Health and Consumers
Scientific Committees

Scientific Committee on Consumer Safety

SCCS

OPINION ON

Toluene-2,5-diamine and its sulfate

COLIPA n° A5

The SCCS adopted this opinion at its 15th plenary meeting

of 26 – 27 June 2012

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.).

Scientific Committee members

Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Qasim Chaudhry, Gisela Degen, Elsa Nielsen, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Jan van Benthem, Jacqueline van Engelen, Maria Pilar Vinardell, Rosemary Waring, Ian R. White

Contact
European Commission
Health & Consumers
Directorate D: Health Systems and Products
Unit D3 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-SCCS-Secretariat@ec.europa.eu

© European Union, 2012

ISSN 1831-4767 ISBN 978-92-79-30766-9

Doi:10.2772/8437 ND-AQ-12-016-EN-N

The opinions of the Scientific Committees present the views of the independent scientists who are members of the committees. They do not necessarily reflect the views of the European Commission. The opinions are published by the European Commission in their original language only.

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

ACKNOWLEDGMENTS

Prof. J. Angerer
Dr. C. Chambers
Dr. W. Lilienblum (associated scientific advisor)
Prof. T. Platzek (chairman, rapporteur)
Dr. S.C. Rastogi
Dr. C. Rousselle
Prof. T. Sanner
Dr. J. van Benthem
Prof. M.P. Vinardell
Dr. I.R. White

External experts

Dr. Mona-Lise Binderup National Food Institute, Denmark

Keywords: SCCS, scientific opinion, hair dye, toluene-2,5-diamine, A5, directive 76/768/ECC, CAS 95-70-5 (free base), 615-50-9 (sulfate), 6369-59-1 (sulfate 1:x), EC 202-442-1 (free base), 210-431-8 (sulfate), 228-871-4 (sulfate 1:x)

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on toluene-2,5-diamine, 26-27 June 2012

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.

TABLE OF CONTENTS

1. BACKGROUND

Submission I on toluene-2,5-diamine was submitted in December 1979 by COLIPA[1] according to COLIPA.

Submission II on toluene-2,5-diamine was submitted in July 2005 by COLIPA.

Submission III on toluene-2,5-diamine and its sulfate salt was submitted in October 2010 by COLIPA.

The Scientific Committee on Consumer Safety (SCCS) adopted at its 10th plenary meeting the 22 of March 2011 the opinion (SCCS/1390/10) with the following conclusion:

Based on the low Margin of Safety using the conventional risk assessment approach, toluene-2,5-diamine and its sulphate salt cannot be considered safe for use as an oxidative hair dye with a concentration on-head of maximum 4.0% (calculated as free base) or 7.2% (calculated as sulfate salt).
The kinetics-based approach for MoS calculation proposed by the applicant, using the AUC determined in a human *in vivo* exposure study, was not accepted due to the shortcomings of the underlying study which was not performed according to modern standards. In order to come to a final conclusion, the SCCS is of the opinion that a state of the art human exposure study *in vivo* would be required.
Toluene-2,5-diamine is at least a strong skin sensitiser.

The substance and its salts is currently regulated in entry 9a of Annex III to the Cosmetics Directive on the list of substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission IV on toluene-2,5-diamine was submitted by COLIPA in February 2012.

This submission contains the results and conclusions obtained in the new performed study in which the applicant assesses the safety of toluene-2,5-diamine at on head concentration of up to 1.5% (expressed as free base) in oxidative hair colouring products and conclude that concentration up to 2.0% toluene-2,5-diamine (expressed as free base) would also meet the requirements for a sufficient Margin of safety.

2. TERMS OF REFERENCE

1. *Does the SCCS consider toluene-2,5-diamine and its sulfate salt, safe for use as an oxidative hair dye with a concentration on-head of maximum 2.0% (3.6% calculated as sulfate salt) taken into account the scientific data provided?*

2. *If not, does the SCCS recommend any other concentration limit with regard to the use of toluene-2,5-diamine and its sulfate salt as an oxidative hair dye?*

3. *And/or does the SCCS recommend any further restrictions with regard to the use of toluene-2,5-diamine and its sulfate salt, in oxidative hair dye formulations?*

[1] COLIPA – The European Cosmetics Association

3. OPINION

3.1. Chemical and Physical Specifications

Toluene-2,5-diamine is used in hair dyes in the form of its free base or its sulfate salt.

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Toluene-2,5-diamine (INCI)
Toluene-2,5-diamine sulfate (INCI)

3.1.1.2. Chemical names

Free Base
1,4-Benzenediamine, 2-methyl- (CA INDEX NAME, 9CI)

Sulfate
2,5-Diaminotoluene sulfate
2-Methyl-p-phenylenediamine sulfate
Toluenediamine sulfate
p-Toluenediamine sulfate
p-toluylenediamine sulfate

3.1.1.3. Trade names and abbreviations

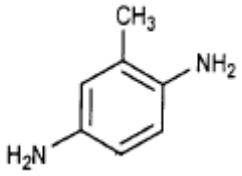
Free base
Imexine OD
COLIPA A005
Colour Index no 76042

Sulfate
Colorex 25DTS (Chemical Compounds, Inc.)
Jarocol TDS (Robinson)
Rodol BLFX (Lowenstein)
COLIPA no A005
Colour Index no 76043

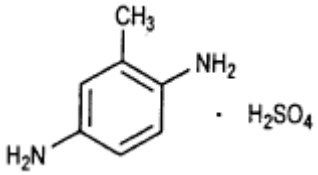
3.1.1.4. CAS / EC number

	Free Base	*Sulfate*
CAS:	95-70-5	615-50-9 (sulfate 1:1); 6369-59-1 (sulfate 1:x)
EC:	202-442-1	210-431-8 (sulfate 1:1); 228-871-4 (sulfate 1:x)

3.1.1.5. Structural formula



Free base



Sulfate

3.1.1.6. Empirical formula

	Free base	Sulfate
Formula:	$C_7H_{10}N_2$	$C_7H_{10}N_2.H_2O_4S$

3.1.2. Physical form

Free base: light yellow to light pink
Sulfate: grey to white powder

3.1.3. Molecular weight

	Free base	*Sulfate*
Molecular weight:	122.17	220.25

3.1.4. Purity, composition and substance codes

Batches used (survey on all the files of Submission II)

Toluene-2,5-diamine (50% aqueous solution)

This name and the respective data below were found only in the "A5 SUMMARY submission II 2005.doc" (pages 8-10). Instead of batch numbers, in page 9 it is noted "*See Annex I for summary and Reference: 3"*. However, Annex 1 and ref. 3 refer only to the sulfate salt.

Toluene-2,5-diamine sulfate

- Batch 2346/01-R99053665 (abbreviations: Batch 2346 or Batch R99053665)
- Batch EFH 290394
- Batch CH1143
- Batch 46847
- Lot 16825DR Sigma Aldrich (see Study 2, human hepatic metabolism *in vitro)*
- Batch präp. 139 (Purity: 98.2%; see 3.3.8.1. Two generation reproduction toxicity)
- Batch 23005

A complete characterization is provided for the first two batches only.

Radioactive toluene-2,5-diamine sulfate

- 3362-259 [ring-U-^{14}C]-toluene-2,5-diamine sulfate (radiochemical purity 99.3% by HPLC)
- CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate (radiochemical purity 98.2% by HPLC)

Purity, accompanying contaminants, and batch codes

Toluene-2,5-diamine (50% aqueous solution)		Toluene-2,5-diamine Sulfate	
Purity		Purity	
HPLC relative	> 99%	HPLC quantitative	> 96.3 weight%
Potentiometric Titer:	48-52%	NMR quantitative	> 97.3 weight%
Potential impurities		Potential impurities	
o-Toluidine *	< 20 ppm	o-Toluidine	< 8 ppm
Solvent residues Solvents (i.e. solvents such as methanol, ethanol, isopropanol, n-propanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone and monochlorobenzene < 100 ppm) were not detected.			

* EU CMR classification: carcinogen category 1B (regulation (EC) No. 1272/2008)

Ref.: 3

Material used in the market (Deduced specifications)

Toluene-2,5-diamine (50% aqueous solution)		Toluene-2,5-diamine sulfate	
Purity		Purity	
HPLC qualitative:	> 99%	HPLC quantitative:	> 98% w/w
Potentiometric Titer:	48-52%	HPLC qualitative (254 nm)	> 99%
		Solvent content:	< 1%
Potential impurities		Potential impurities	
o-Toluidine	< 50 ppm	o-Toluidine	< 50 ppm

Comparison of two different batches of toluene-2,5-diamine sulfate

	batch EFH 290394 29.03.1994	batch 2346 (/01, R99053665) 09.12.1999
NMR content / weight%	97.3	99.5
HPLC purity / area% 210 nm 254 nm 303 nm	 99.3 99.0 99.6	 99.5 99.7 (at 290 nm)
HPLC quantitative (compared to R99053665)	101.6%	
Loss on drying / weight%	*	0.20
Water content / weight%	*	0.04
Sulfated ash / weight%	*	0.11
o-Toluidine	< 1 ppm (detection limit)	< 1 ppm (detection limit)
UV spectra (ethanol)		
ε_{mol} (242 nm) / l cm^{-1} mol^{-1}	9820	*
ε_{mol} (308 nm) / l cm^{-1} mol^{-1}	2419	*

* not determined

3.1.5. Impurities / accompanying contaminants

See point 3.1.4.

3.1.6. Solubility

Solubility of toluene-2,5-diamine

A005 50% aqueous solution:

in water	≥ 10 g/100ml
in ethanol 100%	≥ 10 g/100ml
in DMSO	≥ 10 g/100ml

Ref.: 115

Solubility of toluene-2,5-diamine has not been determined according to standard methods (e.g. EU - A.6)

Solubility of toluene-2,5-diamine sulfate

in water:	5.03 g/l (20 °C) (EU - A.6)	Ref.: 6
in acetone / water 1:1:	< 1 g/l	
in DMSO:	5 < S < 15 g/l	
in ethanol:	1 < S < 10 g/l	(taken from submission summary)

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}:	0.74 (sulfate)	(HPLC method, EU method A.8)
	- 0.32 (free base, 50% aqueous solution)	(shake-flask method, EU method A8)
	0.160 (free base)	(ref. 115)

3.1.8. Additional physical and chemical specifications

Physical properties of toluene-2,5-diamine sulfate

Appearance:	grey to white powder		
Particle size distribution:	mean particle diameter: 46 µm (CIPAC MT59)		Ref. 4
Particle size distribution:	median particle size L50 = 6.5 µm (by laser diffraction ; OECD 110,CIPAC MT 187)		Ref. 5
pH-value:	2.47 (20 °C ; saturated aqueous solution)		Ref. 6
pKa-value:	6.39 and 2.77 (calculated, Pallas Software)		Ref. 7
Melting point:	not detectable, decomposed at 240 °C	(EU – A.1)	Ref. 8
Boiling point:	not detectable, decomposed at 240 °C	(EU – A.2)	Ref. 8
Density:	1.366 g/ml (20 °C)	(EU - A.3)	Ref. 9
Vapour pressure:	<1.0 exp - 7 hPa (20 °C)	(EU - A.4)	Ref. 10
Flammability (solids):	not highly flammable	(EU - A.10)	Ref. 12
Relative self-ignition Temperature:	327 °C	(EU - A.16)	Ref. 13
Surface tension (in water):	69.7 mN/m (20 °C)	(EU - A.5)	Ref. 11
Refractive index:	/		
UV_Vis spectrum	λ_{max} = 210 nm, 254 nm, 303 nm		

Physical properties of toluene-2,5-diamine (free base)

Melting point:	64 °C
Boiling point:	273.5 °C
Vapour pressure:	3.40E-03 (0.0034) mm Hg at 25 °C

Henry's Law Constant: 7.43E-09 (7.43 10^{-9}) atm-m³/mole at 25 °C

Ref.: 115

3.1.9. Stability

The stability of the test substance in aqueous and water:acetone (4:1, v/v) solution was monitored over a time period of 8 days. During the test procedure, the aqueous and water:acetone stock solutions were stored at ambient temperature in the absence of light. The recoveries of the test substance in both solvents were 99.7–109%.

General Comments on physico-chemical characterisation

* Batch 46847, used in 3 mutagenicity studies (ref. 38, 40 and 41), batch 23005, used in the teratogenicity studies (ref. 53 and 54) as well as batches CH1143, präp. 139 and Lot 16825DR Sigma Aldrich were not characterised
* The stability of toluene-2,5-diamine and its sulfate in typical hair dye formulations was not reported.
* The impurity o-toluidine is classified by the EU as carcinogenic category 1B (regulation (EC) No. 1272/2008).
* No documentation was provided to support the reported data on the free base.
* Solubility of toluene-2,5-diamine has not been determined according to standard methods (e.g. EU - A.6)

3.2. Function and uses

Toluene-2,5-diamine and its sulfate are used as an oxidative hair colouring agent (precursor). The intended maximum on-head concentration is 2.0% (calculated as free base), or 3.6% (calculated as sulfate).

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline: /
Species/strain: rat, CFY strain
Group size: 5 males and 5 females per dose group
Test substance: toluene-2,5-diamine
Batch: /
Purity: /
Dose: 0, 64, 100, 160 and 250 mg/kg bw
Route: oral, gavage
Exposure: once
GLP: /

The test substance was diluted at 10% in aqueous sodium sulphite (0.05%) and administered once by oral gavage. During the observation period of 14 d mortalities and signs of toxicity were recorded and body weight was measured weekly.

Results
Lethargy, piloerection, ataxia and increased salivation were observed shortly after dosing. At 100 mg/kg bw increased respiratory rate and above 100 mg/kg bw decreased respiratory rate were observed. The acute median lethal oral dose and its 95% confidence limits were calculated to be 102 (69 – 152) mg/kg bw.

Dosage (mg/kg bw)	Mortality (number deaths/number dosed)	
	Male	Female
0	0/5	0/5
64	3/5	0/5
100	4/5	3/5
160	0/5	5/5
250	5/5	4/5

Ref.: 16

Comment
Despite the lack of data on the batch used and although the study does not conform to OECD guidelines, it is useful for evaluation.

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

Guideline:	OECD 404 (1992)
Species/strain:	Rabbit / New Zealand White
Group size:	3 Males
Test substance:	Imexine OD (free base)
Vehicle:	None (test substance consisted of active ingredient with water)
Batch:	op T 784
Purity:	50.6% active (in water)
GLP:	In compliance

A single dose of 0.5 ml of the test substance (pH 9.71) was prepared on a dry compress and then applied to a 6 cm^2 clipped area of the skin and covered with a semi-occlusive dressing for 4 h. Skin reactions were evaluated 1 h, 24 h, 48 h, and 72 h after removing the dressing and then daily until day 6.

Results
No oedema, ulceration or necrosis was noted. Evaluation of erythema was not possible due to the black colouration of the treatment site.

Conclusion
Although the application of a 50.6% aqueous solution of Imexine OD produced no evidence of an oedematous response after topical application under semi-occluded conditions in New Zealand White rabbits, an erythematous response could not be excluded due to black colouration of the treatment site by the test substance.

