EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

Basic Violet 2

COLIPA n° B115

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 12th plenary meeting

of 20 September 2011

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.

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

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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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.

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1. BACKGROUND

Submission I for Basic Violet 2 with the chemical name 4-((4-Amino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl)-2-methylphenylamine monohydro-chloride was submitted in September 2003 by COLIPA [1] according to COLIPA.

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers adopted 23 April 2004 an opinion (SCCNFP/0784/04) with the conclusion that:

"The SCCNFP 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;*
* *sub-chronic toxicity study providing a NOAEL;*
* *percutaneous absorption study in accordance with the SCCNFP Notes of Guidance;*
* *data on the genotoxicity/mutagenicity following the relevant SCCNFP-opinions and in accordance with the Notes of Guidance."*

Basic Violet 2 is identical with CI 42520 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 Basic Violet 2 was submitted by COLIPA in July 2005. According to this submission Basic Violet 2 is proposed to be used as a non-reactive hair colouring agents ("direct" dye) in oxidative and semi-permanent hair dye formulations at a maximum concentration of 0.25 and 0.5% in the finished cosmetic product, respectively.

An additional submission III was submitted in May 2008 applying for use of a higher concentration of this substance in oxidative hair dyes. The concentration applied for increase from 0.25% up to 1.0% on the scalp.

2. TERMS OF REFERENCE

1. *Does SCCS consider Basic Violet 2 safe for use as an oxidative hair dye with a maximum concentration up to 1.0% on the scalp taken into account the scientific data provided?*

2. *Does SCCS consider Basic Violet 2 safe for use as a non-oxidative hair dye with a maximum concentration of 0.5% in the finished cosmetic product taken into account the scientific data provided?*

3. *And/or does the SCCS have any further scientific concerns with regard to the use of Basic Violet 2 in hair dye formulations?*

[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

Basic Violet 2 (INCI name)

3.1.1.2. Chemical names

4-[(4-amino-m-tolyl)(4-imino-3-methylcyclohexa-2,5-dien-1-ylidene)methyl]-o-toluidine monohydrochloride
4-((4-Amino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl)-2-methylphenylamine-monohydrochloride
Benzenamine, 4,4'-[(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methylene]bis[2-methyl-, monohydrochloride
Benzenamine, (4-amino-3-methylphenyl)(4-imino-3-methyl-2,5-cyclohexadien-1-ylidene)methyl]-2-methyl-, monohydrochloride

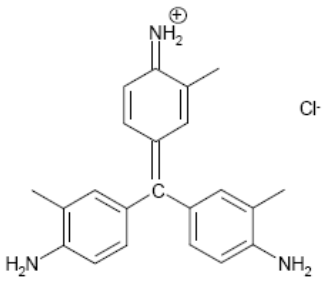
3.1.1.3 Trade names and abbreviations

Lowacryl Violet 2 (Lowenstein)
New Magenta
CI 42520
COLIPA B115

3.1.1.4 CAS / EC number

CAS: 3248-91-7
EC: 221-831-7

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_{22}H_{23}N_3$. HCl

3.1.2 Physical form

Green powder

3.1.3 Molecular weight

Molecular weight: 365.91 g/mol

3.1.4 Purity, composition and substance codes

Chemical identity of batch 063-01/42-14 of the test substance with the code A 000945 has been tested using NMR and HPLC/MS/UV/VIS.
1H-NMR proved the identity of the test substance. The content of the test substance was 85.7% w/w.
HPLC/ MS/ UV/VIS (254, 550nm) confirmed the identity of the test substance.

Ref.: 1

Batch 063-01/41-06 had been tested in the same way as described above. The content of the test substance was 83.7%.

Ref.: 2

For purity testing HPLC/MS/UV_VIS (DAD) has been used. Moreover water content (Karl Fischer); loss on drying (50°C; P2O5) and sulphated ash (NH4SO4; H2SO4) have been determined.
Aniline, o- and p-toluidine were used for calibration and for the quantification of these possible impurities (254 nm).
For the quantification of the homologues the sum of all HPLC peaks were set as 100% (550nm).

Ref.: 1/2

3.1.5 Impurities / accompanying contaminants

Batch063-01/42-14

4-(Bis(4-amino-3-methylphenyl)methylene)-l-imino-2,5-cyclohexadiene-monohydrochloride (Carbol Fuchsin)	5.6% (550nm)
4-(Bis(4-aminophenyl)methylene)-l~imino»2-methyl-2,5-cyclohexadiene~monohydrochloride (Basic Violet 14)	0.1% (550nm)
l-(Bis(4'-aminophenyl)methylene)-4-imino-2,5~cyclohexadiene~monohydrochloride (Basic Fuchsin)	< 100 ppm
o-Toluidine[2]	0.001% (average)
p-Toluidine	<0.0015%
Aniline	<0.001%
Sulphated ash	0.33%
Loss on drying	6.5%
Water content	7.0%
Methyl acetate(for purification)	2.4%

Ref.: 1(2000)

Batch 063-01/41-06

4-(Bis(4-amino-3-methylphenyl)methylene)-l-imino-2,5-cyclohexadiene-monohydrochloride (Carbol Fuchsin)	5.6% (550nm)

[2] o-Toluidine is a carcinogen cat 1B. This low concentration would be of no concern in a hair dye formulation.

4-(Bis(4-aminophenyl)methylene)-l~imino»2-methyl-2,5-yclohexadiene~monohydrochloride (Basic Violet 14)	0.2% (550nm)
l-(Bis(4'-aminophenyl)methylene)-4-imino-2,5~cyclohexadiene~monohydrochloride (Basic Fuchsin)	< 100 ppm
o-Toluidine	0.0039% (average)
p-Toluidine	<0.0015%
Sulphated ash	0.33%
Loss on drying	6.2%
Water content	8.5%
Methyl acetate(for purification)	1.5%

Ref.: 2 (1999)

Taken from summary submission III 2008

Heavy metal content:

Arsenic	<5ppm
Antimony	<5ppm
Lead	<20ppm
Cadmium	<10ppm
Mercury	<5ppm

Comment
The concentrations given for the homologues of Basic Violet 2 are relative ones, resulting from a comparison of peak areas in the HPLC chromatogram.

Both batches according to the applicants were purified by GST (according to later information by the applicant, the purified batches (063-01/41-06, 063-01/41-14) were prepared by crystallization with a solvent mixture) before testing physico-chemical properties.

In the submitted data there was no information, if the batches of B115 used for toxicity testing and for commercial products are purified in the same way before use. Corresponding questions of SCCS were answered by the applicants (see discussion).

