage to three groups of 10 male and 10 female Sprague Dawley rats at doses of 50, 150 and 450 mg/kg body weight 5 days per week for 15 weeks. A further group of 10 male and 10 female rats were given vehicle alone (control group).

Results

No adverse effects or mortalities occurred at doses of 50 and 150 mg/kg body weight. Mortalities occurred at 450 mg/kg body weight, either following general or CNS signs of toxicity, or without previous abnormal observations.

Histological examination of the liver revealed individual pigment inclusions within Kupffer cells of some female rats given 50 mg/kg bw. All other organs were free of foreign material. At 150 mg/kg bw deposits were seen in a number of tissues but there was no accompanying degenerative or inflammatory changes.

Examination of satellite groups of animals, maintained for a further 7 weeks without treatment, showed that the deposits were persistent at 150 and 450 mg/kg bw per day, but not at 50 mg/kg bw per day.

Remarks

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The histological examination of the liver as part of a subchronic toxicity study of Basic Brown 17 in rats revealed pigment inclusions within Kupffer cells even in the lowest dose group of 50 mg/kg bw/day. Examination of the recovery group showed that in the 50 mg/kg bw/day group the deposits were not persistent. The deposit of pigments is considered a normal defence process and is not judged as being adverse. The NOAEL of the study can be established as 150 mg/kg bw/day, the NOEL as 50 mg/kg bw/day.

Ref.: 6

Mouse 90-day feeding study

Guideline	:	pre-dated OECD guideline 408
Species	:	mouse (CF 1)
Route	:	diet
Group sizes	:	10 female
Substance	:	Basic Brown 17
Batch no	:	not stated
Dose levels	:	0, 1250, 2500, 5000 mg/kg diet
Exposure	:	13 weeks
GLP	:	not in compliance

Ten female mice per dose level were used except for the control group where 20 animals were used. The controls were given a control diet for 13 weeks. 1250, 2500 and 5000 mg/kg of the Basic Brown 17 was given daily mixed with the diet for a total of 13 weeks. Both the test diet and the control diet were given fresh daily.

Results

All mice, with the exception of one animal in the highest group (5000 mg/kg), survived the treatment period. Compared to the control animals, no alteration of the behaviour of the test group animals was noted. Food intake and the results of haematological and biochemical determinations gave no indication of any toxic effects but a decrease in body weight gain in all treated groups suggested a possible effect. The data are presented graphically and do not show percentage changes but suggest that the effect was not dose-related. No differences were found in the organ weights (absolute or relative) between control and treated animals.

Coloured (yellow-brown) urine was seen in all treated animals indicating absorption of the test material.

Only discolorations (yellow-brown) of stomach and intestines were observed macroscopically which was explained by the dyeing properties of Arianor Sienna Brown. Histologically, fatty infiltration of the liver and slight haemosiderosis in the spleen were observed in all the treated but not in the control animals.

Dietary administration of 1250 mg/kg day was considered to be on the borderline for possible toxic effects in mice.

Ref.: 7

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity <i>in vitro</i>
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Bacterial Reverse Mutation Test

Guideline	:	OECD 471 (1983).
Species/strain	:	<i>S. typhimurium</i> , TA98, TA100, TA1535, TA1537, TA 1538
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	Brown KS 5156 in DMSO solution
Batch no	:	KS 5156 (purity not stated in this study)
Concentrations	:	Test # 1 : 4, 20, 100, 500, 2500 µg/plate Test # 2 : 8, 40, 200, 1000, 5000 µg/plate
GLP	:	in compliance

Basic Brown 17 has been investigated for gene mutation in several *S. typhimurium* tester strains using the direct plate incorporation method.

Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guideline. The concentration range 4 - 5000 µg/plate was selected on the basis of a preliminary toxicity indicating moderate cytotoxicity at 2500 - 5000 µg/plate.

Results

A dose related and biologically relevant increase in revertant numbers was observed, in the *S. typhimurium* TA 100, TA 1537, TA 1538 and TA98 tester strains in the presence or absence of S9 mix and in the 2 independent experiments conducted.

The test is acceptable for evaluation.

Based on the reversion rate, and under the conditions of the assays performed, it is concluded that the test agent Brown KS 5156 is positive in *S. typhimurium* in the bacterial reverse mutation test.