Ref.: 17

Guideline:	/
Species/strain:	Rabbit / New Zealand White
Group size:	3 Males
Test substance:	toluene-2,5-diamine
Vehicle:	water
Batch:	/
Purity:	/
GLP	/

0.5 ml of a 2.5% w/v solution of toluene-2,5-diamine in aqueous 0.05% sodium sulphite (pH 7.0) was tested on intact and abraded skin of three New Zealand White rabbits under occlusive patches. Cutaneous reactions were observed at 24 h (immediately after patch removal) and again at 72 h.

Results
Slight erythema with and without very slight oedema was observed in the intact and abraded sites, respectively, of one animal at the 24 h evaluation. At 72 h no signs of irritation were observed.

Conclusion
The test substance was considered to be mildly irritating to rabbit skin under the conditions of this test.

Ref.: 18

Comments
In an *in vivo* study in rabbits, a 50.6% Imexine OD applied under semi-occlusive conditions did not produce evidence of oedema and could not be evaluated for erythema due to black colouration of the skin. In the second experiment, which did not conform to guidelines or GLP, the test substance was irritant to rabbit skin under occlusive conditions.

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405
Species/strain:	Rabbit / New Zealand White
Group size:	1 Male
Test substance:	Imexine OD (free base)
Vehicle:	None (test substance consisted of active ingredient with water)
Batch:	op T 784
Purity:	50.6% active (in water)
GLP:	In compliance

A volume of 0.1 ml of the test substance (pH 9.71) was applied into the conjunctival sac of the left eye of one male rabbit; the right eye served as a control. The eye was not rinsed, and was evaluated and scored according to the Draize scoring system at 1, 24, 48, and 72 h after application and then daily until Day 8.

Results
The test substance induced marked conjunctival irritation with chemosis and redness, slight iridial irritation and moderate to slight corneal opacity. All of these effects were reversible within 7-8 days.

Conclusion
Under the test conditions, in which the test substance was applied undiluted and was not rinsed from the eye, the test substance was irritating to the rabbit eye. The high pH of the test solution may have contributed to the observed irritation.

Ref.: 19

Guideline:	/
Species/strain:	Rabbit / New Zealand White
Group size:	3 Males
Test substance:	toluene-2,5-diamine
Vehicle:	water
Batch:	/
Purity:	/
GLP:	/

0.1 ml of a 2.5% w/v solution of toluene-2,5-diamine in 0.05% aqueous sodium sulphite (pH 7.0) was instilled into one eye of each of three rabbits. After 10 seconds the eye was rinsed with 50 ml of lukewarm water. Eyes were evaluated and scored according to the Draize scoring system at 1 h and then at Days 1, 2, 3, 4, and 7.

Results
Mild conjunctival irritation was observed in 2 animals on days 1 and 3 respectively.

Conclusion
Under the conditions of this test, a 2.5% toluene-2,5-diamine solution caused slight irritation to rabbit eyes.

Ref.: 20

Acute eye irritation potential in vitro: HET-CAM

Guideline: /
Species/strain: White Leghorn chicken eggs, freshly fertilized
Group size: 6 eggs
Test substance: p-toluenediamine sulfate (code SAT 010935)
Batch: 46847
Purity: 99.9%
GLP: /

The test substance was tested undiluted for its irritation potential on the chorioallantoic membrane of fertilized chicken eggs using the endpoint assessment for non-transparent solid test items.

Texapon ASV 70 (sodium magnesium laurylmyristyl-6-ethoxy-sulfate) at a test concentration of 5% was used as reference substance with this internal benchmark being defined to be moderately irritating to the rabbit eye *in vivo*.

The undiluted test substance was applied to the chorioallantoic membrane and then rinsed off with physiological saline after 30 sec. Endpoints (haemorrhage, coagulation, and blood vessel lysis) were assessed and semi-quantitatively scored at 30 sec (reference substance) or 180 sec (test substance) after rinsing.

Results
For the relevant endpoints of haemorrhage, coagulation and lysis, scores of 0, 0, and 0, respectively, were obtained with p-toluenediamine sulfate. With Texapon ASV 70 scores of 12, 9, and 0, respectively, were obtained.

Conclusion
Based on the results of this test, p-toluenediamine sulfate was predicted to be 'no more than slightly irritating to mucous membranes', including the eye. The results with the reference substance, Texapon ASV 70, were indicative of a moderately irritating effect.

Ref.: 21

Comment on status of HET-CAM
The HET-CAM (Hen's Egg Test-Chorio Allantoic Membrane) provides only supportive evidence for cosmetic ingredient safety assessment. This method can be recommended for use as screening tests for the identification of ocular corrosives and severe irritants, the protocol and decision criteria for the identification of ocular corrosives and severe irritants need to be optimized and undergo further validation (SCCS/1294/10).

Comment on eye irritation
Eye irritation studies have demonstrated that undiluted Imexine OD is irritant to the rabbit eye. Some irritant effects were also seen with 2.5% toluene-2,5-diamine solution in rabbits and undiluted toluene-2,5-diamine sulfate in the HET-CAM test. The intended on-head concentration is up to 3.6%.

3.3.3. Skin sensitisation

Animal data

Local Lymph Node Assay (LLNA)

Study 1

Reference 22 is the same study as ref. 24, below. Stimulation indices (SI) of 4.4, 10.4 and 19.4 were obtained from test concentrations of 0.5, 1.5 and 2.8%. An EC_3 value of 0.31% was derived by linear regression, indicating that the substance is a strong skin sensitiser in this experiment.

	Study reference 22	**Study reference 24**
Guideline:	/	OECD 406
Species:	mice, CBA/ca01aHsd	mice, CBA/ca01aHsd
Group:	5 animals per test group	female, 5 animals per test group, 3 test groups, 1 positive control group, 1 negative control group
Substance:	p-toluenediamine sulfate (PTD)	Haarbraun, 2-methyl-1,4-benzenediamine sulphate
Batch:	/	2346
Purity:	/	99.5 area% (254 nm) and 99.7 area% (290 nm)
Dose:	25 µl of PTD at 0.5, 1.5 and 2.8%	25 µl of the substance at 0.5, 1.5 and 2.8%
Vehicle:	Aqua/acetone/olive oil (AAOO) 2:2:1	Aqua/acetone/olive oil (AAOO) 2:2:1
Control:	/	p-phenylenediamine 1% in AAOO
GLP:	/	in compliance

In reference 24, the skin sensitising potential of the test substance was investigated by measuring the cell proliferation in the draining lymph nodes after topical application on the ear. 25 µl containing 0 (vehicle only), 0.5, 1.5 and 2.8% of the test substance in a mixture of aqua/acetone (1:1) with olive oil (4:1) were applied to the surface of the ear to each of five mice per group for three consecutive days. p-Phenylenediamine (PPD) at 1% in AAOO was used as the positive control in parallel under identical test conditions

On day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 20 µCi of [^{3}H]-methyl thymidine. Approximately five hours later, the mice were sacrificed by CO_2-inhalation and the draining auricular lymph nodes were removed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [^{3}H]-methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
Mean stimulation indices (SI) of 4.4, 10.4 and 19.4 were obtained for the test concentrations of 0.5, 1.5 and 2.8%, respectively. No EC_3 value was calculated, since all stimulation indices were above 3. The positive control (PPD 1% in AAOO) caused a stimulation index of 5.3.

Ref.: 22, 24

Comment
The lowest concentration used in this test was too high. An extrapolated EC_3 value was calculated by linear regression in reference 22 (found to be 0.31%).
References 22 and 24 seem to use the same data and describe the same LLNA study, however with a different presentation.

Study 2

Guideline:	OECD 406
Species:	mice, CBA/ca01aHsd
Group:	female, 5 animals per test group, 3 test groups, 1 positive control group, 1 negative control group
Substance:	Haarbraun, 2-methyl-1,4-benzenediamine sulphate
Batch:	2346
Purity:	99.5 area% (254 nm) and 99.7 area% (290 nm)
Dose:	25 µl of the substance at 0.5, 1.5 and 5.0%
Vehicle:	DMSO
Control:	p-phenylenediamine 1% in DMSO
GLP:	in compliance

The skin sensitising potential of the test substance was investigated by measuring the cell proliferation in the draining lymph nodes after topical application on the ear. 25 µl containing 0 (vehicle only), 0.5, 1.5 and 5.0% of the test substance in DMSO were applied to the surface of the ear to each of five mice per group for three consecutive days. p-Phenylenediamine (PPD) at 1% in DMSO was used as the positive control in parallel under identical test conditions

On day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 20 µCi of [^{3}H]-methyl thymidine. Approximately five hours later, the mice were sacrificed by CO_2-inhalation and the draining auricular lymph nodes were removed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [^{3}H]-methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm). The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
Mean stimulation indices (SI) of 4.9, 4.2, and 3.7 were obtained for the test concentrations of 0.5, 1.5 and 5%, respectively. No EC_3 value was calculated, since all stimulation indices were above 3. The positive control (PPD 1% in DMSO) caused a stimulation index of 10.1
Ref.: 25

Comment
The lowest concentration used in this test was too high and therefore the study is inadequate.

Guinea pig studies

Guideline:	/
Species/strain:	Female Hartley strain albino guinea pigs
Group size:	6 animals in each test group and each control group The number of control groups was not given
Test substances:	p-toluenediamine 2HCl (PTD); p-phenylenediamine (PPD) p-aminophenol (PAP); p-aminoazobenzene (PAB); Sudan III
Batch:	/
Purity:	PTD: 98%; PPD: 97%; PAP: 98%; PAB: 98%; Sudan III: 99%
Concentrations:	*Intradermal induction*: 0.1% test substance in saline (PTD and PPD) or in olive oil (PAP, PAB, and Sudan III), and in Freund's complete adjuvant (FCA)/saline *Topical induction*: 1% test substance in petrolatum, occluded Pre-treatment with 10% sodium lauryl sulfate in petrolatum

Challenge: 0.001, 0.01 and 0.1% test substance in acetone or in acetone/distilled water, open application

GLP: /

The aim of the study was to evaluate the skin sensitising potency of PTD, PPD, PAP, PAB and Sudan III, and to study cross-reactivity. Induction was performed according to the guinea pig maximisation test protocol by injections on day 0, and topical application on day 7 for 48 hours. Modifications included that the highest possible elicitation concentrations were not chosen, and that challenge was performed by open application and not closed. Challenge on day 21 by open application for 24 hours. Readings were made at 24, 48 and 72 hours after challenge application.

Results

Only results related to PTD are reviewed here. 100% of the animals induced with PTD (6/6) reacted at challenge with PTD, showing that the test substance was an extremely potent skin sensitizer (Table 1). Positive reactions were recorded in the animals induced with PTD at challenge also with PPD (5/6), PAP (3/6), PAB (5/6) and Sudan III (1/6), indicating cross-reactivity to these substances in animals induced with PTD (Table 1). 100% of the animals induced with PPD (6/6) reacted at challenge with PTD, but none of the animals induced with PAP or PAB (Table 2). The results indicate cross-reactivity to PTD in animals induced with PPD.

Table 1: Sensitisation and cross-reactivity test in guinea pigs induced with PTD. Response at challenge with PTD, PPD, PAP, PAB or Sudan III

	Challenge substance (no. positive at challenge/no. induced)				
Challenge concentration (%)	**PTD**	**PPD**	**PAP**	**PAB**	**Sudan III**
0.1	6/6	5/6	3/6	5/6	1/6
0.01	5/6	2/6	0/6	5/6	1/6
0.001	0/6	0/6	0/6	1/6	0/6

Table 2: Sensitisation and cross-reactivity test in guinea pigs induced with PPD, PAP, PAB or Sudan III. Response at challenge with PTD

	Induction substance (no. positive at challenge/no. induced)		
PTD challenge concentration (%)	**PPD**	**PAP**	**PAB**
0.1	6/6	0/5	0/6
0.01	0/6	0/5	
0.001	0/6		

Conclusions

Although not performed according to guideline, the results indicate that PTD is an extreme skin sensitiser. The results indicate also that cross-reactivity in animals induced with PTD occurs to PPD and PAB; and to PTD in animals induced with PPD. As contaminants in test substances were not analysed, conclusions concerning cross-reactivity remain limited.

Ref.: 26

Guideline: /
Species/strain: Hartley albino guinea pigs
Group size: 10 animals in each pre-test group
The number of animals in test groups was not given, no control group
Test substances: toluene-2,5-diamine sulfate
Batch: /

Purity: /
Concentrations: *Topical induction*: 1% in petrolatum, occluded
Challenge: 0.01, 0.05, 0.1, 0.2, 0.5 and 1% test substance, occluded
GLP: /

The aim was to assess the skin sensitising potency in the guinea pig of ten dye intermediates, including toluene-2,5-diamine sulphate, and to compare the results with results from patch testing hair colouring dermatitis patients in Japan. The study was performed by a non-guideline method. Pre-tests were performed by occluded exposure to determine the irritancy threshold. Topical induction was performed by occluded exposure for 48 hours on the nape, 3 times per week for two weeks. Following a 2 week rest period, challenge was performed by occluded exposure for 48 hours on the flank. Readings were made at 24 and 48 hours after removal of the test material. It was reported that 40% of the animals reacted positively to toluene-2,5-diamine sulfate at challenge with 1%, and 10% at challenge with 0.10%.

Ref.: 27

Comment
The results of the study are of limited use.

Human data
Diagnostic patch testing

66 dermatitis patients (hairdressers) were patch tested with the North American patch test standard tray and a hairdresser series. 7.5% were positive to toluene-2,5-diamine sulfate, 46% were positive to p-phenylenediamine, 5% to p-aminodiphenylamine, 3% to o-nitro-p-phenylenediamine. (Table 1)

Ref.: 63

597 dermatitis patients (hairdressers), of which 61.8% were current hairdressers, were patch tested in an IVDK multi-centre study in Germany with the patch test standard series and the hairdressers' series. 21.4% were positive to toluene-2,5-diamine, 18.1% were positive to p-phenylenediamine, 4.0% to p-aminophenol, 3.4% to m-aminophenol. Results from previous periods were also presented - 14.3% were tested positive to toluene-2,5-diamine in 1990-1991 and 16.2% in 1993-1995. (Table 1)

Ref.: 64

106 dermatitis patients (hairdressers) in Greece (102 females and 4 males) were patch tested with the patch test standard series and the hairdressers' series. 10.3% were positive to toluene-2,5-diamine sulfate, 30.2% were positive to p-phenylenediamine, 8.4% to o-nitro-p-phenylenediamine, 4.7% to resorcinol, 4.3% to p-aminodiphenylamine, 2.8% to p-aminophenol. (Table 1)

Ref.: 65

In a multi-centre study by the European Environmental and Contact Dermatitis Research Group (EECDRG), a total of 809 dermatitis patients (hairdressers) were patch tested with hairdresser allergens in 9 centres. 7.6% were positive to toluene-2,5-diamine sulfate, 14.8% to p-phenylenediamine, 4.1% to o-nitro-p-phenylenediamine, 0.6% to resorcinol and 3.6% to p-aminodiphenylamine hydrochloride (Table 1).
In the same study, a total of 104 dermatitis patients identified as hairdressers' clients were patch tested with hairdresser allergens in 4 centres. 8.7% were positive to toluene-2,5-diamine sulfate, 19.2% to p-phenylenediamine, 7.7% to o-nitro-p-phenylenediamine, 1.9% to resorcinol and 3.9% to p-aminodiphenylamine hydrochloride (Table 4).