3.1.6 Solubility

Taken from summary submission II 2005:

Soluble	in water	2.2 weight % (pH 5.8)
	inDMSO	5 weight %
	in Methanol	8 weight %
	in Propylene glycol	5.5 weight %

The solubility in water was determined according to EC guideline 92/69/EC A.6 (ref 5)

3.1.7 Partition coefficient (Log P_{ow})

Log $P_{o/w}$: 0.03 at pH 8.0 (buffered solution; 21 °C)

Partition coefficient was determined according to EC directive 92/69/EC A.8

Ref.: 3

3.1.8 Additional physicochemical specifications

Melting point:	280 °C (decomposition)
Boiling point:	/
Rel. self ignition:	> 400 °C
Vapour pressure:	< 1.0 x 10^{-7} hPa (20 °C)
Density:	1.147 g/ml (20 °C)
Surface tension (water):	59.5 mN/m (20 °C)
Flammability:	not highly flammable
pH:	4.51 (saturated aqueous solution at 20 °C)
oxidising properties:	not expected to be oxidising
Refractive index:	/

3.1.9. Stability

A solution of B115 in water (2%) has been tested for stability using UV spectroscopy. Up to seven days at room temperature in the absence of light the solutions showed recoveries of 98.5% to 101.5%

Ref.: 2

In the receptor fluids of skin absorption experiments B115 decomposed under the influence of light. Within 7 day the concentration decreased by 53%. This, however, was considered in the calculation of absorption values.

Ref.: 23

The applicants report that B115 was stable for 6 months in a common market formulation of the dye stuff (0.5%) in a common packaging at 40 °C. The concentrations measured after 3 and 6 months were 0.54 and 0.44% respectively. HPLC/DAD was used for the determinations.

Basic Violet 2 was also tested with respect to its H_2O_2 stability (under acidic conditions). Using in a 1:1 mixture of the cream formulation and welloxon 6%, the recoveries were 100.0% at t=0; 100.7% at t=30 min; and 98.9% after t=60 min.

Ref.: 13

Answering a corresponding question of SCCS, the applicants stated, that the application of B115 under oxidative conditions takes place under neutral to acidic conditions (pH 6.8 – 2.6).

General Comments on Physico-chemical characterisation

- No reference substances were used for the quantification of homologues of Basic Violet 2. The concentrations given are only relative semiquantative ones.
- o-Toluidine, which is present as an impurity, is a human carcinogen which penetrates skin (skin notation DFG). According to the two batches of the test substance which have been used for testing the physico–chemical properties, the formulations applied to the scalp would contain only minimal o-toluidine concentrations of up to 0.000042%.
- The batches used for identity- and purity- testing have been purified before analytical quantification.
- According to the applicant, Basic Violet 2 is a non reactive dye.

3.2. Function and uses

Basic Violet 2 is a non-reactive hair colouring agent. It is used up to 0.5% in non-oxidative hair dye-formulation.

Basic Violet 2 is used up to an on-head concentration of 1.0% in oxidative hair dye formulations.

It is also used as a colorant (CI 42520) in cosmetic products (intended to come into contact only briefly with the skin (5 ppm maximum concentration in the finished product)[3]).

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCNFP/0784/04

Guideline:	OECD 401 (1987) - limit test
Species/strain:	Rat, Sprague Dawley Hsd: SD strain
Group size:	5 males + 5 females
Batch:	GST 063-01/41-06
Dose:	2000 mg/kg bw–(limit test)
Vehicle:	0.5% CMC aqueous solution
GLP:	in compliance
Study date:	2002

Results
Mortalities: 3 males died within 5 days of dosing.
Clinical signs: Piloerection, breathing difficulties, staining of fur, urine and faeces.

Guideline:	OECD 401 (1987)
Species/strain:	Rat, Sprague Dawley Hsd: SD strain
Group size:	5 males + 5 females per group
Batch:	GST 063-01/41-06
Dose:	500, 1000 and 2000 mg/kg bw
Vehicle:	0.5% CMC aqueous solution
GLP:	in compliance

Results
Mortalities: At 2000 mg/kg bw, 1 male and 1 female died within 48 hours of dosing. No mortalities at lower dose levels.
Clinical signs at 2000 mg/kg bw: piloerection, reduced activity, hunched posture, breathing difficulties, staining of fur, urine and faeces, swollen abdomen.

Conclusion
The oral LD50 in rats exceeded 2000 mg/kg bw.

Ref.: 15

[3] Council Directive 76/768/EEC of 27 July 1976 as amended, Annex IV, entry 67

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 SCCNFP/0784/04

Guideline:	OECD 404 (1992)
Species/strain:	New Zealand albino white rabbit
Group size:	3 females
Observ. Period:	72 hours
Test substance:	Basic Violet 2, 500 mg, mixed with 1 ml of water
Batch:	GST 063-01/41-06
Purity:	94%
Dose level:	0.5 g (4h contact under semi-occlusion)
GLP:	in compliance
Study date:	2003

A gauze square with 0.5 g test material mixed to a paste with sterile water was placed on the shaved skin of three female rabbits and covered with a semi-occlusive dressing for 4 hours. After the 4-hour application time, the area was wiped with cotton wool soaked with water. The animals were checked daily for mortality and systemic symptoms. Skin reactions were evaluated 1, 24, 48, and 72 hours after removing the patches according to the Draize scoring system.

Results
No mortality and no systemic symptoms were recorded in any of the test animals. Erythema could not be assessed due to intense staining of the skin. No oedema was noted in any of the test animals. Measurement of skin-fold thickness after 72 hours revealed no differences between treated skin and untreated (naive) skin. Erythema assessments after a longer period (when discolouration of the skin had disappeared) have not been performed.

Conclusion
The mean irritation scores 24 to 72 hours after application were below the thresholds defined in Commission Directive 2001/59/EC for classification of the test compound as irritating to the skin.

Ref.: 17

3.3.2.2. Mucous membrane irritation

Taken from SCCNFP/0784/04

<u>Undiluted test compound</u>

Guideline:	OECD 405 (1987)
Species/strain:	New Zealand albino white rabbit
Group size:	1 female
Observ. Period:	24 hours
Test substance:	Basic Violet 2

Batch: GST 063-01/41-06
Purity: 94%
Dose level: 100 mg
GLP: in compliance
Study date; 2003

A 100 mg aliquot of the test substance was placed into the conjunctival sac of the right eye of the animal. Lids were then held together for a few seconds. The untreated left eye served as control. Ocular reactions were evaluated approximately 1 and 24 hours after instillation of the test article.

Results
Intense staining of the eye and adnexa occurred. Chemosis score 2 of the conjunctiva was reported 1 hour after dosing, increasing to score 4 at 24h. Discolouration of the nictitating membrane, possibly indicating corrosive actions of the test substance, was also noted at the 24h observation time. Marked to severe corneal opacity was apparent after 24 hours. The study was terminated after 24h. No indication of a systemic effect could be detected.

Conclusion
Based on these results, the undiluted test compound was irritant to eyes.