Ref.: 10

***In vitro* Mammalian Cell Gene Mutation Test**

Guideline	:	/
Cells	:	Chinese Hamster V79 cells (HPRT locus)
Replicates	:	2 independent tests
Test substance	:	Brown KS 5156 in DMSO solution
Batch no	:	KS 5156 (purity: dye content 68 % as chloride)
Concentrations	:	Test # 1 : 20, 30, 100, 250, 500 µg/ml Test # 2 : 100, 200, 300, 1000, 2000 µg/ml
Exposure time	:	24 hours without activation : 2 hours with activation.
GLP	:	in compliance

Basic Brown 17 has been investigated for gene mutation at the HPRT locus in Chinese Hamster V79 cells. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

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Exponentially growing suspension cultures of in Chinese Hamster V79 cells were treated with the test agent for 2 hours in the culture medium in the presence of S9 mix and for 24 hours in the absence of S9 mix.

The concentration range 20 - 2000 µg/ml was selected on the basis of a preliminary toxicity study in the presence of S9 mix, a precipitate occurred at any concentration tested.. Negative and positive controls were in accordance with the OECD guideline.

Results

A precipitate occurred in the presence of S9 mix. pH measurement of post-treatment medium was not performed.

Cytotoxicity

In the range finding experiment

- without S9 mix: the toxicity as evidenced by 73 % reduction in the cloning efficiency (CE) was noted as compared to the controls.
- with S9 mix: the toxicity as evidenced by 63 % reduction in the cloning efficiency (CE) was noted as compared to the controls.

Mutant frequency

- without S9 mix: some sporadic and mainly not statistically significant increases in mutant frequency were observed over the concurrent solvent controls in both tests.
- with S9 mix: some sporadic but not statistically significant increases in mutant frequency were observed over the concurrent solvent controls in both tests.

From the results generated in 2 experiments it may be concluded that Basic Brown 17 induced some increased mutant frequencies. Moreover, the precipitates that were observed in the presence of S9 mix (at acceptable levels of toxicity) lead to an error in the assessment of the doses. Therefore, it cannot be excluded that the absence of significant increases in mutant frequencies may result from exposure to lower doses than expected. Therefore, the assay is considered unsuitable for genotoxicity evaluation.

Ref.: 7

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vitro*

Guideline	:	OECD 482
Species/strain	:	Wistar rat hepatocytes
Test substance	:	Basic Brown 17 dissolved in 0.9% NaCl
Batch no	:	KS 5156 (purity dye content: 68 % as chloride).
Dose levels	:	Exp # 1 25, 100, 333.33, 10000, 2500 µg/ml Exp # 2 3.33, 10.0, 33.33, 1000, 333.33 µg/ml Exp # 3 0.03, 0.10, 0.33, 1.0, 3.33 µg/ml.
Exposure time	:	3 hours in the presence of ³ H-thymidine.
GLP	:	in compliance

Basic Brown 17 batch No KS 5156 was tested for induction of UDS in freshly isolated hepatocytes in three independent experiments. Hepatocytes were incubated with the test material and ³H-thymidine for three hours, then nuclear DNA was isolated and the incorporation of ³H-thymidine was determined by liquid scintillation counting. The solvent, DMSO, was the negative control. 2-Acetylaminofluorene served as positive control.

Results

- Exp #1: a reduction in the incorporation of radioactivity, occurred at every concentrations (negative ctrl 178 ± 35.4 dpm/ μ g DNA; top dose of 2500 μ g/ml: 29.0 ± 22.7 dpm/ μ g DNA).
- Exp #2: a reduction in the incorporation of radioactivity, occurred at every concentrations (negative ctrl 266 ± 36.4 dpm/ μ g DNA; top dose of 333.33 μ g/ml: 59.7 ± 46.5 dpm/ μ g DNA).
- Exp #3: no substantial reduction in the incorporation of radioactivity, occurred for any concentrations (negative ctrl 143.4 ± 38.3 dpm/ μ g DNA; top dose of 3.33 μ g/ml: 106.0 ± 19.9 dpm/ μ g DNA).

From the results the authors concluded that Basic Brown 17 did not induce a significant increase in DNA repair in freshly isolated rat hepatocytes.