Ref.: 67

In a multi-centre study by the Italian Contact Dermatitis Research Group (GIRDCA), a total of 302 dermatitis patients (hairdressers) (259 females and 43 males) were patch tested with hairdressers' allergens in 9 Italian centres. 13.2% were positive to toluene-2,5-diamine sulfate, 16.6% to p-phenylenediamine base (in 1989-1990), 7.6% to p-phenylenediamine dihydrochloride (in 1985-1988), 7.9% to o-nitro-p-phenylenediamine, 1.3% to resorcinol and 10.6% to p-aminodiphenylamine. (Table 1)

Ref.: 68

In a multi-centre study by the German Contact Dermatitis Group (DKG), 178 dermatitis patients (hairdressers) were patch tested with hairdressers' allergens in 11 centres. 18.0% were test positive to toluene-2,5-diamine, 8.4% to toluene-2,5-diamine sulfate, 18.0% to p-phenylenediamine base, 0.6% to resorcinol, 1.1% to 3-aminophenol, 2.2% to p-aminodiphenylamine hydrochloride, 3.4% to 4-aminophenol and 6.2% to o-nitro-p-phenylenediamine. (Table 1)

Ref.: 69

103 hairdressers (not dermatitis patients) in the Netherlands (96 females and 8 males) were patch tested with a special series including standard allergens and hairdressers' allergens. 2% were positive to toluene-2,5-diamine sulfate, 6% to p-phenylenediamine and 4% to 2-nitro-4-phenylenediamine. (Table 1)

Ref.: 66

The degree and pattern of hand eczema in hairdresser trainees and hairdressers was compared in Norway. 75 hairdressers affected by hand eczema and 74 hairdresser trainees with or without hand eczema were examined and patch tested with a hairdressers' series and some additional substances from the standard series. 2.7% of the hairdressers affected by hand eczema were test positive to toluene-2,5-diamine sulfate, compared to 0% of the hairdresser trainees. (Table 2)

Ref.: 71

In a German multi-centre study by the IVDK, hairdressing cosmetics and hair care products were considered causative of contact dermatitis in a total of 2328 dermatitis patients (92% female). 884 of the cases were currently or had been working as hairdressers. 1217 had not been hairdressers (in the publication called clients). All were patch tested in 1995-2002. Among the hairdressers, 24.8% were test positive to toluene-2,5-diamine, 22.0% to p-phenylenediamine, 6.1% to p-aminophenol and 3.6% to m-aminophenol (Table 1). Among the non-hairdressers, 13.2% were test positive to toluene-2,5-diamine, 14.7% to p-phenylenediamine, 6.5% to p-aminophenol and 4.2% to m-aminophenol (Table 4)

Ref.: 72

209 dermatitis patients (hairdressers) in Italy (182 females and 27 males) were patch tested with a standard series and a hairdressers' series. 13.8% were positive to toluene-2,5-diamine sulfate, 36.8% to p-phenylenediamine base, 3.8% to p-aminodiphenylamine, 4.7% to o-nitro-p-phenylenediamine and 0.9% to resorcinol. (Table 1)

Ref.: 73

1000 dermatitis patients in Germany were patch tested with a standard series in 1970-1972 and another 1000 dermatitis patients were tested in 1976-1979. In 1970-1971, 2.3% were test positive to toluene-2,5-diamine (female 1.9%, male 2.9%), and 5.2% to Ursol (=p-phenylenediamine) (female 4.2%, male 6.6%). In 1997-1979, 3.4% were test positive to toluene-2,5-diamine (female 2.1, male 5.0%) and 7.0% to p-phenylenediamine (female 7.1%, male 6.8%). (Table 3)

Ref.: 74

5348 dermatitis patients were patch tested with a standard series in Hamburg, Germany. 2.7% were test positive to toluene-2,5-diamine (females 1.9%, males 3.8%) and 4.1% were test positive to p-phenylenediamine (females 4.2%, males 3.9%). (Table 3)

Ref.: 75

5202 dermatitis patients were patch tested in Belgium with a standard series and many patients were tested also with supplementary substances. 1.6% were test positive to toluene-2,5-diamine, 7.2% to p-phenylenediamine, 0.2% to resorcinol, 1.8% to o-nitro-PPD, 2.1% to p-aminodiphenylamine, 0.1% to p-toluene sulfate. (Table 3)

Ref.: 76

1385 dermatitis patients (824 females, 561 males) were patch tested in Vienna, Austria with a standard series. 2.5% were test positive to toluene-2,5-diamine, 3% to p-phenylenediamine and 0.4% to resorcinol. (Table 3)

Ref.: 77

261 dermatitis patients identified as hairdressers' clients, for whom treatment with hair dyes or permanent wave solutions was suspected to be the cause of the dermatitis (256 females, 5 males), were patch tested in Bologna, Italy with the Italian standards series for patch testing and with a hairdressers' screening series. 4.6% were test positive to toluene-2,5-diamine sulfate, 7.3% to p-phenylenediamine, 4.2% p-aminodiphenylamine, 4.6% to o-nitro-p-phenylenediamine and 0.4% to resorcinol. (Table 4)

Ref.: 78

154 dermatitis patients with a positive patch test reaction to p-phenylenediamine were tested further with para compounds frequently used in hair dyes, in Amsterdam, the Netherlands. 9.7% were positive to toluene-2,5-diamine sulfate, 15% to p-aminoazobenzene, 3.2% to p-aminophenol, 3.2% to o-nitro-p-phenylenediamine, 2.6% to p-aminodiphenylamine and 0.6% to resorcinol. (Table 4)

Ref.: 79

475 dermatitis patients for whom contact allergy to cosmetic ingredients had been shown by patch testing in 5 European centres in the UK, Germany and Belgium, were included in a retrospective study. 11 cases (possibly 2.3%) were tested positive to toluene-2,5-diamine, 33 cases to p-phenylenediamine, 8 cases to 2-nitro-p-phenylenediamine, 2 cases to n-phenyl-p-phenylenediamine, 1 case to resorcinol. It was not stated if all patients had been tested with all substances. (Table 4)

Ref.: 80

613 dermatitis patients had been patch tested with the German Contact Dermatitis Group (DKG) para-amino compounds test series. 10.0% were tested positive to toluene-2,5-diamine, 14.1% to p-phenylenediamine, 3.1% to p-aminophenol and 16.2% to p-aminoazobenzene. (Table 4)

Ref.: 81

819 dermatitis patients (589 females, 230 males, 1-93 years) in Belgium were patch tested with the standard series and from 16 years of age also with a complementary cosmetic-medicinal series, and depending on clinical history with additional tests. 0.6% were test positive to toluene-2,5-diamine, 2% to p-phenylenediamine, 0.2% to 3-aminophenol, 2-nitro-phenylenediamine and to 4-aminophenol. (Table 3)

Ref.: 82

Table 1: Contact allergy to toluene-2,5-diamine in patch tested dermatitis patients who were, or had been hairdresser. Test substance: toluene-2,5-diamine (TDA) or toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	No. tested patients	Positive patch test (%)	Year	Country	Ref.
TDAs	66	7.6	1973-1981	Canada	63 Lynde
TDA	597	21.4	1996-1998	Germany	64 Uter
TDAs	106	10.3	1985-1994	Greece	65 Katsarou

TDAs	781	7.6	1988-1991	9 European centres	67 EECDRG
TDAs	302	13.2	1985-1990	Italy	68 Guerra
TDA TDAs	178	18.0 8.4	1988-1989	Germany	69 Frosch
TDA	884 a)	24.8	1995-2002	Germany	72 Uter
TDAs	209	13.8	2002	Italy	73 Iorizzo
SUMMARY	**3123**	**Mean: 16.8%**			

a) hairdresser dermatitis patients with dermatitis from hair cosmetics

Table 2: Contact allergy to toluene-2,5-diamine in patch tested hairdressers and hairdresser trainees. Test substance: toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	Population	No. tested	Positive patch test (%)	Year	Country	Ref.
TDAs	Hairdressers in saloons	103	2%	1989-1992	The Netherlands	66 van der Walle
TDAs	Hairdressers with hand eczema	75	2.7%	1994	Norway	71 Holm
TDAs	Hair-dresser trainees, with or without hand eczema	74	0%	1994	Norway	71 Holm

Table 3: Contact allergy to toluene-2,5-diamine in patch tested unselected dermatitis patients. Test substance: toluene-2,5-diamine (TDA) or toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	No. tested patients	Positive patch test (%)	Country	Year	Ref.
TDA	1000 1000	2.3 3.4	Germany	1970-1971 1976-1979	74 Schwarz
TDA a)	5348	2.7	Germany	1976-1980	75 Kuhlwein
TDA	5202	1.6	Belgium	Not specified	76 Broueckx
TDA	1386	2.5	Austria	1972-1976	77 Jarisch
TDA	819	0.6	Belgium	1998-1999	82 Kohl
SUMMARY	**14755**	**Mean: 2.5%**			

a) 0.25% pet.

Table 4: Contact allergy to toluene-2,5-diamine in patch tested dermatitis patients selected due to symptoms or exposure related to cosmetics. Test substance: toluene-2,5-diamine (TDA) or toluene-2,5-diamine sulphate (TDAs) 1% in petrolatum

Test substance	No. tested patients and selection criteria	Positive patch test (%)	Country	Year	Ref.
TDAs	104 Hairdressers' clients	8.7	4 European centres	1988-1991	67 EECDRG
TDA	1217 Dermatitis from hair cosmetics, not hairdressers	13.2	Germany	1995-2002	72 Uter
TDAs	261 Hairdressers' clients	4.6	Italy	1985-1990	78 Guerra
TDAs	154 Patch-test pos. to PPD	9.7	The Netherlands	1996-1999	79 Koopmans

Test substance	No. tested patients and selection criteria	Positive patch test (%)	Country	Year	Ref.
TDA	475 Contact allergy to cosmetic ingredients	2.3	5 European centres	1996	80 Goossens
TDA	613 Tested with para amino compounds series	10.0	Germany	1995-1999	81 Uter
ALL	**2824**	**Mean: 9.5%**			

Conclusions
Results from several diagnostic patch studies in dermatitis patients show a high rate of contact allergy to toluene-2,5-diamine and toluene-2,5-diamine sulphate. The highest rate was found in dermatitis patients being hairdressers (16.8%, Table 1), followed by dermatitis patients selected due to symptoms or exposure related to cosmetics (9.5%, Table 4), and unselected dermatitis patients (2.4%, Table 3). The rate of contact allergy to toluene-2,5-diamine sulphate in hairdressers (not patients) was 2-2.7% (Table 2).

Due to different selection criteria and different patch test substances used (Table 1-4), conclusions cannot be drawn concerning the trend over time of contact allergy to toluene-2,5-diamine and toluene-2,5-diamine sulphate. The results indicate that patch test reactivity is higher to toluene-2,5-diamine than toluene-2,5-diamine sulphate (Table 1, particularly ref 69 Frosch).

In all publications (except ref Holm), results from patch testing with p-phenylenediamine is given and in several publications also results from tests with additional hair dye substances. In the majority of publications, the rate of contact allergy to p-phenylenediamine was the highest, followed closely by toluene-2,5-diamine, both generally much higher than to other hair dye substances. In some publications, the order between p-phenylenediamine and toluene-2,5-diamine was reversed.

The results do not allow further conclusions concerning concomitant patch test reactions - whether they were the result of multiple sensitisation, or if the result of cross-reactivity to different compounds was due to chemical similarity. Conclusions concerning cross-reactivity require animal studies where induction and elicitation are controlled.

Overall Conclusion Sensitisation
Toluene-2,5-diamine is a well recognised contact sensitizer in humans from both occupational and consumer exposure. Although an EC3 value of 0.31% from a LLNA assay suggests that it is a strong allergen, results from the GPMT indicates an extreme allergen. This latter classification is used in the Opinion.

3.3.4. Dermal / percutaneous absorption

Submission III, 2010

In Vitro Percutaneous Absorption of toluene-2,5-diamine sulfate

Guideline:	OECD guideline 428 (2004)
Tissue:	Human skin (abdomen, breast, or back; thickness: ca 400 µm)
Number of chambers:	18 from 3 donors for each dose formulation
Method:	Automated Teflon flow-through diffusion cells (0.64 cm^2 exposed area)
Integrity:	Tritiated water
Test substance:	1) Hair dye Color Cream formulation containing 0.25, 0.8, 2.4, or 7.2% toluene-2,5-diamine sulfate;

	2) Hair dye Color Cream formulation containing 0.5, 1.6, 4.8, or 14.4% toluene-2,5-diamine sulfate and 0.25, 0.793, 2.38, or 7.135% m-aminophenol mixed 1:1 with a solution containing 6% hydrogen peroxide prior to application.
Batch:	2346 (toluene-2,5-diamine sulfate) DTF0440082CF00 (for formulations)
Purity:	>99.5% HPLC
Radiolabel Batch:	CFQ40199 batch 1: 2,5-diamino[ring-U-14C]-toluene sulfate 72 mCi/mmol 2346: (non-labelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity (HPLC): 99.0% 99.7 area% at 290 nm (non-labelled compound)
Area Dosed:	20 mg formulation/cm²
Receptor fluid:	Saline (0.9% NaCl) with 0.01% sodium azide
Solubility:	10 g/l in water
Stability:	8 days
Analysis	Liquid Scintillation Counting
GLP:	In compliance
Date	October 2008

The skin absorption of toluene-2,5-diamine sulfate at concentrations of 0.25, 0.8, 2.4, or 7.2% was investigated with human skin (abdomen, breast, back, thickness ca 400 µm). An area dose of 20 mg/cm² of the final formulation was applied once to the skin (0.64 cm²) in a commercial oxidative hair dye formulation in either the presence or absence of hydrogen peroxide for 30 min.

Automated PTFE flow-through chambers were used. The receptor fluid (0.9% sodium chloride (w/v) containing 0.01% sodium azide, pH ca 6.5) was pumped through the receptor chamber at a rate of 1.6 ml/h. Eighteen chambers were investigated per formulation.

Thirty minutes after substance application, the test item was removed by washing the skin ten times with 0.32 ml water, then once with 0.32 ml washing solution (2% (v/v) sodium dodecyl sulfate) and again ten times with 0.32 ml water. The skin surface was dried with three cotton swabs. Cotton swabs were pooled per skin membrane and extracted with ethanol for at least 24 h. Receptor fluid was collected during the following intervals: 0-0.5 h, 0.5-1 hr, 1-2 h, followed by 2 h intervals until 24 h after application. After 24 h, the diffusion cells were dismantled and the receptor and donor compartments were washed twice with 1 ml ethanol. Each skin membrane was tape stripped 15 times. Skin membranes were digested in a 1.5 M KOH solution with 20% ethanol for at least 24 h.