Ref.: 18

Diluted test compound

Guideline: OECD 405 (1987)
Species/strain: New Zealand albino white rabbit
Group size: 3 females
Observ. Period: 14 days
Test substance: Basic Violet 2, 1% aqueous solution
Batch: GST 063-01/41-06
Purity: 94%
Dose level: 100 mg
GLP: in compliance
Study date: 2002

0.1 ml of a 1% solution of the test article in distilled water was placed into the conjunctival sac of the right eye of each animal. Lids were then held together for a few seconds. The untreated left eye served as control. The animals were checked daily for mortality and signs of systemic toxicity. Ocular reactions were evaluated 1, 24, 48, and 72 hours after instillation of the test article. Further examinations were performed 7 and 14 days after instillation.

Results
There were no mortality and no clinical signs. Slight conjunctival irritation (discharge and chemosis) was seen in all three animals (all scores = 1.0), while conjunctival redness could not be evaluated 1 h post treatment due to a diffuse colour produced by the test substance. Conjunctival irritation was still present on day 7, but the animals were completely recovered on day 14. Iris and cornea were not affected.

Conclusion
Since the mean irritation scores 24 to 72 hours after application were below the thresholds defined in Commission Directive 2001/59/EC, a 1% solution of the test compound is considered not to be irritating to the eye.

Ref.: 19

3.3.3. Skin sensitisation

Taken from SCCNFP/0784/04

Magnusson & Kligman Maximisation test

Guideline:	OECD 406 (1992)	
Species/strain:	Dunkin-Hartley guinea pig	
Group size:	20 females in test group, 10 females in control group	
Observ. Period:	25 days	
Test substance:	Basic Violet 2	
Batch:	GST 063-01/41-06	
Purity:	94%	
Dose levels:	intradermal injection:	1% solution,
	dermal induction:	50% solution,
	dermal challenge:	50% solution
	(preliminary screening study available)	
GLP:	in compliance	
Study date:	2003	

The test group consisted of 20 female Guinea pigs, two control groups of ten female Guinea pigs each. In the first week of induction, the test group was treated with single intradermal injections of complete Freund's adjuvant/water mixture 1:1 (v/v), 1% of the test substance in sterile water and with 1% of the test substance emulsified in Freund's complete adjuvant. The negative control groups were treated with the adjuvant and the vehicle (sterile water) in the same manner. Seven days after injection, a 50% solution of the test substance in sterile water was dermally applied under occlusive dressing for 48 h to the area of the intradermal injections. The negative control group was treated with the vehicle alone. After a period of 2 weeks without treatment, sensitisation reactions were challenged in the test group as well as in one negative control group by dermal administration of the test substance in sterile water (50%, on one flank and vehicle alone on the contralateral flank) under occlusive dressing for 24 hours. 24 and 48 hours after removal of the patches the skin reactions were scored. Following the 48 hour examination at challenge, skin fold thickness of the treated sites was measured using a digital micrometer. Body weights were recorded on days 1 and 25 (termination of the study). Body weights were not affected by the test compound.

Results
As marked coloration prevented formal assessment of erythema at any time point during the main study, assessment of skin fold thickness was performed. With the exception of one animal, values of the vehicle treated sites and test substance treated sites were in the same range in animals of the test group compared to animals of the control group.

Ref.: 20

Comment
The test is inconclusive due to staining of the skin.

Buehler test

Guideline:	OECD 406 (1992). No justification is given for the performance of the Buehler test.
Species/strain:	Dunkin-Hartley guinea pig
Group size:	20 females in test group, 10 females in control groups
Observ. Period:	25 days
Test substance:	Basic Violet 2
Batch:	GST 063-01/41-06

Purity:	94%
Dose levels:	dermal inductions: 75% solution, dermal challenge: 50% solution (preliminary screening study available)
GLP:	in compliance
Study date:	2003

The test group consisted of 20 female Guinea pigs, two control groups of ten female Guinea pigs each. During the induction phase, the test group was treated with the test substance in sterile water at 75% at the left flank. The negative control groups were treated with the vehicle (sterile water) in the same manner. The gauze patches with test substance or vehicle under occlusive dressing were removed after 6 hours. Approximately 24 hours after removal of the patches, skin reactions were scored. These procedures were repeated at weekly intervals (days 8/9 and 15/16 of the study).
On study day 29, sensitisation reactions were challenged in the test as well as in one negative control group by topical administration of the test substance in sterile water (50% on one side and vehicle alone on the contralateral flank) under occlusive dressing for 6 hours.
Twenty-four and 48 hours after removal of the patches the skin reactions were scored. Following the 48 hour examination at challenge, skin fold thickness of the treated sites was measured using a digital micrometer.
Body weights were recorded on days 1 and 31 (termination of the study). Body weights were not affected by the test compound.

Results
As intense staining of the skin prevented formal assessment of erythema at any time point during the main study, assessment of skin fold thickness was performed. No differences between values of the test group compared to values of the control group were apparent. Slightly thicker skin at vehicle treated sites compared to test substance treated sites in both treated and control animals was regarded to be due to the different location (vehicle sites being anterior to test substance site).
Two reliability checks with mercaptobenzothiazole are stated at the end of the test: one generated 30% response in the test group and 0% response in the control group and the second check revealed 70% response in the test group and 70% response in the control group. Both were found acceptable by the performing laboratory.

Conclusion
The performing laboratory concludes that the test compound is not sensitising.

Ref.: 21

Comment
Due to intense staining of the skin, both experiments are inconclusive.

3.3.4. Dermal / percutaneous absorption

Non-oxidative conditions

Guideline:	OECD 428
Tissue:	pig skin
Group size:	1 donor, 5 chambers
Skin integrity:	penetration tritiated water
Diffusion cell:	flow through Teflon-chambers
Test substance:	Basic violet 2
Batch:	GST063-01/41-06
Purity:	HPLC: 94.2 area% (m/z 330) at 550 nm HPLC: 5.6 area% (m/z 316) at 550 nm HPLC: 0.2 area% (m/z 302) at 550 nm

Radiochemical:	tritiated water
Test item:	Color cream formulation for direct hair dyeing (0.5% Basic violet 2)
Dose volume:	100 mg/cm²
Receptor fluid:	phosphate buffer
Solubility receptor fluid:	0.116 mg/ml (pH 7.3)
Stability receptor fluid:	significant decomposition of 53% after 7 days
Method of Analysis:	HPLC
GLP:	in compliance
Study date:	30 May – 6 June 2005

The cutaneous absorption of Basic Violet 2 in a typical hair dye formulation for direct hair dyeing was investigated in vitro using pig skin preparation. 400 mg of a hair dye formulation containing 0.5% Basic Violet 2 were applied to 4 cm^2 pig skin. The formulation was removed after 60 minutes

Results
All receptor fluid samples were below the limit of detection (1.5 ng per injection). The applicant considered for the worst case assumption a maximal amount of 0.01 ± 0.00 µg/cm^2 of Basic Violet 2 as biologically available.

Ref.: 23

Comment
The amount applied was very high and only 5 chambers from one donor were used. There was a significant decomposition of the test substance in the receptor fluid which was corrected by the recovery rate.
The study is inadequate.