The study is unsuitable for genotoxicity evaluation for the following reasons:

- no explanation is given regarding the decrease in incorporation of ^3H -thymidine seen at every concentrations in 2 tests.
- only one exposure period has been evaluated (2 h) and no indication about the potential effects due to a long term exposure (14 – 16 h) is presented.
- although not specifically disallowed by the OECD guideline, the use of liquid scintillation counting is considered inferior to autoradiographic scoring of UDS, because of potential interference from cells undergoing replicative DNA synthesis.

Ref.: 13

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474
Species/strain	:	CFW 1 mice
Group size	:	5 males + 5 females
Test substance	:	Basic Brown 17 dissolved in 0.9% NaCl
Batch no	:	KS 5156 (not stated in this report but same batch was used in the <i>Mammalian Cell Gene Mutation Test</i> , purity: 68 % as chloride)
Dose levels	:	0 and 5000 mg/kg bw by a single gavage.
Sacrifice times	:	24, 48 and 72 hours after dosing
GLP	:	in compliance

Basic Brown 17 has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined by a preliminary range finding study in which no observable toxic effects were seen at 5000 mg/kg bw/day in either sex. All animals had coloured urine within 2 hours post treatment. The substance was administered by a single intragastric gavage and groups of animals sacrificed 24, 48 and 72 hours after the administration for harvest of bone marrow cells. Negative and positive controls were in accordance with the OECD guideline.

Results

Maximum Tolerated Dose (MTD)

The top dose of Basic Brown 17 batch No KS 5156 was chosen on the basis of the lack of clinical signs. 5000 mg/kg

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Test doses

Basic Brown 17 batch No KS 5156 was administrated by 1 single oral dose of 5000 mg/kg.

1 sacrifice times was chosen: 24 h after the last dosing.

Bone marrow smears were obtained from the positive control group only 24 hours after intraperitoneal injection.

Number of cells scored

A total of at least 1000 erythrocytes were examined from each animal; the incidence of micronucleated erythrocytes and the ratio of polychromatic erythrocytes to normo-chromatic erythrocytes were calculated.

Results

Reactions to treatment

No signs of clinical toxicity were observed, but all animals had coloured urine within 2 hours post treatment.

Mean values of micronucleated PCE

No statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values were observed

Females : negative ctrl PCE 1.6 per 1000 / 24 h: 1.8 per 1000 / 48 h: 1.2 per 1000, 72 h: 2.2 per 1000. (+ ctrl: 9.2 per 1000)

Males : negative ctrl PCE 3.0 per 1000 / 24 h: 2.0 per 1000 / 48 h: 2.2 per 1000, 72 h: 2.2 per 1000. (+ ctrl: 10.6 per 1000).

PCE/NCE ratio

No statistically significant reduction in the PCE/NCE ratio was observed in the dosage group of mice treated with Basic Brown 17 batch No KS 5156. However, a slight reduction ratio was observed amongst time both in male or female mice.

PCE/NCE ratio Females : negative ctrl 1.08 - 24 h only
24 h: 1.34 / 48 h: 1.19 / 72 h: 1.09.
positive ctrl: 1.16 - 24 h only.

PCE/NCE ratio Males : negative ctrl: 1.10 - 24 h only
24 h :1.23 / 48 h :1.03 / 72 h : 0.93.
positive ctrl : 1.18 - 24 h only.

Under the conditions of the test it can be concluded that Basic Brown 17 batch No KS 5156 at doses at which no signs of clinical toxicity were recorded, but with slightly changed PCE/NCE ratio were observed, does not induce statistically significant increase in the frequency of PCE.

The negative and positive controls gave the expected results. Therefore, Basic Brown 17 is not clastogenic and/or aneugenic in this mouse bone marrow micronucleus test.

Ref.: 12

Remark

The purity of the hair dye Basic Brown 17 batch No KS 5156 is estimated by dye content and is equal to 68 % as chloride.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

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3.3.8.1. Two generation reproduction toxicity

See point 3.3.8.2.

3.3.8.2. Teratogenicity

Guideline	:	/
Species	:	Sprague Dawley rat, CD strain
Route	:	oral
Group sizes	:	30 females, prior to mating
Substance	:	Basic Brown 17 in aqueous solution
Batch no	:	KS 5156
Dose levels	:	0 and 50 mg/kg bw/day in a volume of 10 ml/kg
Administration	:	days 6-15 of gestation
GLP	:	in compliance

Pregnant rats of Sprague-Dawley CD strain were used. There were 26 rats in the control group and 24 in the treatment group.