Ultima GoldTM scintillation liquid was added to samples of the receptor fluid, the diffusion cell washes, the pooled cotton swab extracts, individual tape strips, and digested skin membrane, and radioactivity was determined by liquid scintillation counting.

Results and Discussion
The mean recovery of [^{14}C]-toluene-2,5-diamine sulfate ranged from 99.18 + 3.21% to 102.80 + 5.05% of the applied dose across the eight test groups.

The results of the study provided below are the mean values of 18 samples from 3 donors per formulation tested:

0.25% toluene-2,5-diamine sulfate

	non-oxidative	oxidative

Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	47.9 ± 0.4	100 ± 0.8	60.9 ± 9.3	100 ± 15.3
Skin wash	47.03 ± 2.54	98.13 ± 5.20	56.61 ± 9.66	92.69 ± 3.77
Stratum corneum	0.17 ± 0.03	0.36 ± 0.07	1.60 ± 0.32	2.72 ± 0.86
Skin	0.19 ± 0.10	0.40 ± 0.21	0.79 ± 0.43	1.32 ± 0.77
Receptor fluid	1.55 ± 0.47	3.24 ± 0.98	1.29 ± 0.26	2.15 ± 0.51
Total recovery	48.98 ± 2.40	102.18 ± 4.93	60.47 ± 9.61	99.18 ± 3.21
Total absorption*	**1.75 ± 0.52**	**3.65 ± 1.08**	**1.83 ± 0.46**	**3.49 ± 1.02**

0.8% toluene-2,5-diamine sulfate

	non-oxidative		oxidative	
Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	173.4 ± 23.9	100 ± 13.8	181.5 ± 31.2	100 ± 17.2
Skin wash	159.10 ± 21.80	91.94 ± 5.54	171.48 ± 32.18	94.25 ± 3.13
Stratum corneum	1.48 ± 0.64	0.89 ± 0.46	3.59 ± 1.40	1.98 ± 0.68
Skin	2.16 ± 0.89	1.25 ± 0.49	2.28 ± 1.33	1.23 ± 0.61
Receptor fluid	14.09 ± 2.78	8.29 ± 2.06	4.61 ± 1.36	2.64 ± 0.91
Total recovery	177.54 ± 20.45	102.80 ± 5.05	182.30 ± 32.93	100.30 ± 2.48
Total absorption*	**16.27 ± 2.94**	**9.55 ± 2.17**	**6.92 ± 1.56**	**3.88 ± 0.92**

2.4% toluene-2,5-diamine sulfate

	non-oxidative		oxidative	
Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	514.2 ± 66.7	100 ± 13.0	566.9 ± 83.7	100 ± 14.8
Skin wash	463.15 ± 66.67	89.98 ± 3.59	548.56 ± 89.60	96.62 ± 3.73
Stratum corneum	3.55 ± 1.87	0.69 ± 0.36	9.64 ± 3.94	1.78 ± 0.93
Skin	5.30 ± 2.20	1.05 ± 0.47	5.81 ± 2.42	1.07 ± 0.55
Receptor fluid	41.87 ± 11.66	8.19 ± 2.15	15.96 ± 4.52	2.83 ± 0.79
Total recovery	515.64 ± 68.90	100.25 ± 1.84	581.01 ± 88.22	102.49 ± 3.06
Total absorption*	**47.23 ± 13.35**	**9.25 ± 2.54**	**21.81 ± 4.87**	**3.90 ± 0.93**

7.2% toluene-2,5-diamine sulfate

	non-oxidative		oxidative	
Amount in	µg/cm² skin surface	% of applied dose	µg/cm² skin surface	% of applied dose
Applied formulation	1599.1 ± 285.6	100 ± 17.9	1574.4 ± 303.6	100 ± 19.3
Skin wash	1497.17 ± 278.24	93.58 ± 2.94	1483.91 ± 295.61	94.19 ± 3.79
Stratum corneum	9.03 ± 3.48	0.57 ± 0.20	32.62 ± 10.95	2.12 ± 0.70
Skin	10.06 ± 3.79	0.63 ± 0.21	11.25 ± 6.42	0.71 ± 0.36
Receptor fluid	90.82 ± 33.76	5.79 ± 2.20	42.63 ± 11.23	2.76 ± 0.75
Total recovery	1616.29 ± 284.15	101.14 ± 1.27	1577.34 ± 302.13	100.26 ± 3.37
Total absorption*	**101.03 ± 36.67**	**6.43 ± 2.36**	**54.03 ± 15.38**	**3.49 ± 0.92**

* Total absorption is defined as the amount in the receptor fluid, the receptor compartment wash and skin membrane (epidermis and dermis), excluding tape strips.

Under both oxidative and non-oxidative conditions, a linear increase in the amount of toluene-2,5-diamine sulfate in the receptor fluid and the total amount absorbed (receptor fluid plus skin (epidermis plus dermis) was observed. Under oxidative conditions, the amount of radioactivity recovered from the tape strips (i.e., stratum corneum) was considerably higher when compared to non-oxidative conditions, whereas the total amount absorbed was lower under oxidative conditions when compared to non-oxidative conditions with the exception of the lowest concentration tested (0.25% toluene-2,5-diamine sulfate).

Ref.: 11 (subm III)

Comment
These were well performed studies. Therefore, mean + 1SD may be used as the amount absorbed in calculating the MOS. Experiments were conducted with toluene-2,5-diamine sulfate in a range of dilutions from 0.25% to 7.2%.
For 2.4% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 47.23 + 13.35 = 60.58 µg/cm². Under oxidative conditions, the amount absorbed is 21.81 +4.87 = 26.68 µg/cm². The latter value may be used for the conventional MOS calculation after correction (26.68 x 3.6 / 2.4 = 40.02 µg/cm²) since the intended on head concentration is 3.6%.

Taken from SCCP/1084/07

Percutaneous absorption/penetration in-vitro, human skin

Guideline:	/
Tissue:	Human abdominal skin (thickness 350 µm) from women, 3 donors
Method:	Dynamic diffusion cells, surface area of application 2.0 cm^2
Test substance:	Formulation 175307/Water: *toluene-2,5-diamine sulfate* (**4.5%** final applied concentration) in a hair dye formulation mixed with water prior to application Formulation 175307/peroxide: *toluene-2,5-diamine sulfate* (**4.5%** final applied concentration) in a hair dye formulation mixed with hydrogen peroxide prior to application Formulation 175308/peroxide: *toluene-2,5-diamine sulfate* (**4.5%** final applied concentration) in a hair dye formulation containing m-aminophenol coupler and mixed with hydrogen peroxide prior to application
Batch:	CFQ9920 (^{14}C-labelled substance) CH1134 (non-labelled substance)
Purity:	Radiochemical purity: 97.2% (HPLC) Non-labelled substance: 99.7% (titrated); o-toluidine < 100 ppm (HPLC)
Dose levels:	0.9 mg *toluene-2,5-diamine sulfate* / cm^2
Dosing schedule:	30 min application
Replicates:	Two different *in vitro* systems were used: dermatomed human skin (8 replicates) and isolated human epidermis (8 replicates)
Study year:	1997
GLP:	not in compliance

[^{14}C]-*Toluene-2,5-diamine sulfate* and non-labelled substance were added to a final concentration of 9% to the hair dye bases no. 175307 and no. 175308. The formulation no. 175308 contained an equimolar amount of the coupler m-aminophenol.

Three different mixtures were then prepared:

1. formulation no. 175307 mixed with water (50:50)
2. formulation no. 175307 mixed with hydrogen peroxide (50:50)
3. formulation no. 175308 mixed with hydrogen peroxide (50:50) and coupler m-aminophenol.

These mixtures were applied in amounts of 20 mg/cm^2 (equals 0.9 mg toluene-2,5-diamine sulfate/cm^2) to the surface of either human skin samples of 350 µm thickness or samples of isolated epidermis mounted in perfusion cells with 2 cm^2 application area. For each *in vitro* model, 8 perfusion cells were initially prepared and a skin integrity test was performed. After 30 min the test substance was rinsed off with water and sodium lauryl sulfate solution. The intradermal distribution, the amounts of penetrated substance and the kinetics of the dermal penetration of [^{14}C]-toluene-2,5-diamine sulfate were analysed over a period of 24 h. Results for the dermatomed skin are presented below.

Results
The number of cells which could be used for data analysis was 7, 6 and 4 respectively for the three formulations using isolated skin samples and 8, 7 and 7 respectively for the dermatomed skin.

The majority of the test substance (93.5% of the applied dose) was recovered from the skin by rinsing 30 min after application. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, approached a plateau at approximately 5 hours. The presence of peroxide diminished the absorbed amount of radioactivity while the amount adsorbed in the stratum corneum was increased. As a consequence the sum of the amounts of radioactivity in the total skin and in the receptor fluid was about the same for the three formulations.

The systemically available amount of [^{14}C]-toluene-2,5-diamine sulfate was calculated as the amount found in the receptor fluid plus the residual amount in the skin, excluding the amount adsorbed to the stratum corneum. The results for the dermatomed skin samples were as follows:

	Total absorption	
	µg$_{eq}$/cm^2	**% of applied dose**
Formulation 175307/water	40.31 ± 23.77 (19.48-78.03)	4.17 ± 2.54 (2.04-8.56)
Formulation 175307/peroxide	32.03 ± 26.58 (12.54-76.04)	3.44 ± 2.84 (1.29-8.12)
Formulation 175308/peroxide/coupler	31.26 ± 11.64 (16.54-51.82)	3.41 ± 1.32 (1.88-6.07)

Ref.: 28

Percutaneous absorption/penetration in vitro, pig skin (study 1)

Guideline:	OECD Draft Guideline - Skin absorption: *in vitro* method (2000)
Tissue:	Porcine skin (thickness: 560-750 µm); 2 donors, 1 male, 1 female
Test substance:	Formulation A: toluene-2,5-diamine sulfate (5.4% final applied concentration) in a hair dye formulation containing resorcinol coupler; mixed with water prior to application Formulation B: 5.4% toluene-2,5-diamine sulfate (5.4% final applied concentration) in a hair dye formulation containing coupler; mixed with a solution of hydrogen peroxide (3% final concentration) prior to application Formulation C: toluene-2,5-diamine sulfate (5.4%) in an aqueous solution
Method:	Static diffusion cells, surface area of application 1.0 cm^2
Batch:	3362-259 [Ring ^{14}C (U)]-toluene-2,5-diamine sulfate 1.49 GBq/mmol (40.37 mCi/mmol)

	46847 (non-labelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity (HPLC): 99.3% 99.9% (non-labelled substance)
Dose Applied:	1.08 mg toluene-2,5-diamine sulfate/cm^2 (20 mg of formulation)
Replicates:	2 experiments, 6 replicates for each pig for each experimental group in each experiment
GLP:	In compliance

The percutaneous penetration of toluene-2,5-diamine sulfate at a concentration of 5.4%, below the maximum concentration intended for hair colorants, was investigated with pig skin prepared from animals and stored at -20 °C until use.

The test substance was applied in a formulation containing resorcinol (coupler) which was mixed with either water or hydrogen peroxide (final concentration 3%) prior to application. A preparation of 5.4% toluene-2,5-diamine sulfate in water was also tested. Skin integrity was determined at the start and at termination by measurement of the transdermal electrical resistance.
20 mg of the respective formulation (corresponding to 1.08 mg toluene-2,5-diamine sulfate and ca. 0.5 MBq/cm^2) was applied to the skin surface, and rinsed off after 30 minutes. Samples of the receptor fluid were taken before application of the test substance and after 0.5 h, 1 h, 2 h, 4 h, 6 h, 24 h, 29 h, 48 h. At the end of the experiment the skin was rinsed, and the stratum corneum was stripped with adhesive tape. Radioactivity in the receptor fluid, skin, tapes, and rinsings was measured by liquid scintillation counting.

Results
The majority of the test substance (86.9-95.8% of the applied dose) was recovered from the skin by rinsing 30 min after application. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, approached a plateau at approximately 24 hours. Percutaneous absorption was calculated by adding the amounts of radioactivity measured in epidermis to those in dermis and in receptor fluid (see table below). The total recovery was 98.4%, 94.4%, and 96.2% for Formulations A, B, and C, respectively.

5.4% final			Total absorption	
	Receptor Fluid ($\mu g_{eq}/cm^2$)	Epidermis plus dermis ($\mu g_{eq}/cm^2$)	$\mu g_{eq}/cm^2$	% of applied dose
Formulation A (with coupler + water)	10.7	10.1	20.84 ± 5.1 (13.84–32.06)	1.72 ± 0.32 (1.23-2.44)
Formulation B (with coupler + peroxide	14.9	13.5 $\mu g_{eq}/cm^2$	28.46 ± 9.5 (15.68- 44.90)	2.39 ± 0.79 (1.25-3.88)
Formulation C (aqueous vehicle)*	13.3	28.6 $\mu g_{eq}/cm^2$	41.98 ± 19.9 (18.20-75.99)	3.94 ± 2.07 (1.60-8.27)

* The higher values for epidermis plus dermis for Formulation C were attributed to a methodological problem with the first experiment run with this formulation.

Ref.: 29

Dermal absorption / penetration in vitro, pig skin (study 2)

Guideline:	OECD Draft Guideline – Skin absorption: *in vitro* method (2000)
Tissue:	Porcine back skin (thickness: 0.9 ± 0.1 mm); 1 female donor
Method:	Dynamic Teflon diffusion chambers, surface area of application 4.0 cm^2
Test substance:	4.6% toluene-2,5-diamine sulfate in a hair dye formulation not containing coupler or hydrogen peroxide
Batch:	CFQ13783 batch1 [ring-^{14}C (U)]-toluene-2,5-diamine sulfate (55 mCi/mmol) R0017930 (Ondal) non-labelled toluene-2,5-diamine sulfate
Purity:	[ring-^{14}C (U)]-toluene-2,5-diamine sulfate HPLC: 98.2 area% (254 nm)

	Non-labelled-toluene-2,5-diamine sulfate HPLC: 99.9 area% (254 nm); 99.7 weight% by NMR
Dose applied:	4.6 mg toluene-2,5-diamine sulfate / cm^2 (100 mg of formulation)
Replicates:	6
GLP:	In compliance

Dermatomed skin preparations of ~1000 µm thickness were mounted into Teflon diffusion cells with an application area of 4 cm^2. An integrity test using tritiated water was performed before the skin samples were covered with the test substance (400 mg of the hair dye formulation containing 4.6% of toluene-2,5-diamine sulfate per 4 cm^2). After 30 min the formulation was removed by intensive washing and fractions of the receptor fluid were collected after 16 h, 24 h, 40 h, 48 h, 64 h and 72 h. Epidermis and upper dermis were separated by heat treatment and extracted for analysis.