Submission 2011

Non-oxidative conditions

Guideline:	OECD 428
Tissue:	human skin (abdomen or breast; thickness 380-400 µm)
Group size:	10 chambers (from 5 donors)
Skin integrity:	penetration tritiated water
Diffusion cell:	Automated PTFE flow-through chambers (0.64 cm²)
Test substance:	Basic violet 2
Batch:	GST063-01/41-06
Purity:	HPLC: 93.8 area% at 250 nm
Radiochemical:	tritiated water
Test item:	Hair dye formulation under non-oxidative conditions (0.5% Basic violet 2)
Dose volume:	20 mg/cm²
Receptor fluid:	phosphate buffered saline (containing sodium azide 0.01%, ethanol 5%)
Solubility receptor fluid:	22.2 mg/ml in water
Stability receptor fluid:	/
Method of Analysis:	LC-MS/MS
GLP:	in compliance
Study date:	22-25 October 2010

The percutaneous absorption of Basic Violet 2 in representative commercial hair dye formulation with an on-head concentration of 0.5% was performed under non-oxidative conditions using human skin in vitro. The receptor fluid was pumped through the receptor chamber at a rat of 1.5 ml/h. Thirty minutes after application, the test item was removed by

washing the skin with water. Receptor fluid was collected in 30 min fractions from 0 to 1 h post dose and hourly fractions from 1 to 6 h post dose and then in 2 hourly fractions from 6 to 72 h post dose.

Results

	Cell Number and Donor Number													
	Cell 1 0264	*Cell 3 0294*	Cell 4 0286	Cell 5 0317	*Cell 6 0264*	Cell 7 0267	Cell 8 0264	Cell 11 0286	Cell 12 0317	Cell 13 0294	Cell 14 0286	Cell 16 0267	Mean	SD
Skin Wash 30 min	74.57	*67.02*	71.77	84.54	*67.95*	78.00	75.82	80.13	70.95	77.61	74.61	82.56	77.06	4.42
Tissue Swab 30 min	3.63	*0.32*	0.64	0.27	*0.46*	0.08	0.26	0.06	0.44	0.48	0.08	0.12	0.61	1.08
Pipette Tips 30 min	0.40	*0.16*	0.21	0.18	*0.25*	0.12	0.30	0.22	0.31	0.31	0.22	0.31	0.26	0.08
Dislodgeable Dose 30 min	78.59	*67.50*	72.62	84.99	*68.67*	78.20	76.39	80.41	71.70	78.40	74.91	82.99	77.92	4.23
Skin Wash 72 h	0.06	*0.04*	0.04	0.03	*0.06*	0.04	0.03	0.03	0.04	0.05	0.03	0.04	0.04	0.01
Tissue Swab 72 h	*0.01	**0.01*	°0.00	0.05	*°0.00*	*0.01	°0.00	*0.00	*0.01	*0.03	°0.00	*0.01	0.01	0.01
Pipette Tips 72 h	*0.00	**0.00*	°0.00	°0.00	*°0.00*	*0.00	°0.00	°0.00	*0.00	*0.01	°0.00	*0.00	0.00	0.00
Cell Wash	*0.05	**0.06*	*0.01	*0.02	**0.04*	*0.01	0.08	*0.01	*0.01	0.09	*0.01	°0.01	0.03	0.03
Dislodgeable dose 72 h	0.13	*0.11*	0.06	0.10	*0.10*	0.07	0.12	0.05	0.06	0.18	0.05	0.06	0.09	0.04
Total Dislodgeable Dose	78.72	*67.61*	72.68	85.09	*68.76*	78.27	76.50	80.46	71.76	78.58	74.96	83.05	78.01	4.24
Stratum corneum 1-5	0.16	*0.12*	0.13	*0.02	*0.10*	0.07	0.13	0.08	*0.02	0.07	0.07	0.07	0.08	0.05
Stratum corneum 6-10	0.11	*0.05*	*0.02	*0.01	**0.01*	0.04	*0.02	*0.02	*0.01	0.07	*0.03	0.06	0.04	0.03
Stratum corneum 11-15	0.04	**0.01*	°0.00	°0.00	**0.01*	*0.03	*0.00	*0.00	*0.01	0.03	°0.00	*0.03	0.02	0.02
Stratum corneum 16-20	*0.01	**0.01*	°0.00	*0.01	*°0.00*	*0.02	°0.00	°0.00	°0.00	*0.01	°0.00	*0.01	0.01	0.01
Stratum Corneum	0.33	*0.18*	0.15	0.05	*0.12*	0.16	0.16	0.11	0.04	0.19	0.10	0.17	0.15	0.08
Unexposed Skin	°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 Unabsorbed	79.05	*67.79*	72.83	85.14	*68.89*	78.43	76.66	80.57	71.80	78.78	75.06	83.22	78.15	4.24
Epidermis	*0.01	*°0.00*	°0.00	*0.01	*°0.00*	*0.03	°0.00	°0.00	*0.01	*0.02	°0.00	*0.01	0.01	0.01
Dermis	*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
Receptor Fluid	0.02	*0.02*	0.02	0.02	*0.02*	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00
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	0.02	*0.02*	0.02	0.02	*0.02*	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00
Dermal Delivery	0.03	*0.02*	0.02	0.03	*0.02*	0.05	0.02	0.02	0.03	0.04	0.02	0.03	0.03	0.01
Mass Balance	79.08	*67.82*	72.86	85.16	*68.91*	78.48	76.68	80.60	71.82	78.81	75.09	83.25	78.18	4.25

Cell 3 and 6 was rejected from the mean ± SD due to mass balance being outwith 100 ± 15%
° = Value not quantifiable, limit of detection (LOD) value entered. See Section 6.19 for LOD of each matrix.
* = Value less than the lower limit of quantification (LLOQ). Extrapolated value entered. See Section 6.19 for LLOQ of each matrix.

The dermal absorption of Basic violet 2 in non-oxidative conditions was 0.03 ± 0.01 µg/cm².

Ref.: 1 (subm 2011)

Comment
The mean + 1 SD (0.04 µg/cm²) should be used for the calculation of the Margin of Safety.

Oxidative conditions

Guideline:	OECD 428
Tissue:	porcine ear skin, 12 donors
Group size:	6/group (2 groups)
Skin integrity:	conductivity
Diffusion cell:	glass diffusion chamber
Test substance:	Basic violet 2
Batch:	FD019ST
Purity:	90% (HPLC, rel.)
Test item:	Basic violet 2 (3%) in formulation
Dose volume:	20 µl/cm^2
Receptor fluid:	saline (0.9% NaCl)
Solubility receptor fluid:	/
Stability receptor fluid:	/
Method of Analysis:	HPLC
Validation:	limit of detection 2 ng/ml and limit of quantitation 2.5 ng/ml
GLP:	in compliance
Study date:	23 – 27 April 2007

Two independent experiments were performed on fresh dermatomed pig skin samples under static conditions with 6 diffusion cells per experiment. Per diffusion cell, the skin of one animal was used. 20 µL of the mixed test item formulation were applied on each skin sample, left on the skin for 30 minutes and then washed off. The dermal absorption was monitored over 24 hours under non-occluded static conditions.