The material was administered daily to the pregnant rats by gavage from the 6th to the 15th day post coitum at a dose level of 50 mg/kg. Control animals were dosed with the vehicle alone (distilled water). On day 20 post coitum, the dams were sacrificed and Caesarean sections were performed. The number of implantation sites, resorptions, living foetuses and the number of corpora lutea were counted in each litter. The weight of placenta, uterus, foetuses, dams and the sex of the foetuses were recorded. About one third of each litter was prepared and examined for soft tissue anomalies. The remaining foetuses were examined for skeletal abnormalities after staining with alizarin red S. The body weight gains were determined for each dam.

Results

Dams: The applied dose of 50 mg/kg of Basic Brown 17 was tolerated by the dams. No mortalities were reported. No differences in the mean body weight gain were seen during the course of gestation in any group.

Foetuses: There were no treatment related effects concerning reproduction data and malformations of the foetuses. The level of skeletal variation or ossification in the test and control group was regarded as similar. Thus, the test material was considered to produce no embryotoxic or teratogenic effects under the employed test conditions at a dose of 50 mg/kg body weight.

Ref.: 8

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

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3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)
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CALCULATION OF THE MARGIN OF SAFETY

Not applicable

3.3.14. Discussion

Basic Brown 17 was found to possess a very low acute oral toxicity in mice and rats. After repeated oral administration of Basic Brown 17 by gavage (50, 150 and 450 mg/kg and day) in a 90-day test to rats, the NOAEL can be established as 150 mg/kg bw/day, the NOEL as 50 mg/kg bw/day.

The eye irritation potential has not been examined at the in-use concentration, although there was no marked effect in the rabbit eye at the tested concentration of 0.5%.

In a skin irritation test in rabbits, undiluted Basic Brown 17 provoked no dermal irritation after a contact time of 24 hours under occlusive conditions.

Too low an intradermal induction concentration was used in the guinea pig dermal sensitisation test. The study is considered to be inadequate.

Basic Brown 17 was regarded as a non-sensitiser in an Local Lymph Node Assay.

A cutaneous absorption study carried out with human volunteers and by repeated stripping with adhesive tapes after application to the skin of the forearm showed no diffusion into the skin. However, the test seems not to be adequate for estimating low levels of skin penetration.

A new study has been performed in compliance to GLP, using pig dermatomed skin *in vitro*. The total amount absorbed calculated from the epidermis, the dermis and the receptor fluid contents was 0.106 ± 0.106 % of the applied dose for the aqueous-alcoholic solution, and 0.066 ± 0.024 % of the applied dose for the standard formulation at the end of 48 hours of diffusion after a contact with the skin of 30 minutes.

Basic Brown 17 (batch No KS 5156) was tested in procaryotic cells for gene mutation in several tester strains of *S. typhimurium*. The test agent is positive in the *S. typhimurium* tester strains in the presence or absence of S9 mix and in the 2 independent experiments conducted.

Basic Brown 17 (batch No KS 5156) is at least equivocal in the *in vitro* Mammalian Cell Gene Mutation Test. Moreover, the precipitates that were observed in the presence of S9 mix may have induced important bias in the final dose estimated. Therefore, the assay is considered unsuitable for genotoxicity evaluation.

The *In vitro* mammalian chromosomal aberration test is missing

The Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vitro* is considered unsuitable for evaluation (lack of second sampling time, unexplained and unknown decrease in incorporation of ³H-thymidine seen at every concentration in 2 tests, liquid scintillation counting instead of autoradiographic scoring of UDS).

The mammalian Erythrocyte Micronucleus Test is acceptable and gave negative results.

Therefore, on the basis of the data evaluated, the substance can be considered non-clastogenic *in vivo* and mutagenic *in vitro*.

4. CONCLUSION

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

Before any further consideration, the following information is required:

- * complete physico-chemical characterisation of the test substances used, including data on stability.
- * data on the genotoxicity/mutagenicity following the relevant SCCNFP-opinions and in accordance with the Notes of Guidance.

5. MINORITY OPINION

Not applicable

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7. ACKNOWLEDGEMENTS

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