Results
The majority of the test substance (92.8%) was removed from the skin by rinsing 30 min after application. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, approached a plateau at approximately 24 hours. Percutaneous absorption was calculated by summing the cumulative amounts of substance measured in the receptor fluid and upper dermis. The results were as follows:

Receptor Fluid	Upper dermis	Total absorption
5.6 ± 1.7 (4.0-8.2)	4.9 ± 1.9 (3.3-8.0)	10.5 ± 3.2 (0.2% of applied dose) A_{max} 16.2 (0.4% of applied dose)

There was no tape stripping of the skin to remove stratum corneum from the epidermis, and epidermis was not included in the calculation of total percutaneous absorption. The amount present in the epidermis corresponded to 7.0 ± 1.4 $\mu g_{eq}/cm^2$. Total recovery of radioactivity was 93.2%.

Ref.: 30

Comment
The test formulation contained only 4.6% toluene-2,5-diamine sulfate where the maximum in-use concentration is 7.2%. The amount of formulation applied (100 mg/cm²) is not in accordance with the SCCP Notes of Guidance (20 mg formulation/cm²).

Dermal absorption / penetration in vitro, pig skin (study 3)

Guideline:	OECD Draft guideline: Skin absorption: *in vitro* method (1999)
Tissue:	Porcine skin (thickness: 1 mm); 1 male donor
Method:	Dynamic diffusion chambers, surface area of application 0.785 cm²
Test substance:	0.6% 2-methyl-1,4-benzenediamine sulphate in acetone/H_2O/olive oil (2:2:1)
Batch:	R0054969
Purity:	> 99% (HPLC)
Dose applied:	0.59 mg 2-methyl-1,4-benzenediamine sulphate /cm^2 (78µl of solution applied)
Replicates:	6 (5 used for analysis)
GLP:	In compliance

The objective of this study was to examine dermal penetration from the vehicle used in a Local Lymph Node Assay (LLNA).

A solution of 2-methyl-1,4-benzenediamine sulphate (corresponding to 0.59 mg/cm²) in acetone/H_2O/olive oil (2:2:1) was applied to the skin samples and rinsed off after 30 min

using water and a shampoo. The receptor fluid was sampled after 16, 24, 40, 48, 64 and 72 h and analysed for 2-methyl-1,4-benzenediamine sulphate by HPLC. Subsequently upper skin (stratum corneum + upper *stratum germinativum*) and lower skin (lower *stratum germinativum* + upper dermis) were separated by heat treatment. Extracts of skin layers were prepared and analysed by HPLC.

Results
The majority of the test substance (>100%) was removed from the skin by rinsing 30 min after application. Total recovery was 107.7%. The cumulative penetration, based on the amount of radioactivity in the receptor fluid, had reached a plateau at 16 hours. Percutaneous absorption was calculated by summing the cumulative amounts of substance measured in the receptor fluid and upper dermis. The results were as follows:

Receptor Fluid $\mu g_{eq}/cm^2$	**Lower skin (lower *stratum germinativum* + upper dermis) $\mu g_{eq}/cm^2$**	**Total absorption $\mu g_{eq}/cm^2$**
24.7 $\pm$ 7.2 (18.8-36.9)	7.2 $\pm$ 3.3 (3.94-12.64)	31.9 (5.35% of applied dose) A_{max} 42.2 (7.08% of applied dose)

Ref.: 31

Overview of the *in vitro* percutaneous absorption studies that have been performed with t*oluene-2,5-diamine sulfate:*

Studies from submission II		
Human skin	^{14}C-labelled	Formulation (4.5%) without coupler; mixed with water Formulation (4.5%) without coupler; mixed with hydrogen peroxide Formulation (4.5%) containing coupler (m-aminophenol); mixed with peroxide
Pig skin (study 1)	^{14}C-labelled	Formulation (5.4%) with coupler (resorcinol); mixed with water Formulation (5.4%) with coupler (resorcinol); mixed with hydrogen peroxide Aqueous solution (5.4%)
Pig skin (study 2)	^{14}C-labelled	Formulation without coupler or hydrogen peroxide
Pig skin (study 3)	Non-labelled	0.6% solution of 2-methyl-1,4-benzenediamine sulphate in acetone/water/olive oil
Studies from submission III		
Human skin	^{14}C-labelled	Formulation containing 0.25, 0.8, 2.4, or 7.2% toluene-2,5-diamine sulfate without coupler Formulation containing 0.5, 1.6, 4.8, or 14.4% toluene-2,5-diamine sulfate containing coupler (m-aminophenol); mixed with peroxide

General conclusion on percutaneous absorption studies *in vitro*
Percutaneous absorption studies have been conducted in both human skin and pig skin *in vitro.*
In the first study with human skin (submission II), using formulations containing 4.5% toluene-2,5-diamine sulfate + coupler + hydrogen peroxide, the percutaneous absorption was 31.26 ± 11.64 (A_{max} 51.82) $\mu g_{eq}/cm^2$ (3.41 ± 1.32 (A_{max} 6.07) % of applied dose). The value of 51.82 $\mu g_{eq}/cm^2$ (A_{max}, formulation + hydrogen peroxide + coupler) after extrapolation to 7.2% concentration (51.82 x 7.2/4.5 = 82.9 $\mu g_{eq}/cm^2$) was used for the calculation of the Margin of Safety in the previous opinion SCCP/1084/07.

Studies with pig skin employed various application conditions (e.g., formulations containing coupler with or without hydrogen peroxide, formulations without coupler and peroxide, aqueous solution, or acetone/water/olive oil solution). The percutaneous absorption values for formulations containing 4.6 - 5.4% toluene-2,5-diamine sulfate (including studies of formulations with and without coupler + peroxide) ranged from 10.5 to 28.5 $\mu g_{eq}/cm^2$ (0.2 to 2.39% of applied dose). The study involving 0.6% toluene-2,5-diamine sulfate in an acetone/water/olive oil vehicle showed the greatest penetration (31.9 (A_{max} 42.2) $\mu g_{eq}/cm^2$ or 5.35 (A_{max} 7.08) % of applied dose), including the greatest amount present in the receptor fluid, but the application vehicle was not considered representative of human use conditions. These results did however confirm the adequacy of the vehicle used in the LLNA.

The percutaneous absorption values obtained in these *in vitro* experiments were comparable to those calculated from a study conducted in human volunteers with a ^{14}C-toluene-2,5-diamine sulfate-containing hair dye (20 or 71 $\mu g_{eq}/cm^2$ see Section 3.3.9.2.).

The data obtained in the different percutaneous absorption studies vary in the range of approximately 10 to 70 µg/cm². Such a variation is expected in view of the differences in design and data evaluation across studies. Factors which influenced the results were related to the test formulation (test substance concentration, presence of hydrogen peroxide and reaction partner, vehicle) to test model details (species/source and skin type) and to the number and type of compartments included in the calculation of systemic exposure.

In submission III of 2010 an additional well performed dermal absorption study with human skin *in vitro* was included. From this study the mean + 1SD may be used as the amount absorbed. The intended use of toluene-2,5-diamine was at a maximum of 4% 'on head' (7.2% calculated as sulphate salt). Experiments with toluene-2,5-diamine sulfate were conducted in a range of dilutions from 0.25% to 7.2%. For 7.2% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) was 137.7 µg/cm². Under oxidative conditions, the amount absorbed is 69.4 µg/cm², which was used for MOS calculation in a previous opinion (SCCS/1390/11).

For 2.4% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 47.23 + 13.35 = 60.58 µg/cm². Under oxidative conditions, the amount absorbed is 21.81 + 4.87 = 26.68 µg/cm². The latter value may be used for the conventional MoS calculation after correction (26.68 x 3.6 / 2.4 = 40.02 µg/cm²) since the intended on head concentration is 3.6%.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral / dermal / inhalation toxicity

Range finding study

Guideline:	/
Species/strain:	Sprague-Dawley rats, Crl:CD(SD)BR
Group size:	10 animals per sex per dose
Test substance:	toluene-2,5-diamine sulfate in deionised water
Batch:	CH 1143
Purity:	99.7%
Dose:	0, 7.5, 15, 30, 60 mg/kg bw/d
Route:	oral, gavage
Exposure:	once daily for 14 d
GLP:	in compliance

Doses of 0, 7.5, 15, 30, 60 mg/kg bw/d of toluene-2,5-diamine sulfate in deionised water were given once daily to 10 rats of each sex. Animals were observed twice daily for mortality and daily for clinical signs, body weights and food intake were recorded weekly. At

the end of treatment period blood samples for the investigation of haematology and biochemistry were taken, the animals were sacrificed and subjected to necropsy, organs were weighed and tissues were examined microscopically.

Results
One animal died after blood withdrawal which was not associated with the test substance. No treatment related clinical observations were recorded and body weight and food intake were not changed. While no haematological parameters were altered several biochemistry parameters were influenced at and above 30 mg/kg bw/d (AST, CPK, LDH, ALT (only at 60 mg/kg bw/d)). The mean absolute and relative (to body weight) liver weights of both sexes at 60 and males only at 30 mg/kg bw/d were increased. At necropsy no macroscopic abnormalities were noted. Myocyte degeneration was noted in the heart, skeletal muscle, tongue and diaphragm in both sexes in all dose groups.

Ref.: 32

Peer review of two external experts
The histological slides from relevant tissues (diaphragm, skeletal muscle, tongue and heart) from this study were reviewed by two independent pathologists. Both pathologists agreed that treatment-related myofiber necrosis, degeneration, and/or inflammatory change were present in skeletal muscle, tongue, and diaphragm of both males and females given 30 or 60 mg/kg bw/d in this study and that the NOAEL for muscle degenerative change in this study was 15 mg/kg bw/d.

Ref.: 4, 5 (subm III)

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

Study 1

Guideline:	OECD 408 (1981)
Species/strain:	Sprague-Dawley rats, Crl:CD(SD)BR
Group size:	15 animals per sex per dose
Test substance:	toluene-2,5-diamine sulfate in deionised water
Batch:	CH 1143
Purity:	99.7%
Dose:	0, 2.5, 5, 10, 20 mg/kg bw/d
Route:	oral, gavage
Exposure:	once daily for 13 weeks
GLP:	in compliance

Doses of 0, 2.5, 5, 10, 20 mg/kg bw/d of toluene-2,5-diamine sulfate in deionised water were given once daily to 15 rats of each sex for 13 weeks. Animals were observed daily for mortality and clinical signs, body weights and food intake were recorded weekly. Ophthalmoscopy was performed on all animals before the start of treatment and during week 13. Blood and urine samples were taken during weeks 4 and 12/13.

At the end of treatment period the animals were sacrificed and subjected to necropsy, organs were weighed and tissues were examined microscopically.

Results
While 2 males were killed *in extremis* no treatment related deaths were observed. No treatment-related clinical signs were observed. Body weights and body weight gains as well as food consumption were not affected by treatment. Changes in haematology were not considered to be test substance related. In blood chemistry significant increases in AST levels were seen in females from 5 mg/kg bw/d upwards and urinalysis revealed increased urine levels associated with decreases in specific gravity at 10 (females) and 20 mg/kg bw/d (males and females). Ophthalmoscopic examination revealed retinal hyper-reflectivity in 2 males given 20 mg/kg bw/d, 1 male given 2.5 mg/kg bw/d and 1 female given 5 mg/kg

bw/d. During histopathology retinal degeneration was diagnosed only for the males. The results were re-evaluated in a pathology peer review and it was concluded that this linear focal retinopathy was similar to the spontaneous incidence of focal linear degeneration of around 3% in this rat strain. No dose-response relationship was seen. At 20 mg/kg bw/d an increased incidence of abnormally shaped pituitary glands was observed.

Conclusion
The NOAEL is considered to be 2.5 mg/kg bw/d (free base: 1.4 mg/kg bw/d), based on an increase in AST levels.

Ref.: 33

Comment of the SCCP
The myocyte degeneration observed in the dose range finding study was not reported in the main study. Both evaluations were made by the same evaluator in the same time period. No comment on these conflicting results was given in discussion of the study results as well as in the dossier.
A further 12-week oral toxicity study in rats was cited in Ref. 52 and also referenced in the dossier (Ref. 93) but not provided to the SCCP. This study should be checked with regard to myopathies.

Peer review of two external experts
The pathology experts reviewed the clinical chemistry data from this 90-day study in order to clarify the toxicological relevance of those findings. Both pathologists agreed that the minimal elevations in AST concentration observed in females, but not in males, at week 4 at doses of 5, 10, and 20 mg/kg bw/d in the 90-day study were not related to treatment. These changes were not dose-related, of minimal magnitude (not higher than 1.1-fold the mean control value), and individual values were essentially within the historical control range. No elevation in AST concentration was observed at the end of the study in males or females at doses of up to 10 mg/kg bw/d. At 20 mg/kg bw/d, mean AST concentrations were elevated in males and females at the end of the study as some animals clearly exceeded historical control ranges. One of the reviewers noted, in contrast to the original evaluation and the second reviewer, muscle degeneration in multiple organs at 20 mg/kg bw/d. Considering the AST results as well as other clinical pathology results, both pathologists concluded that the NOAEL for clinical pathology was 10 mg/kg bw/d.

Comment of the SCCS
AST (aspartate aminotransferase, also known as glutamate oxalacetate transaminase, GOT) release is closely related to myotoxicity and, therefore, changes in the plasma level have to be considered with a substance known to induce myodegenerative changes. It is well known that p-phenylenediamine and several derivatives including toluene-2,5-diamine induce such effects on skeletal muscles. Toluene-2,5-diamine was effective already after a s.c. 3-day treatment with 22 mg/kg bw/d in rats (e.g. Munday et al. 1990; Munday, Manns 1999). However, the slightly increased values observed at week 4 in the dose groups 5, 10, and 20 mg/kg bw (89, 89, 87) were not dose-related and well within historical controls (61-105). In contrast, at 20 mg/kg bw/d in week 13 the figure (135) was clearly exceeding controls and according to the one peer reviewer also degenerative changes were seen at this dose.

Ref.:AR1, AR2

Study 2 (Submission III, 2010)

Guideline:	OECD Guideline 408 (1981)
Species/strain:	Sprague Dawley Rats, Crl:CD(SD)
Group size:	15 animals per sex per dose level plus 5 animals per sex per dose level for 28-day recovery in control and 20 mg/kg bw/d groups
Test substance:	toluene-2,5-diamine sulfate
Batch:	01057257RB-03

Purity:	99.8%
Vehicle:	Reverse osmosis deionized water, pH 5.0 ± 0.3
Dose levels:	0, 2.5, 5, 10, and 20 mg/kg bw/d
Dose volume:	10 ml/kg bw
Route:	Oral, gavage
Exposure period:	Once daily over 91 days, followed by a 28-day recovery period (control and 20 mg/kg bw/d groups only)
Date:	07/2009 – 08/2010 (performed outside the EU)
GLP:	In compliance

Doses of 0 (control group), 2.5, 5, 10, and 20 mg/kg bw/d of toluene-2,5-diamine sulfate dissolved in vehicle (reverse osmosis deionized water, pH 5.0 ± 0.3) were administered once daily to groups of 15 male and 15 female Sprague Dawley rats by oral gavage in a total volume of 10 ml/kg bw for a periods of 91 consecutive days followed by a 28-day recovery period (control and 20 mg/kg bw/d groups only). The control group received vehicle only. Dose formulations were prepared daily. Animals were housed individually.