The test item was mixed with the colorant lotion at the ratio 1 to 2 prior to treatment. The mixture, corresponding to a final concentration of B115 of 1% was applied to the skin

Results

Experiment 1						
	Chamber					
	1	2	3[4]	4	5	6
Amount of Basic Violet 2 (reference material) applied (ng) [1]	153931	152561	154357	155348	147294	150425
Total amount of Basic Violet 2 (reference material) measured (ng)	162549	141004	127785	135411	130266	170896
Recovery (%)	105.6	92.4	82.8	87.2	88.4	113.6
Dermal absorption of Basic Violet 2 (reference material) penetrated (ng/cm^2) [2]	8.88	8.59	21.2	5.93	27.4	35.1
Dermal absorption (%) [3]	0.00577	0.00563	0.0137	0.00382	0.0186	0.0233
Experiment 2						
	Chamber					
	1	2	3	4	5	6
Amount of Basic Violet 2 (reference material) applied (ng) [1]	170450	163131	167035	161367	164184	170972
Total amount of Basic Violet 2 (reference material) measured (ng)	160160	163707	158126	158290	175425	171934
Recovery (%)	94.0	100.4	94.7	98.1	106.8	100.6
Dermal absorption of Basic Violet 2 (reference material) penetrated (ng/cm^2) [2]	6.80	14.2	11.9	26.3	19.9	342
Dermal absorption (%) [3]	0.00399	0.00868	0.00710	0.0163	0.0122	0.200

	Experiment 1					
Chamber	1	2	3[1]	4	5	6
Dermal Absorption (ng/cm^2)	8.88	8.59	21.2	5.93	27.4	35.1
Dermal Absorption (%)	0.00577	0.00563	0.0137	0.00382	0.0186	0.0233
	Experiment 2					
Chamber	1	2	3	4	5	6
Dermal Absorption (ng/cm^2)	6.80	14.2	11.9	26.3	19.9	342
Dermal Absorption (%)	0.00399	0.00868	0.00710	0.0163	0.0122	0.200
Mean dermal absorption experiment I-II ± SD	**0.0278 ± 0.0575 %** **0.0461 ± 0.0986 µg/cm^2**					

Conclusion

The dermal absorption of Basic Violet 2 in oxidative conditions is 0.0461 ± 0.0986 µg/cm^2 or 0.0278 ± 0.0575%.

Ref.: 30

Comment
The mean + 1SD (0.145 µg/cm^2 (0.046 + 0.099)) should be used for the calculation of the Margin of Safety.

The sponsor stated that the study was performed in oxidative conditions by adding 2 parts of a commercially available colour lotion, but the composition of the lotion was not reported.

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

Taken from SCCNFP/0784/04

Guideline:	OECD 408 (1998)
Species/strain:	rat, Sprague Dawley Hsd: SD strain
Group size:	10 males + 10 females per group
Batch:	GST 063-01/41-06
Purity:	94%
Dose levels:	3, 10 and 30 mg/kg bw/day
Vehicle:	Water (10 ml/kg bw/day)
Exposure:	once daily for 13 weeks
Route:	oral, gavage
GLP:	in compliance
Study date:	2002

Results

Mortality:	Two high-dose females died on day 57, probably due to misdosing.
Clinical signs:	Staining of the faeces in all test groups. Occasional hunched posture and dyspnoea in a few high-dose rats
Neurotoxicity:	Motility impairment in a few high-dose rats
Ophthalmoscopy:	No treatment-related findings
Body weight:	Reduced body weight in high-dose females from day 8 of treatment
Food intake:	Reduced food intake in high-dose females in the last week

Haematology, clinical chemistry and organ weights:

Dose (mg/kg bw/d)	3		10		30		dose related
	m	f	m	f	m	f	
Haematology							
haematocrit	dc	-	dc	-	dc	-	yes
red blood cell count	d	d	dc	d	dc	dc	yes
haemoglobin	-	-	-	dc	-	dc	yes
MCH	-	-	ic	-	ic	-	yes
MCV	-	-	-	-	ic	-	
Clinical chemistry							
sodium	i	ic	ic	ic	ic	ic	yes (males)
calcium	ic	ic	ic	ic	ic	ic	yes (males)
potassium	ic	-	ic	-	ic	-	
cholesterol	-	-	-	-	ic	ic	
glucose	-	ic	-	i	-	ic	
ASAT activity	-	-	-	dc	dc	dc	yes
ALP activity	-	-	-	dc	-	dc	yes
Organ weights							
relative liver weight	-	-	-	-	ic	ic	
relative kidney weight	-	-	-	-	ic	ic	
relative heart weight	-	-	ic	-	ic	ic	

dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased, but not statistically significantly compared to the controls

Macroscopy: Dark and firm areas in the liver in three high-dose males
Histopathology: Centrilobular hepatocytic hypertrophy in 5 mid-dose males and in most high-dose males and females. Hepatocytic necrosis in one high-dose male. Nephropathy (tubular basophilia/dilatation and/or chronic inflammation) in 5 mid-dose males and 6 high-dose males.

Conclusion
The changes in haematology and clinical chemistry in the low-dose group were stated to be 'within the range of historical control data', and a NOAEL of 3 mg/kg bw/day was concluded by the authors. However, taking into account the effects observed at higher dose levels and the dose-related responses, the SCCNFP concluded in 2004 that the NOAEL is below 3 mg/kg bw/day (SCCNFP/0784/04).

Ref.: 16

In the updated submission II of 2005, the 90-d study was re-evaluated by the applicant.

- With regard to body weight a statistically significant reduction was observed in females at 30 mg/kg/bw and at 10 mg/kg bw/d also a reduction was noted. In males only a slight reduction was observed at 30 mg/kg/bw. This was accompanied by reduced body weight gains in all these groups. In females at 30 mg/kg/bw also food consumption was reduced.
- Terminal body weights were reduced in females at 30 mg/kg bw (statistically significant difference to controls). Relative organ weights were statistically significantly increased in males and females at 30 m/kg bw/d for liver, kidneys and heart, the latter being additionally elevated (statistically significant) in males at 10 mg/kg bw/d. In addition, at 30 mg/kg bw slight increases were observed in relative testes weights in males and relative brain weights in females (both statistically significant). Despite the results of the statistical analyses of differences to controls, the differences were slight and all mean values remained well within the range of historical control data. The only organ in which a histopathological correlate was observed was the liver (centrilobular hepatocytic hypertrophy in 5 mid-dose males and in most high-dose males and females. Hepatocytic necrosis in one high-dose male).
- Haematological observations were reduction in red blood cells counts in both males and females which were statistically significant at 10 and 30 mg/kg bw/d for males and at 30 mg/kg bw/d for females. Furthermore, haematocrit values were slightly reduced in all

dose groups in females. Further, the values for mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) were slightly increased in males and females of all dose groups. However, all changes were in the range of the historical controls of the test institute.
- In the clinical biochemical investigations some differences were observed with regard to Na, Ca, K, glucose, cholesterol as well as in the levels of alkaline phosphatase and aspartate aminotransferase. It was argued that all values were well within the range of historical controls.

Comment of the SCCS on sub-chronic toxicity
At the doses 30 and/or 10 mg/kg bw/d histopathological findings in liver and kidney indicate organ toxicity which is supported by changes in body and organ weights as well as in biochemical parameters (cholesterol, aspartate aminotransferase, alkaline phosphatase). The NOAEL is considered to be 3 mg/kg bw/d.

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

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium*: TA98, TA100, TA102, TA1535, TA1537
Replicates:	triplicates in two independent experiments
Test substance:	Basic Violet 2
Batch:	GST063-01/41-06
Purity:	HPLC: 94.2%; NMR: 96.8%;
Vehicle:	DMSO
Concentration:	experiment 1: 1, 10, 100, 300, 1000 and 5000 µg/plate with and without S9-mix experiment 2: 3, 10, 30, 100, 300, 1000 and 3000 µg/plate with and without S9-mix
Treatment:	direct plate incorporation with 48 h incubation without and with S9-mix
GLP:	in compliance
Study period:	14 July 2000 – 2 August 2000

Basic Violet 2 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Both experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline

Results
Toxicity observed as growth inhibition, clearing of the background lawn and strong reduction in the number of spontaneous revertants, was found in the first experiment in the absence of S9-mix for TA98 and TA102 (1000 and 5000 µg/plate), TA100, TA1535 and TA1537 (100, 1000 and 5000 µg/plate); in the presence of S9-mix for TA98 (5000 µg/plate), TA100 and TA1535 (100, 1000 and 5000 µg/plate) and TA1537 and TA102 (1000 and 5000 µg/plate). This toxicity was observed also in the second experiment.
An increase in the number of revertants was observed on TA102 strain only in the presence of S9-mix at non-toxic concentrations. However, the effect never reached a factor of 2 compared to the control. This effect was not considered biologically relevant. The study

must be considered inadequate for the evaluation of this substance, because of the substantial toxicity observed which made an adequate evaluation of the mutagenic effect of this substance on *Salmonella typhimurium* impossible.

Conclusion
Under the experimental conditions used Basic Violet 2 was not mutagenic in this gene mutation tests in bacteria.

Ref.: 26

In Vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)	
Species/strain:	mouse lymphoma cell line L5178Y $tk^{+/-}$	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	Basic Violet 2	
Batch:	GST 063-01/41-06	
Purity:	94 % (HPLC)	
Vehicle:	DMSO	
Concentrations:	experiment 1:	0.0625, 0.125, 0.25, 0.5, 0.75, 1 and 1.5 µg/ml without S9-mix 0.156, 0.313, 0.625, 1.25, 2.5 and 2.75 with S9-mix
	experiment 2:	0.025, 0.05, 0.1, 0.2, 0.3 and 0.4 µg/ml without S9-mix 1.5, 2, 2.5, 2.6 and 2.7 µg/ml with S9-mix
Treatment:	experiment 1:	3 h both without and with S9-mix; expression period 7-9 days, selection growth 13-14 days
	experiment 2:	24 h without S9-mix; expression period 7-9 days, selection growth 13-14 days 3 h with S9-mix; expression period 7-9 days, selection growth 13-14 days
GLP:	in compliance	
Study period:	29 October 1999 – 4 July 2003	

Basic Violet 2 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Basic Violet 2 test concentrations were based on the results of a cytotoxicity assay with a wide range of concentration levels both in the presence and absence of S9-mix measuring cell survival relative to the concurrent vehicle control cell cultures. In the main test cells were treated for 3 h or 24 h (experiment 2 with S9-mix only) followed by an expression period of 7-9 days to fix the DNA damage into a stable *tk* mutation.

Toxicity was measured as percentage relative total growth of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the cytotoxicity test both in the absence and presence of S9-mix and at 3 and 24 h treatment, severe toxicity was observed at the highest dose levels tested, reducing survival below 5% of the negative control level. On the basis of these findings 1.5 and 2.75 µg/ml were selected as maximum dose level used in the first experiment with and without S9-mix respectively and 0.4 µg/ml in experiment 2 for the 24 h treatment group.
In the 2 experiments in the absence of S9-mix the appropriate level of toxicity (reduction of the relative total growth after the highest dose to 10-20%) was reached; in the experiments

in the presence of S9-mix the appropriate level of reduction in relative total growth was not reached.
No biologically relevant and dose dependent increases in the mutant frequency following treatment with Basic Violet 2 were found at any dose level, in the absence or presence of S9-mix at any treatment time.

Conclusion Basic Violet 2
Under the experimental conditions used, Basic Violet 2 was not mutagenic in the mouse lymphoma assay at the *tk* locus.

Ref.: 27

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

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

Guideline:	OECD 474 (1997)
Species/Strain:	Swiss CD-1 Mice
Group size:	5 mice/sex/dose
Test substance:	Basic Violet 2
Batch:	GST 063-01/41-06
Purity:	HPLC: 94% (HPLC)
Vehicle:	sterile distilled water
Route:	Intraperitoneal injection
Dose level:	0, 3, 6 and 12 mg/kg bw
Sampling:	24 and 48h control and high dose group only)
GLP:	in compliance
Study date:	3 December 1999 – 19 June 2003

Basic Violet 2 was evaluated for its potential to induce micronuclei in bone marrow after ip administration of the test article to mice. Test doses were based on acute toxicity in a pre-test with 2 animals per sex/group. In the micronucleus test groups of 5 male and 5 female mice were treated with test material dissolved in sterile distilled water. The test article was administered intraperitoneally at 3, 6 and 12 mg/kg bw. Animals were inspected for signs of reaction to treatment daily throughout the study. Bone marrow cells were collected 24 h or 48 h (control and highest dose only) after dosing and stained with May-Gruenwald and Giemsa. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/PCE+NCE) over the negative control value. Negative and positive controls were in accordance with the OECD guideline.