Dose levels were selected on the basis of a previously conducted 13 week oral toxicity study included in Submission II (Ref. 33). A NOAEL was considered by the SCCP to be 2.5 mg/kg bw/d (free base: 1.4 mg/kg bw/d), based on an increase in AST levels and conflicting results on myocyte degeneration observed in the dose range finding and the main study (SCCP/1084/07).

In-life evaluations included twice daily mortality/morbidity checks, daily recording of clinical signs, weekly recording of clinical observations, body weight and food consumption, abbreviated functional observational battery (FOB) (days 26 and 54) and full FOB (days 89-90), and ophthalmology examination during week 13. Blood and urine were collected for clinical pathology at the end of week 13 and at the end of the recovery period. All animals were subjected to a complete gross necropsy examination. Organ weights were measured and tissues were taken from animals in all treatment groups and recovery groups and preserved in 10% neutral buffered formalin. Histopathological investigation was performed on all collected tissues from the animals in the control, high dose, and recovery group. The skeletal muscle (thigh and diaphragm), tongue, heart, and periocular muscle were identified as possible target tissues in the high dose treatment group (20 mg/kg bw/d), and therefore, these tissues were processed and examined from the other treatment groups (2.5, 5, and 10 mg/kg bw/d).

Results
Dose formulations prepared and analyzed on Days 1, 8, 50, and 85 verified that the target concentrations were achieved (± 15%).

No treatment-related mortality occurred and there were no adverse treatment-related effects on clinical signs, functional observational battery findings, body weights, body weight changes, food consumption, ophthalmic findings, haematology parameters, coagulation parameters, urinalysis parameters, gross necropsy findings, or organ weights.

The only treatment-related clinical chemistry change was an elevation in aspartate aminotransferase (AST) at both males and females at 20 mg/kg bw/d, although the increase in the mean value in males was not statistically significant and substantial elevations were observed in only 4/15 animals whereas in females 12/15 AST values were substantially elevated. The elevation in AST correlated with microscopic changes in skeletal muscle. At the end of the 28-day recovery period, AST levels in 20 mg/kg bw/d animals were comparable to controls.

Treatment-related microscopic changes were observed in males and females at 20 mg/kg bw/d and were found in skeletal muscle of the thigh, diaphragm, and tongue and in the periocular muscle of the eye. These changes consisted of mononuclear cell infiltrates (eye,

diaphragm, thigh, and tongue), muscular degeneration (diaphragm, thigh, and tongue), and muscular regeneration (diaphragm). After a 28-day treatment-free period, the treatment-related findings noted at the terminal sacrifice were not present at the recovery group sacrifice.

Conclusion
Administration of toluene-2,5-diamine sulfate once daily by oral gavage at a dose of 20 mg/kg bw/d was associated with microscopic changes in the skeletal muscle of the thigh, diaphragm, tongue, and periocular muscle that correlated with elevations in AST. These target organ effects resolved following the 28-day recovery period. No treatment-related findings were reported at 10 mg/kg bw/d. Based on these results, the no-observed-adverse-effect level (NOAEL) was determined to be 10 mg/kg bw/d.

Ref.: 13 (subm III)

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Taken from SCCP/1084/07

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537
Replicates:	triplicates in 2 individual experiments both in the presence and absence of S9-mix.
Test substance:	toluene-2,5-diamine sulfate
Solvent:	DMSO
Batch:	R99053665
Purity:	99.2 - 99.8%
Concentrations:	3 - 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with at least 48 h incubation without and with S9-mix
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment for toxicity and mutation induction with all strains and both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as experiment I. Both experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Toxic effects evident as reduction in the number of revertants were observed at 2500 µg/plate and above with the exception of TA100 and TA102 without S9-mix (5000 µg/plate) and TA102 with S9-mix where no toxicity were seen. All incubated plates showed normal background growth up to 5000 µg/plate.

In both experiments in the presence of S9-mix a dose dependent increase in revertant colonies was observed in TA98, TA100, TA1535 and TA1537. In TA102 and in the absence of S9-mix in all five tester strains toluene-2,5-diamine sulfate did not induce a biologically relevant increase in revertant colonies.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate was genotoxic (mutagenic) in this gene mutation test in bacteria.

Ref.: 34

In Vitro* Mammalian Cell Gene Mutation test (*tk* locus*)

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	triplicates in 2 independent experiments
Test substance:	A 5 (toluene-2,5-diamine sulfate)
Solvent:	NH_4OH (1%)
Batch:	EFH 290394
Purity:	97.3% (technical product)
Concentrations:	1.0 - 15.0 µg/ml (without S9-mix) 10.0 - 100.0 µg/ml (with S9-mix)
Treatment	4 h treatment without and with S9-mix; expression period 72 h and selection period of 11-13 days
GLP:	in compliance

Toluene-2,5-diamine sulfate was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth. In the main tests, cells were treated for 4 h followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment on toxicity distinct toxic effects could be observed with concentrations above 3.0 µg/ml. In both experiments the required toxicity was reached (10-20% survival compared to the concurrent negative controls) without S9-mix. In the presence of S9-mix the required level of toxicity was not achieved.
Occasionally an increase in mutant frequency was observed in both experiments with and without S9-mix. However, these results appeared not reproducible and are, therefore, considered as not biologically relevant.

Conclusion
Under the experimental conditions used, toluene-2,5-diamine sulfate did not induce gene mutations in this gene mutation test in mammalian cells.

Ref.: 35

Comments
The required toxicity (10-20% survival compared to the concurrent negative controls) was not reached in the experiments with S9-mix.

***In vitro* Chromosome Aberration Test**

Guideline:	OECD 473
Replicates:	duplicate cultures
Cells:	V79
Test substance:	SAT 010935 (toluene-2,5-diamine sulfate)
Solvent:	culture medium (Minimum essential medium, MEM)
Batch:	46847
Purity:	99.9%
Concentrations:	2.5, 5.0 and 10.0 µg/ml without S9-mix 100, 200, 300 and 400 µg/ml with S9-mix
Treatment:	4 h treatment and harvest time 18 after start of treatment both in the absence and presence of S9-mix
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in V79 cells. Test concentrations were based on the results of a range finding pre-test on cell number and cell morphology with 4 h and 24 h treatment. The highest dose in the pre-test was the prescribed maximum concentration (2210 µg/ml ≈ 10 mM). Cells were treated for 4 h and harvested 18 h after the start of treatment. 2.5 h before harvest, each culture was treated with colcemid (final concentration 0.2 µg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring the decrease in the mitotic index. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations and the mitotic index. Negative and positive controls were in accordance with the OECD draft guideline.

Results
In the pre-test, precipitation was observed at doses far above the test doses of the main test. No relevant influence of toluene-2,5-diamine sulfate on the osmolarity was observed. A slight pH shift was adjusted with NaOH.
Biologically relevant increases in polyploid metaphases were not found. At the concentrations evaluated, no clear toxic effects indicated by reduced mitotic indices or reduced cell numbers were found. The required 50% decrease in MI compared to the concurrent control at the highest dose tested was not reached.
Both in the absence and presence of S9-mix toluene-2,5-diamine sulfate induced a more or less dose dependent and biologically relevant increase in cells with chromosomal aberrations.

Conclusion
Under the experimental conditions used the increase in cells with structural chromosomal aberrations indicates a genotoxic (clastogenic) activity of toluene-2,5-diamine sulfate in V79 cells *in vitro*.

Ref.: 36

***In vitro* unscheduled DNA synthesis test**

Guideline:	/
Cells:	hepatocytes from male Sprague-Dawley caesarean-derived rats (Crl:COBS®(SD)BR) or Golden Syrian hamsters (LAK: LVG(SYR))
Replicates:	2 independent experiments
Test substance:	2,5-diaminotoluene
Solvent:	DMSO
Batch:	/
Purity:	95%

Concentrations: 10^{-4}, 10^{-5}, 10^{-6} and 2 x 10^{-7} M
Treatment: 4 h treatment; fixation of the cells after overnight culture.
GLP: not in compliance

2,5-Diaminotoluene was investigated for the induction of unscheduled DNA synthesis (UDS) in primary hepatocytes isolated from rats and hamsters.
Cells were treated for 4 h with 2,5-diaminotoluene and (me-^{3}H)-thymidine (specific activity 70 -80 Ci/mmol) and further cultured overnight. Slides were then progressed for autoradiography.
Evaluation of autoradiography was done after 10 days exposure and methyl-green Pyronin Y staining. UDS was measured by counting the number of grains in each nucleus and subtracting the average number of grains present in 3 equal-sized adjacent cytoplasmic areas (net nuclear grain). The average net nuclear grain count for 60 cells per slide was calculated and the percentage of cells with > 5 net nuclear grains was determined.

Results
2,5-Diaminotoluene produced an increased average net nuclear grain count in the hepatocytes isolated from rat and hamster at the highest concentration tested compared to the untreated control cultures. Also the percentage of cells with > 5 net nuclear grains increased in a dose dependent manner.

Conclusion
Under the experimental conditions used 2,5-diaminotoluene induced unscheduled DNA synthesis and, consequently, is genotoxic in rats in this *in vitro* UDS test.

Ref.: 37

Comments
The present assay is reported in a paper from the open literature in which 7 azo dyes and their reduction products were tested in the *in vitro* unscheduled DNA synthesis test with hepatocytes isolated from rats and hamsters. Consequently, the raw data were not available. The results can only be used as supportive evidence.
The paper was published before the implementation of OECD guidelines.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline: OECD 474
Species/strain: Crl:NMRI BR
Group size: 5 mice/sex/group
Test substance: SAT 010935 (toluene-2,5-diamine sulfate)
Batch: 46847
Purity: 99.9%
Dose level: 0, 25, 50 and 90 mg/kg bw
Route: i.p.
Vehicle: ethanol/deionised water (20/80 v/v)
Sacrifice times: 24 h after treatment for all concentrations, 48 h for the control and mid dose
GLP: in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the findings of two range finding studies to the highest tolerable dose. The LD50 was estimated to be about 123 mg/kg bw. 75% of this LD50 (90 mg/kg bw) was chosen to be the highest dose.
Therefore, in the main experiment mice were exposed to single i.p. doses of 0, 25, 50 and 90 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (control, mid and high dose

only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE).
Bone marrow preparations were stained with a slightly modified Pappenheim method and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the high dose group 65% of the male and female mice died within 24 h after administration of toluene-2,5-diamine sulfate. From the "48 h sacrifice" group all animals died. The "48 h sacrifice" group was replaced by 5 male and 5 female mice treated with the mid dose. All other animals survived until the scheduled sacrifices.
In the high dose animals reduced motor activity and sedation was noted from toluene-2,5-diamine sulfate administration until premature death or sacrifice. In the mid dose animals reduced motor activity was noted on the day of toluene-2,5-diamine sulfate administration. No adverse effects were noted in the low dose group.
The amount of nucleated cells was slightly below the range of historical negative control data in the high dosed group and the mid dosed females of the "48 h sacrifice" group.
Treatment with toluene-2,5-diamine sulfate resulted in decreased PCE/NCE ratios compared to the untreated controls indicating that toluene-2,5-diamine sulfate had cytotoxic properties in the bone marrow.
Biologically relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 or 48 h after treatment and neither for male and females.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate did not induce micronuclei in bone marrow cells of treated mice and, consequently, toluene-2,5-diamine sulfate is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 38

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	NMRI
Group size:	5 mice/sex/group
Test substance:	A 5 (toluene-2,5-diamine sulfate)
Batch:	EFH 290394
Purity:	> 98%
Dose level:	0, 15, 50 and 150 mg/kg bw
Route:	orally
Vehicle:	PEG 400
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only.
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on a preliminary study on acute toxic syndromes (like death, reduced spontaneous activity, eyelid closure, apathy) at various intervals of 1, 6, 24 and 48 h after start of treatment. In the main experiment mice were exposed to single oral doses of 0, 15, 50 and 150 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results

In the preliminary study with oral exposure to 50 - 400 mg/kg bw toluene-2,5-diamine sulfate, death, reduction of spontaneous activity, eyelid closure and apathy were found. The seriousness of these clinical effects decreased with dose resulting in only eyelid closure in the 150 mg/kg bw group, the highest dose in the main experiment. At doses below 150 mg/kg bw, no clinical effects were observed.
Treatment with toluene-2,5-diamine sulfate did not result in decreased PCE/NCE ratios compared to the untreated controls indicating that toluene-2,5-diamine sulfate had no cytotoxic properties in the bone marrow. Therefore, sufficient exposure of bone marrow cells is questionable.
Biologically relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate did not induce an increase in the number of micronucleated PCEs in bone marrow cells of treated mice and, consequently, toluene-2,5-diamine sulfate is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 39

Comment
Because the PCE/NCE ratio was not decreased, there are no indications for bone marrow cell exposure. In the preliminary study clinical effects indicated systemic availability of toluene-2,5-diamine sulfate. However, in the main experiment at the doses used these effects were not found. Therefore, this study is of limited value and can only be used as supportive evidence.

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline:	OECD 486
Species/strain:	male Sprague Dawley rats
Group size:	3 rats per dose and sacrifice time
Test substance:	SAT 010935 (toluene-2,5-diamine sulfate)
Batch:	46847
Purity:	> 99.9%
Dose level:	0, 20, 40 and 80 mg/kg bw
Route:	oral gavage
Vehicle:	sterile water
Sacrifice times:	2 h and 14 h after dosing
GLP:	in compliance

Toluene-2,5-diamine sulfate was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test concentrations were selected on the basis of information supplied by the sponsor. Clinical observations were carried out approximately 30 minutes after dosing and before sacrifice for the 14 h sampling time. Hepatocytes for UDS analysis were collected approximately 2 h and 14 h after administration of toluene-2,5-diamine sulfate. At least 90 minutes after plating the cells were incubated for 4 h with 10 µCi/ml ^{3}H-thymidine followed by overnight incubation with unlabelled thymidine. Evaluation of autoradiography was done after 14 days.
UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. The percentage of cells in repair (defined as cells with a net grain count of at least +5) was calculated for each animal. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat. Negative and positive controls were in accordance with the OECD guideline.

Results
Mortality was observed in animals dosed with 80 mg/kg bw for the 14 h sampling time. Therefore, slides prepared from animals treated with 80 mg/kg bw for both sampling times were not evaluated. Cytotoxic effects were not seen in rats treated with 20 and 40 mg/kg bw.
Neither a biologically relevant increase in mean net nuclear grain count nor in the percentage of cells in repair as compared to the untreated control was found in hepatocytes of any treated animal both for the 2 h and the 14 h treatment time. A positive (> 0) net nuclear grain count was found for one animal which was attributable to a cytotoxic effect of toluene-2,5-diamine sulfate as indicated by a reduced cytoplasm count.