Results
Three toxicity studies with decreasing doses were performed in order to identify the doses to be used in the main study. All mice died at doses ≥ 125 mg/kg bw. All mice treated with lower doses survived. Clinical signs observed were swollen abdomen, ataxia, hunched posture, lethargy, reduced activity, semi closed eyes and piloerection. Mice treated with 3.91 mg/kg bw did not show any adverse reactions. At doses ≥ 15.6 mg/kg bw the PCE/PCE+NCE ratio was markedly depressed. Therefore 12 mg/kg bw was chosen as top dose.
In the micronucleus test no animals died. Animals from the intermediate treatment group showed piloerection; those from the high dose group hunched posture, swollen abdomen, closed eyes, piloerection and pink spots in the cage litter indicative for excretion of coloured urine.
In the micronucleus test a reduction in the PCE/(PCE+NCE) ratio was observed for both sexes and both time points. Biological relevant increases in the number of bone marrow cells with micronuclei compared to the concurrent vehicle controls were not found at any dose tested, either 24 or 48 h after treatment or for males and females.

Conclusion

Under the experimental conditions, Basic Violet 2 did not induce an increase in bone marrow cells with micronuclei and, consequently, Basic Violet 2 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 28

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Taken from SCCNFP/0784/04

Guideline:	OECD 414 (1983)
Species/strain:	rat, Sprague Dawley Hsd: SD strain
Group size:	24 females (mated) per group
Batch:	GST 063-01/41-06
Dose levels:	2, 10 and 50 mg/kg bw/day
Vehicle:	distilled water (10 ml/kg bw/day)
Treatment period:	days 6-15 of gestation
GLP:	in compliance
Study date:	2001

Results

Clinical signs:	Violet coloured faeces in mid- and high-dose groups. Dyspnoea in high-dose group
Body weights:	reduced in high-dose group from day 8
Food intake:	reduced in high-dose group during treatment
Necropsy F0:	final body weight, uterus weight and corrected body weight were decreased in high-dose group.
Litter data:	The number of implantations was decreased in high-dose group. Foetal weight was decreased in the high-dose group
Foetal visceral exam.:	no treatment related effects
Foetal skeletal exam.:	no treatment related effects

Conclusion

Foetal visceral and skeletal examination did not reveal teratogenic effects. Maternal toxicity and a delay of foetal development were observed at 50 mg/kg bw/day. The NOAEL for maternal and developmental effects was 10 mg/kg bw/day.

Ref.: 22

3.3.9. Toxicokinetics

In vitro study with human intestinal epithelial cells

Guideline	:	/
Cells	:	Human intestinal epithelial cell line TC-7
Test substance	:	Basic Violet 2
Batch no	:	GST063-0Í/41-O6
Purity	:	93.8 area % (HPLC, 254 nm)

Test concentration	:	50 µM in HBSS buffer containing 1 % DMSO
Incubation time	:	60 min
GLP	:	/
Study date:		2005

The bioavailability of Basic Violet 2 across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro* in 2 independent experiments. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37 °C in 96-well transwell plates with shaking for a 60 min contact time. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of HLPC-MS/MS and the apparent permeability coefficient (P_{app}) was calculated. ^{14}C-mannitol (about 4 µM) was used to demonstrate the integrity of the cell monolayer. Only monolayers revealing a permeability of < 2.5 x 10^{-6} cm/sec were used. Propranolol, atenolol, vinblastine and ranitidine were analysed concurrently to demonstrate the validity of the test system.
According to the laboratory's classification system, a low permeability is considered for test items revealing a P_{app} < 2 x 10^{-6} cm/sec. A P_{app} of 2 - 20 x 10^{-6} cm/sec and a P_{app} ≥ 20 x 10^{-6} cm/sec classify a substance to have a moderate and a high permeability, respectively. As recommended by FDA, ranitidine (50 % absorption in humans) was used as the low permeability reference compound and propranolol (90 % absorption in humans) was used as the high permeability reference compound.

Results
The total recovery for the reference substances and Basic Violet 2 ranged from 82 to 118 % (mean of 2 values). The permeability figures for the reference substances propranolol (P_{app} = 50.6 and 52.7 x 10^{-6} cm/sec), a high permeability reference compound with 90% absorption in humans, and ranitidine (P_{app} = 0.3 and 0.3 x 10^{-6} cm/sec), known to be absorbed at about 50 % in humans, were within acceptance range of 20 - 60 x 10^{-6} cm/sec and 0 - 2 x 10^{-6} cm/sec, respectively and demonstrated the validity of the assay.
For Basic Violet 2 a P_{app} of 2.8 x 10^{-6} cm/sec (2.9 and 2.6 x 10^{-6} cm/sec) was determined and thus the test substance was classified to be of medium permeability, indicating a substantial absorption from the gastrointestinal tract.

Conclusion
A mean permeability in human intestinal epithelial (TC-7) cells of 2.8 x 10^{-6} cm/sec was obtained with Basic Violet 2 which classifies the test item to be of medium permeability. As the absorption across the intestinal epithelium is considered to be the limiting factor of the uptake through the gastro-intestinal tract, the medium permeability observed in this assay indicates a substantial but not complete absorption of Basic Violet 2 after oral administration.

Ref.: 25

Comment
The study was not performed under GLP conditions, but a statement of the quality assurance unit of the test facility is included. There is no official guideline for this assay.
However, the study was performed according to ECVAM recommendations. The generated data is considered to provide an estimation of the bioavailability of Basic Violet 2 after oral administration.

The substance is expected to be only partially absorbed after oral dosing, which should be considered in the calculation of the Margin of Safety.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Basic Violet 2
(non- oxidative conditions)

Absorption through the skin	**A (µg/cm^2)**	**=**	**0.04 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.023 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.0004 mg/kg bw/d**
NOAEL		**=**	**1.5 mg/kg bw/d**
(sub-chronic toxicity, oral, rat, corrected for 50% oral absorption)*			

MOS	**NOAEL / SED**	**=**	**3750**

(oxidative conditions)

Absorption through the skin	**A (µg/cm^2)**	**=**	**0.145 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.084 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.0014 mg/kg bw/d**
NOAEL		**=**	**1.5 mg/kg bw/d**
(sub-chronic toxicity, oral, rat, corrected for 50% oral absorption)*			

MOS	**NOAEL / SED**	**=**	**1071**

* Standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14. Discussion

Basic Violet 2 is allowed to be used as a colorant at a maximum concentration of 5 ppm in cosmetic products intended to come into contact only briefly with the skin (Annex IV, part 1, CI 42520).

Physico-chemical specification
Basic Violet 2 is used up to 0.5% in non-oxidative hair dye-formulation and up to 1.0% on-head concentration in oxidative hair dye formulations.

No reference substances were used for the quantification of homologues of Basic Violet 2. Their concentrations are relative semiquantative ones.

The batches used for identity- and purity testing have been purified before the analytical determinations. Therefore SCCS raised the question, if the batches, which have been used for toxicity testing, had been purified in the same way before use and if this also applies for commercial products. The applicants stated that the batches used for toxicity testing had been purified in the same way and that the currently used quality shows higher purity compared to the batches described in the dossier.

General toxicity
The oral LD_{50} of Basic Violet 2 in rats exceeded 2000 mg/kg bw. In a subchronic toxicity study in rats at the doses 30 and/or 10 mg/kg bw/d histopathological findings in liver and kidney indicate organ toxicity which is supported by changes in body and organ weights as well as in biochemical parameters (cholesterol, aspartate aminotransferase, alkaline phosphatase). The NOAEL is considered 3 mg/kg bw/d. However, the substance is expected to be only partly absorbed after oral application. Therefore the NOAEL was corrected to 1.5 mg/kg bw/d in the calculation of the Margin of Safety.
In a developmental toxicity study in rats, foetal visceral and skeletal examination did not reveal teratogenic effects. Maternal toxicity and a delay of foetal development were observed at 50 mg/kg bw/day. The NOAEL for maternal and developmental effects was 10 mg/kg bw/day.
No study on reproductive toxicity was submitted.

Toxicokinetics
A mean permeability in human intestinal epithelial (TC-7) cells of 2.8×10^{-6} cm/sec was obtained with Basic Violet 2 which classifies the test item to be of medium permeability. As the absorption across the intestinal epithelium is considered to be the limiting factor of the uptake through the gastro-intestinal tract, the medium permeability observed in this assay indicates a substantial but not complete absorption of Basic Violet 2 after oral administration.

Irritation, sensitisation
The assessment of skin irritation potential was hampered because erythema could not be assessed due to intense staining of the skin. No oedema was noted in any of the test animals. Measurement of skin-fold thickness after 72 hours revealed no differences between treated skin and untreated (naive) skin. The undiluted test substance was therefore not considered a skin irritant.
The ocular irritation test with the undiluted substance showed clear eye damage. A 1% solution of the test compound is considered to be not irritating to the eye.
The sensitisation tests are inconclusive. A sensitisation potential of Basic Violet 2 cannot be excluded.

As a general remark about the skin irritation, eye irritation and sensitisation tests, all performed by the same laboratory, it can be stated that the Quality Assurance statements have dates which do not correspond with the actual dates of the performed studies, indicating that the studies on Basic Violet 2 have never been audited. Moreover, the final signature on GLP-compliance and Quality Assurance statements are dated 3 to 4 years after performance of the tests. Nevertheless, the studies are accepted for evaluation.

Dermal absorption
Under oxidative conditions, the mean + 1SD (0.145 µg/cm²) should be used for the calculation of the Margin of Safety.
For non-oxidative conditions, the mean + 1SD (0.04 µg/cm²) should be used for the calculation of the Margin of Safety.

Mutagenicity
Overall, the genotoxicity of Basic Violet 2 is investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Only gene mutations were tested *in vitro*. Basic Violet 2 treatment did not result in an increase in gene mutations both in bacteria and in mammalian cells. The induction of chromosome aberrations and aneuploidy was only tested in an *in vivo* assay. In an adequate *in vivo* micronucleus test in mice an increase in bone marrow cells with micronuclei was not observed.
Based on the data available, Basic Violet 2 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data submitted

4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of Basic Violet 2 with a maximum on-head concentration of 1.0% in oxidative and 0.5% in non-oxidative hair dye formulations does not pose a risk to the health of the consumer.

A sensitisation potential of Basic Violet 2 cannot be excluded.

5. MINORITY OPINION

Not applicable

6. REFERENCES

1. Tegeler, A.; Identity and purity test of Basic Violet 2; WELLA AG; 2000
2. Tegeler, A.; Identity, stability and purity test of Basic Violet 2; WELLA AG; 1999
3. Lange, J.; Partition Coefficient (n-octanol/water) Shake Flask Method; NOACK; 2004
4. Geffke, Th.; Sieve Analysis; NOACK; 2004
5. Lange, J.; Water Solubility (Flask Method); NOACK; 2004
6. Lange, J.; Prediction of the pKa-value; NOACK; 2004
7. SmeykaS, H.; Melting Point / Boiling Point; SIEMENS AXIV A GMBH & CO. KG; 2004
8. Lange, J.; Determination of the Density; NOACK; 2004
9. Franke, J.; Vapour Pressure; SIEMENS AXIVA GMBH & CO. KG; 2004
10. Lange, J.; Surface Tension; NOACK; 2004
11. Lange, J.; Flammability of Solids; NOACK; 2004
12. Smeykal, H.; Auto-flammability (solids-determination of relative self-ignition temperature); SIEMENS AXIVA GMBH & CO. KG; 2004
13. Waibel, J.; Stability in formulation of Basic violet 2; WELLA AG; 2005

14. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers concerning Basic Violet 2, COLIPA n° B115; SCCNFP; 2004
15. Longobardi, C; Acute oral toxicity study in the rat; RTC\ 2002
16. Longobardi, C; 13 week oral toxicity study in rats; RTC; 2002
17. Longobardi, C; Acute dermal irritation study in the rabbit; RTC; 2003
18. Longobardi, C; Acute eye irritation study in the rabbit; RTC; 2003
19. Longobardi, C; 1% Aqueous Solution of Basic Violet 2 - Acute eye irritation study in the rabbit; RTC; 2002
20. Longobardi, C; Delayed dermal sensitisation study in the guinea pig (Magnusson and Kligman test); RTC; 2003
21. Longobardi, C; Delayed dermal sensitisation study in the guinea pig (Buehler Test); RTC; 2003
22. Cicalese, R.; Oral teratogenicity study in rats; RTC; 2001
23. Beck, H.; Cutaneous Absorption of 0.5% CI 42520 (=WR18006) in a typical hair dye formulation through pig skin in vitro; COSMITAL SA; 2005
24. Simon, G. A.; Maibach, H. L; The pig as an experimental animal model of percutaneous permeation in man: Qualitative and quantitative observations - An overview; SKIN PHARMACOL. PHYSIOL; 13,229-234; 2000
25. Kennedy, K. J.; ADME: Permeability - Study of Basic Violet 2; CEREP; 2005
26. Aeby, P.; Assessment of the potential mutagenicity of Basic Violet 2 in the Ames reversion assay with Salmonella Typhimurium; COSMITAL SA; 2000
27. Cinelli S.; Mutation in L5178Y TK +/- Mouse Lymphoma Cells (Fluctuation Method); RTC; 2003
28. Cineili, S.; Micronucleus Test; RTC; 2003
29. König, P.; Data base search for references for B115 - Basic Violet 2, CÏ 42520: 3248-91-7; WELLA AG;2005
30a. Martina Jäger, 2007; Skin permeability in vitro absorption through porcine ear skin with Basic Violet 2 (3%) in formulation; RCC Project No. 1077802; Test Report, Rossdorf, October 24, 2007
30b. Martina Jäger, 2007; Quantification of Basic Violet 2 (3%) in formulation; RCC 1077801.

Submission 2011

1. Toner, F. The In Vitro Percutaneous Absorption of Basic Violet 2 (B115, WR18006) in Typical Hair Dye Formulations Under Oxidative and Non-Oxidative Conditions through Human Skin. Charles River UK- Edinburgh. Test Facility Study No. 787697, Report No. 31431. 2011