Conclusion
Under the experimental conditions used toluene-2,5-diamine sulfate did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 40, 41

In vivo alkaline single cell gel electrophoresis (Comet) assay in mice and rats

Guideline:	/
Species/strain:	ddY mice and Wistar rats
Group size:	4 male animals/group
Test substance:	2,5-diaminotoluene sulfate
Batch:	/
Purity:	> 98%
Dose level:	0 and 60 mg/kg bw
Route:	oral
Vehicle:	saline
Sacrifice times:	3, 8 and 24 h after start of treatment
Organs studied:	stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow
GLP:	not in compliance

2,5-Diaminotoluene sulfate was investigated for the induction of DNA damage in the alkaline single cell gel electrophoresis (Comet) assay in various tissues of mice and rats. Test concentrations were based on the LD50 of acute toxicity experiments (0.5 x LD50). Mice and rats were orally exposed to 60 mg/kg bw 2,5-diaminotoluene sulfate and sacrificed 3, 8 and 24 h after treatment.
The animals were carefully observed for pharmacotoxic signs after treatment until sacrifice. Histopathological examination was conducted when positive results were obtained. Per organ 50 nuclei were examined for migration which was calculated as the difference between length of the whole comet and diameter of the head.

Results
An increase in DNA damage was exclusively found in the stomach of rats of the 8 h sampling time but not the shorter or longer sampling time. 2,5-Diaminotoluene sulfate did not induce a biologically relevant increase in DNA damage in any of the other tissues tested of the mice and rats.

Conclusion
Under the experimental conditions used 2,5-diaminotoluene sulfate induced significant DNA damage in the stomach of rats and, consequently, 2,5-diaminotoluene sulfate is genotoxic in the Comet assay with rats.

Ref.: 44

Comments

The present assay is reported in a paper from the open literature. The raw data were not available.
Effects observed only in the stomach may be due to localized irritation/toxicity. Since there was no information on histology provided in this study, the impact of localized irritation/toxicity can not be ruled out. The validity of this study, which was part of a large comparative investigation, has been questioned in the scientific community for several reasons. The study performance does not conform to the requirements that were recently published in order to improve the quality of comet assay data (Refs 42 and 43). Specifically, only one dose was evaluated, there is no historical control data to determine validity of each study and aid in interpretation of results, evaluation of only one slide and 50 nuclei per tissue and animal. For some substances, the positive results reported by Sasaki and colleagues could not be verified by others (Ref 43).
Therefore, the value of this single positive result at one sampling time in only one species is unclear.

Mouse spot test

Guideline:	/
Species/strain:	male T stock and female C57BL/6J mice
Group size:	4 mice per dose
Test substance:	2,5-diaminotoluene
Batch:	/
Purity:	/
Dose level:	0 and 30 mg/kg bw
Route:	i.p.
Vehicle:	HBSS
Scoring for mutations:	12 – 15 days after birth
GLP:	not in compliance

2,5-Diaminotoluene sulfate was investigated for the induction of somatic mutations in the mouse spot test. Female C57B1/6J mice were mated with C57B1/10J or T stock males. On day 10-14 of gestation, the female mice were treated *i.p*. with 30 mg/kg bw 2,5-diaminotoluene. At birth the number and morphology of the offspring were recorded. At 12 to 15 days after birth the offspring was scored for coat colour spots. Triethylenemelanine was used as positive control.

Results
There was no significant increase in recessive spots in the 2,5-diaminotoluene exposed pups observed after birth. Nor in the percentage "white midventral spots", which are thought to be related to toxicity of treatment.

Conclusion
Under the conditions of this test, 2,5-diaminotoluene did not induce somatic mutations in foetal cells following transplacental absorption and consequently, 2,5-diaminotoluene is not genotoxic (mutagenic) in mice.

Ref.: 46

Comment
The present assay is reported in a paper from the open literature. The experiment, performed before the development of the OECD guidelines, is a non-GLP study conducted with unspecified test material. It was not reported if the applied dose induced toxic effects in the treated females. Because the test does not comply with the requirements of the currently valid guideline, the result of this experiment can only be used as supportive evidence.

Mouse spot test

Guideline:	/
Species/strain:	male T stock and female C57BL/6 mice
Group size:	5 female mice per dose
Test substance:	Orex 111 (2,5-diaminotoluene dihydrochloride)
Batch:	/
Purity:	/
Dose level:	0, 15, 150 and 1500 mg/kg bw
Route:	dermal
Vehicle:	corn oil
Scoring for mutations:	days 12 and 24 after birth
GLP:	not in compliance

2,5-Diaminotoluene dihydrochloride was investigated for the induction of somatic mutations in the mouse spot test. Female C57B1/6J mice were mated with C57B1/10J or T stock males. On days 9, 10, and 11 post fertilisation 2,5-diaminotoluene dihydrochloride was administered dermally to a shaved patch on the dorsal side of the animal. At 12 and 24 days after birth the offspring was scored for coat colour spots. Benz(a)pyrene was used as positive control.

Results
No toxic effects in the treated animals and no effect on fertility or litter size were observed. There was no significant increase in treated animals with recessive spots as compared to the concurrent controls. 2,5-Diaminotoluene dihydrochloride exposure did not induce an increase in the percentage "white midventral spots" either, which are thought to be related to toxicity of treatment.

Conclusion
Under the conditions of this test, 2,5-diaminotoluene dihydrochloride did not induce somatic mutations in this mouse spot test and consequently, 2,5-diaminotoluene dihydrochloride is not genotoxic (mutagenic) in mice.

Ref.: 47

Comment
The experiment was not in compliance with GLP and was conducted before the development of the OECD guidelines. The test material is not specified. Because the test does not comply with the requirements of the currently valid guideline, the result of this experiment can only be used as supportive evidence.

Adapted from submission II, 2005
Two dominant lethal test have been conducted with toluene-2,5-diamine. Because these are non-GLP studies conducted with unspecified test material, only a brief description is provided. They are included since they add to the assessment of the genotoxicity of toluene-2,5-diamine *in vivo*. Though none of the tests fully comply with the requirements of the currently valid guidelines, taken together the results give further support for the conclusion that toluene-2,5-diamine is not mutagenic *in vivo*.

Dominant lethal test
Toluene-2,5-diamine dissolved in water was administered three times per week intraperitoneally at a dose of 20 mg/kg bw over 8 weeks to 20 male Charles-River rats. Each male was then housed with two females for 5 days. The females were killed 17 days later and the uteri were examined for live and dead foetuses, implantation and resorption sites. In total there were 460 live foetuses (12.4 per litter). Neither the percentages of litters with one or more resorptions, nor the number of mean resorptions per pregnancy or the percent resorptions per litter were different from the vehicle control values. Toluene-

2,5-diamine under the conditions of this test did not induce dominant lethal mutations in male rats. Under the conditions of this test toluene-2,5-diamine did not induce embryonic or foetal deaths by inducing chromosomal aberrations in germinal tissue. The test supports the conclusion that toluene-2,5-diamine sulfate is not mutagenic *in vivo* in germ cells.

Ref.: 48

Dominant lethal test

Toluene-2,5-diamine dihydrochloride in corn oil was applied topically to the shaved dorsal surface at doses of 1.5, 15, 150 mg/kg bw for 5 consecutive days to male mice. A weekly mating sequence with 2 females per week was started 2 days after the last application and was continued for 7 weeks. No attempt was made to prevent ingestion of the test substance during the treatment period. Fourteen days after the midweek of being caged with the male, the females were sacrificed. The final evaluation revealed no indication of dominant lethality. The positive control triethylenemelamine induced a significant dominant lethal response demonstrating the validity of the test system.

Ref.: 49

3.3.7. Carcinogenicity

Oral administration, mice

Guideline:	/
Species:	Mouse/B6C3F1
Group size:	50 animals per sex per dose level
Test substance:	toluene-2,5-diamine sulfate (CAS n° 6369-59-1)
Vehicle:	Diet (Wayne Lab-Blox® meal)
Batch:	Not indicated
Purity:	99% with 25 ppm iron, 0.6% volatiles, max. 0.1% moisture. No impurities were detected by thin-layer chromatography in two solvent systems
Dose level:	0.06, 0.1%
Route:	Oral, diet
Exposure period:	78 weeks, followed by an additional 16 – 19 weeks without treatment
GLP:	In compliance

Toluene-2,5-diamine sulfate was administered in the diet to groups of 50 mice per sex at either 0.06 or 0.2% for a period of 78 weeks followed by an additional 16 to 19 weeks of observation. These doses were selected after completion of a 4 week feeding study in male and female B6C3F1 mice. Because the test substance administration to the high and low dose groups was not begun simultaneously, each dosed group was assigned a separate control group of 50 animals per sex. Body weights were recorded twice weekly for the first 12 weeks and then at monthly intervals. Food consumption was monitored for seven consecutive days once a month for the first nine months and then for 3 consecutive days each month thereafter. Animals were monitored twice daily for mortality. A necropsy was performed on all animals that died, were sacrificed when moribund, or were sacrificed at study termination, and histopathological examinations were performed on major tissues, organs, and gross lesions. Slides were prepared from the following tissues: skin, subcutaneous tissue, lungs and bronchi, trachea, bone marrow, spleen, lymph nodes, thymus, heart, salivary gland, liver, gall bladder, pancreas, oesophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, testis, prostate, seminal vesicle, brain, tunica vaginalis, muscle, ear, uterus, mammary gland, and ovary.

Results

Mean body weight was consistently depressed in high dose female mice compared to the corresponding control. There was no treatment-related effect on survival in either males or females.

A statistically significant increase in lung tumours (alveolar/bronchiolar adenomas or carcinomas) was observed in high dose female mice (high dose 8/45 (17%) vs. 1/45 (2%) in high dose control, P=0.016). The incidence was not significantly increased in low dose female mice (low dose 6/42 (14%) vs. 4/46 (9%) in low dose control). Results from high dose were not considered as convincing evidence of a treatment-related effect because the control for the high dose female mice were very low compared to the control for the low dose female mice (1/45 (2%) vs 4/46 (9%)), moreover no similar effects were observed in the male mice. The incidence of pituitary adenomas or carcinomas was significantly lower in high dose female mice (high dose 0/38 (0%) vs. 6/37 (16%) in high dose control, P=0.012). The incidence in low dose female mice was also lower, although not statistically significant (low dose 1/38 vs. 3/42 in low dose control).

Ref.: 51

Oral administration, rats

Guideline:	/
Species/Strain:	Rat/Fischer 344
Group size:	50 animals per sex per dose level except for high dose control groups which had 25 animals per sex
Test substance:	toluene-2,5-diamine sulfate (CAS n° 6369-59-1)
Vehicle:	Diet (Wayne Lab-Blox® meal)
Batch:	Not indicated
Purity:	99% with 25 ppm iron, 0.6% volatiles, max. 0.1% moisture. No impurities were detected by thin-layer chromatography in two solvent systems
Dose levels:	Low dose: 0.05% for 14 weeks, increased to 0.06% at week 15 (time weighted average = 0.06%) High dose: 0.2%
Route:	Oral, diet
Exposure period:	78 weeks, followed by an additional 28 to 31 weeks without treatment
GLP:	In compliance

Toluene-2,5-diamine sulfate was administered in the diet to groups of 50 rats per sex at either 0.05-0.06 or 0.2% for a period of 78 weeks followed by an additional 28 to 31 weeks of observation. These doses were selected after completion of a 4 week feeding study in male and female Fischer 344 rats. Because the test substance administration to the high and low dose groups was not begun simultaneously, each dosed group was assigned a separate control group of 50 animals per sex (low dose control) or 25 animals per sex (high dose control). Body weights were recorded twice weekly for the first 12 weeks and then at monthly intervals. Food consumption was monitored for seven consecutive days once a month for the first nine months and then for 3 consecutive days each month thereafter. Animals were monitored twice daily for mortality. A necropsy was performed on all animals that died, were sacrificed when moribund, or were sacrificed at study termination, and histopathological examinations were performed on major tissues, organs, and gross lesions. Slides were prepared from the following tissues: skin, subcutaneous tissue, lungs and bronchi, trachea, bone marrow, spleen, lymph nodes, thymus, heart, salivary gland, liver, pancreas, oesophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, testis, prostate, seminal vesicle, brain, tunica vaginalis, muscle, ear, uterus, mammary gland, and ovary.

Results
Mean body weight was consistently depressed in high dose female rats. This trend was not as evident in the other groups of dosed rats. There was no treatment-related effect on survival in either males or females.

A statistically significant increase in the incidence of interstitial cell tumours of the testis was observed in male rats (low dose 43/48 (90%) vs. 33/45 (73%) in low dose control, P=0.039; high dose 47/48 (98%) vs. 19/24 (79%) in high dose control, P=0.014), but this was not considered treatment-related since the spontaneous incidence of these tumours in male rats is very high and variable. The incidence of pituitary adenomas in low dose male rats showed a statistically significant decrease relative to the corresponding control (low dose 3/45 (7%) vs. 12/41 (29%) in low dose control, P=0.006). A similar trend (not statistically significant) was seen in high dose male rats (high dose 3/40 (8%) vs. 3/21 (14%) in high dose control). The incidence of lung tumours (alveolar/bronchiolar adenomas or carcinomas) was significantly lower in high dose male rats (high dose 0/49 (0%) vs. 3/25 (12%) in high dose control, P=0.035), but this difference was not seen in low dose male rats (low dose 1/48 vs. 0/46 in low dose control). No significant increases in neoplasms were observed in female rats. The incidence of thyroid C-cell adenoma or carcinoma was significantly lower in high dose female rats (0/49 vs. 3/21 in high dose control), but the opposite trend (not statistically significant) was seen in low dose female rats (3/48 vs. 1/47 in low dose control).

Ref.: 51

Comment
The conclusion drawn in the NCI report for this study was: *"under the conditions of this bioassay, sufficient evidence was not provided to conclusively demonstrate the carcinogenicity of 2,5-toluenediamine sulphate in either Fischer 344 rats or B6C3F1 mice".*

Toluene-2,5-diamine in hair dye formulations together with hydrogen peroxide

Topical administration, mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	28 Males and 28 females per treatment group and positive control, 14 males and 14 females in vehicle control group and 76 males and 17 females in untreated control group
Test substance:	toluene-2,5-diamine. Two hair dye formulations containing 3% toluene-2,5-diamine, 1.5% p-phenylenediamine and either 0.2% or 0.6% toluene-2,4-diamine. The dye preparation was mixed with equal volume 6% hydrogen peroxide before application.
Batch:	/
Purity:	not given
Dose:	0.05 ml of a solution containing toluene-2,5-diamine and hydrogen peroxide
Route:	Topical, 1 application weekly
Exposure:	2 years
GLP:	not in compliance

Two oxidation hair dye formulations containing 3% toluene-2,5-diamine, mixed with an equal volume of 6% hydrogen peroxide, were tested by topical application in groups of 28 male and 28 female mice weekly for 2 years. 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) was used as positive control (0.05 ml containing 2.5 and 10 µg DMBA). Each week they were weighed. When signs of marked irritation, ulceration, or tumour formation were evident, the application of the chemical was discontinued until the skin looked "normal". Tissue specimens were taken from all major organ systems and tumours of mice found dead during the study or sacrificed when moribund or at 2 year, the termination of the experiment. Body weight gains of mice in treated groups were not significantly different from those of mice in the untreated control groups. It was concluded that the male and female mice in all groups developed both malignant and benign neoplasms. There were no statistical difference between the sexes in the total number of neoplasms or in the incidence

of neoplasms of a particular organ and type. The incidence of skin neoplasms did not show statistically significant differences in any of the groups under test except for the positive control groups exposed to DMBA.

Ref.: 91

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 Males and 50 females per treatment group and vehicle control
Test substance:	toluene-2,5-diamine. Two hair dye formulations containing 3.0% toluene-2,5-diamine, 1.5% p-phenylenediamine with either 0.2% toluene-2,4-diamine, 0.38% 2,4-diaminoanisole or 0.17% m-phenylenediamine prior to mixing with equal volume 6% hydrogen peroxide just prior to use.
Batch:	/
Purity:	not given
Dose:	0.05 ml of a solution containing toluene-2,5-diamine and hydrogen peroxide
Route:	Topical, 1 application weekly or 1 application every second week
Exposure:	18 months
GLP:	not in compliance

Two oxidation hair dye formulations containing 3% toluene-2,5-diamine, mixed with an equal volume of 6% hydrogen peroxide, were tested by topical application in groups of 100 mice weekly or once every two weeks for 18 months. 7,12-dimethylbenz(a)anthracene (DMBA) (0.1%) was used as positive control (0.05 ml containing 50 µg DMBA first 6 months, 10 µg next 4 months and 50 µg last 8 months). The mice were observed daily for signs of toxicity. Each week they were weighed, the skin graded for irritation and papillomas and other gross lesions were noted. Animals that died or that were killed because of general debility were autopsied and examined histopathologically when possible. At termination of the study, all survivors were weighed and killed and a gross autopsy was performed. There were no overt signs of systemic toxicity in any of the dye-treated groups. The survival varied from 58 to 80% except in the positive controls in which only 21% of the mice were alive after 18 months. Average body weights were comparable in all groups throughout the study. It is concluded that no evidence of carcinogenic activity was seen.

Ref.: 90

Topical administration, rats

Three experimental preparations in a carboxymethylcellulose gel were tested: formulation 1, containing 4% toluene-2,5-diamine (calculated as free base but used as sulfate): formulation 2, containing 3% toluene-2,5-diamine and 0.75% 2,4-diaminoanisole; and formulation 3, used as control without added dye intermediates. Each formulation was mixed with an equal volume of 6% hydrogen peroxide immediately before use, and 0.5 g of the mixture was applied to the dorsal skin. Three groups each of 50 male and 50 female Sprague-Dawley rats, 12 weeks old, were treated twice weekly for two years with either formulation 1 or 2 or left untreated. A fourth group of 25 male and 25 female rats were treated with formulation 3. No statistically significant differences were observed in tumour incidence between the experimental and control groups.

Ref.: 89

Comment
Hair dye formulations of toluene-2,5-diamine together with hydrogen peroxide have been tested in three experimental studies after topical application to mice or rats. The sensitivity of these studies may have been low as no response to hair dye formulations containing known carcinogens (toluene-2,4-diamine [EU carcinogenic. category 1B] or 2,4-

diaminoanisole [EU carcinogenic, category 1B] was observed. Thus, no conclusions with regard to carcinogenicity can be drawn from these studies.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Guideline:	OECD 416
Species/strain:	Sprague-Dawley rats Him:OFA
Group size:	24 males and 24 females
Test substance:	p-toluenediamine
Batch:	präp. 139 (not characterised, see general comments)
Purity:	98.2%
Dose:	0, 5, 15 and 45 mg/kg bw/d in deionised water, 50 µl 25% ammonia per g test substance added
Route:	oral, gavage
Exposure:	once daily males: 70 d before mating females: prior to mating for 14 d, during mating period, gravidity, lactation until the end of the experiment F_1: from weaning for approximately 80 days until the end of the experiment
GLP:	in compliance

The test substance was administered to males for 70 days and to females for 14 days until mating. The animals were monogamously mated within the dose groups. Dams were further dosed until weaning of the pups. Starting on day 21 after birth the F_1 generation was dosed for approximately 80 d. After mating the F_2 generation was kept until weaning. The common parameters were evaluated (female sexual cycle, mating, insemination, gravidity, birth and litter data, postnatal weight and physiological development). Histopathology was performed for organs with obvious abnormalities, for parents without live offspring and for all parental animals of the control and the highest dose group. The reproduction organs (pituitary gland, mamma, vulva, vagina, cervix, uterus, tubes, ovaries, penis, testes, epididymides, ducti referentes, coagulation gland, prostate gland, vesicular gland) were examined microscopically.

Results
4 animals (one of P- and 3 of F_1-generation) died due to intubation-induced lesions. Body weight development, feed consumption and observation of the animals did not show substance treatment related differences. Observations and measurements in the pups of both generations until weaning did not show differences in the parameters evaluated. No detrimental effect on male and female fertility was found.

Conclusion
The NOAEL for reproductive toxicity was 45 mg/kg bw/d.

Ref.: 52

3.3.8.2. Teratogenicity

Study in rabbits

Guideline:	/
Species/strain:	New Zealand White Rabbit
Group size:	16 (vehicle control and dose groups), 18 (positive control Vitamin A in rape seed oil)
Test substance:	toluene-2,5-diamine sulfate in distilled water

Batch:	23005
Purity:	/
Dose:	0, 10, 25, 50 mg/kg bw/d, positive control Vitamin A 6 mg/kg bw/d
Route:	oral, gavage
Exposure:	once daily from day 6 to 18 of gestation
GLP:	/

The females were fertilised by natural mating. After coitus HCG was given i.v. to ensure ovulation. The animals were treated with 0, 10, 25, 50 mg/kg bw/d toluene-2,5-diamine sulfate in distilled water, the positive control group received 6 mg/kg bw/d Vitamin A from day 6 to 18 of gestation. The animals were examined daily for mortality and clinical signs. The body weights were determined on days 0, 6, 18 and 28 of gestation. On day 28 of gestation the animals were sacrificed, the foetuses were dissected and examined for congenital abnormalities and macroscopic changes. The common *sectio* parameters were recorded. Half of the foetuses were examined for skeletal and the other half for visceral abnormalities.

Results
1 female at 10 and 1 female at 25 mg/kg bw/d died; at 50 mg/kg bw, 3 females died presumably because of the intubation procedure. No clinical signs were observed. Body weights of the females in the dose groups were similar to the controls. The changes in the incidences of intrauterine death observed were not dose-related. The number and sex of the foetuses as well as the foetal body weights were not influenced by substance treatment. The frequencies of external abnormalities, visceral malformations and variations as well as skeletal defects exhibited no substance-related changes. The positive control (Vitamin A) did not show teratogenicity and only slight embryotoxicity.

Conclusion
The NOAEL of embryotoxicity and teratogenicity of toluene-2,5-diamine sulfate in rabbits is 50 mg/kg bw/d.

Ref.: 53

Study in rats

Guideline:	/
Species/strain:	Sprague Dawley rats
Group size:	23 mated females
Test substance:	toluene-2,5-diamine sulfate in distilled water, Vitamin A in rape seed oil
Batch:	23005
Purity:	/
Dose:	0, 10, 50 and 80 mg/kg bw/d, positive control Vitamin A 15 mg/kg bw/d
Route:	oral, gavage
Exposure:	once daily from day 6 to 15 of gestation
GLP:	/

The test and control solutions were administered to the pregnant females from day 6 to 15 of gestation once daily by oral gavage. The animals were observed once daily for mortality and clinical signs. The body weights were determined on day 0, 6, 15 and 19 of gestation. On day 19 of gestation the animals were sacrificed by chloroform inhalation, the foetuses were dissected and examined for congenital abnormalities. The common *sectio* parameters were evaluated. Half of the foetuses were examined for skeletal and the other half for visceral abnormalities.

Results
2 animals (1 at 10 and 1 at 80 mg/kg bw/d) died possibly due to gavaging. No clinical signs were observed. The maternal body weights were markedly reduced at 80 and slightly

reduced at 50 mg/kg bw/d during the dosing period. For the period d 15-19 these changes were not observed. Post implantation loss was increased at 80 mg/kg bw/d. Number of foetuses, sex distribution and foetal weights were comparable to controls. Visceral and skeletal variations were in the normal range, no malformations were observed. The positive control showed a high incidence of skeletal malformations.

Conclusion
The NOAEL of toluene-2,5-diamine sulfate in rats for maternal toxicity is 50 mg/kg bw/d, the NOAEL of embryotoxicity and teratogenicity 80 mg/kg bw/d.

Ref.: 54

Comment
The use of a positive control is uncommon in teratogenicity studies.

3.3.9. Toxicokinetics

3.3.9.1. Toxicokinetics *in vitro*

Study 1, metabolism in primary hepatocytes of human, rat and mouse

Guideline:	/
Cells:	hepatocytes from humans (pooled from 3 male donors), male Sprague-Dawley rats and male ICR/CD-1 mice
Test substance:	toluene-2,5-diamine sulfate
Batch:	2346
Purity:	98.3% (NMR)
Test concentration:	10 µM
Incubation time:	4 h
Study period:	March 2003
GLP:	in compliance

Phase I and II metabolism of the test substance was examined *in vitro* using comparatively primary human, rat and mouse hepatocytes. Cryopreserved primary human hepatocytes that were phenotyped by the supplier regarding NAT2 activities were pooled in this study to achieve average activities; in summary, a rapid N-acetylation phenotype was expected. SD-rats are rapid metabolizers while CD-1 mice were reported to be a mixed population of rapid and slow metabolizers. The cell viability appeared to be unaffected over the incubation period of 4 h. The functionality of the system was tested using marker reactions for CYP2A6, CYP1A, CYP2A and CYP2B. Additional marker reactions for CYP1A1/2, CYP2E1 and human NAT1/NAT2 were included.

Results
Toluene-2,5-diamine sulfate was extensively metabolized by all hepatocytes (order rat ≈ mouse > human). With human hepatocytes the substance was completely metabolized after 4 h. For all three species N-acetylation was the major metabolic reaction and the results indicate that toluene-2,5-diamine sulfate is substrate for both types on N-acetyltransferases NAT1 and NAT2. Only the mono-N-acetylated metabolite was detected under the conditions of the study. Mouse hepatocytes additionally showed extensive hydroxylation of toluene-2,5-diamine sulfate.

Ref.: 56

Comment
No firm conclusions can be drawn from the study results with regards to the relative activity or preference of human NAT1 and NAT2 towards the substrate toluene-2,5-diamine.

Study 2, human hepatic metabolism *in vitro*

Guideline:	/
Cells:	hepatocytes from humans (pooled from 4 female donors) pooled human liver microsomes bacterially expressed human CYP isozymes CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6, CYP3A4
Test substance:	[ring-U-^{14}C]-toluene-2,5-diamine sulfate
Batch:	CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate Lot 16825DR Sigma Aldrich non-labelled toluene-2,5-diamine sulfate
Purity:	radiochemical purity 98.2% (HPLC) non-labelled toluene-2,5-diamine sulfate 98.3% (NMR)
Test concentration:	10 µM and 100 µM
Incubation time:	hepatocytes 4 h hepatic microsomes 20 min recombinant human isozymes 60 min
Study conduct:	2004
GLP:	/

The human hepatic metabolism *in vitro* of [ring-U-^{14}C]-toluene-2,5-diamine sulfate was investigated in hepatocytes from humans (pooled from 4 female donors, each phenotyped with regards to the isoenzymes investigated), pooled human liver microsomes and bacterially expressed human CYP isozymes CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6, CYP3A4. The formation of metabolites was proven by LC/MS.

Results
With human microsomes (10 µM and 100 µM toluene-2,5-diamine sulfate) there was no evidence for the production of oxidative metabolites while the positive control substance 2-aminofluorene yielded a number of oxygenated metabolites. When toluene-2,5-diamine sulfate (10 µM and 100 µM) was incubated with recombinant CYP isozymes no metabolites were detected while the positive control substance 2-aminofluorene yielded a isozyme-specific pattern of oxidative metabolites. When toluene-2,5-diamine sulfate (10 µM and 100 µM) was incubated with human hepatocytes an indication was found by mass spectrometry (MS) for the formation of a mono-acetylated derivative. No glucuronides, sulphate esters or mono-hydroxylated metabolites were detected.

Ref.: 57

3.3.9.2. Toxicokinetics *in vivo*

Study 1, absorption, distribution, metabolism and excretion in Kyoto rats after a single oral or dermal dose

Guideline:	OECD 417 (1984) OECD 427 (draft 2000)
Species/strain:	Wistar Kyoto rats, WKY/NR Crl BR (inbred)
group size:	4 females in the mass balance groups 6 females in the toxicokinetics groups
Test substance:	toluene-2,5-diamine sulfate oral: in water at pH 7 dermal: in water:acetone 1:1 at pH 7
Batch:	3362-259 [ring-U-^{14}C]-toluene-2,5-diamine sulfate CFQ13783, batch 1 [ring-U-^{14}C]- toluene-2,5-diamine sulfate 2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	99.3% (HPLC) (radiochemical purity) 98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	oral: 2.5, 25 mg/kg bw

	dermal: 0.5 mg/cm^2 equal to 33.3 mg/kg bw
Route:	oral, gavage or dermal (30 min)
Study conducted:	2004
GLP:	in compliance

Rats were dosed orally with 2.5 or 25 mg/kg bw and dermally with 33.3 mg/kg bw (corresponding to 0.5 mg/cm^2). In the oral mass balance study urine and faeces were collected for 0-8, 8-24, 24-48, 48-72, 72-96 h intervals and several tissues were collected. In the dermally dosed group urine and faeces were collected at 24 h intervals and animals were sacrificed at 120 h. In the toxicokinetic groups, blood was sampled at the time points 0.25, 0.5, 1, 2, 4, 6, 24, 48 and 72 h after dosing.

Results
The mean cumulative recovery in the urine after 96 h was 62.2 (low dose) and 72.9% (high dose) while the figures for the faeces were 31.4% and 22.0%, respectively. The mean mass balance (oral) was ca. 98%. The average dermal absorption was 16% (= 0.101 mg/cm^2) but together with the amount remaining in the skin the absorbed amount was calculated as 20% of the applied dose (= 0.126 mg/cm^2). The mean mass balance (dermal) was nearly 100%. After oral dosing three metabolites were observed in the urine, two of them probably mono-N-acetylated. The largest peak was identified as N,N'-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern after dermal dosing was similar to that after oral dosing.
Following oral dosing of 2.5 and 25 mg/kg bw toluene-2,5-diamine the AUC values were 17.59 and 174 mg_{eq} x h/l, respectively. The dermal dose of 33.3 mg/kg bw (equal to 0.5 mg/cm^2) resulted in an AUC of 4.39 mg_{eq} x h/l.

Ref.: 58

Comment
The Kyoto strain was used because it is a slow acetylator phenotype.

Study 2, absorption, distribution, metabolism and excretion in Kyoto rats after a single intravenous dose

Guideline:	OECD 417 (1984)
Species/strain:	Wistar Kyoto rats, WKY/NR Crl BR (inbred)
Group size:	4 females (mass balance), 6 females (kinetics)
Test substance:	toluene-2,5-diamine sulfate in water at pH 7
Batch:	CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate 2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	98.2% (HPLC) (radiochemical purity) 98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	2.5 mg/kg bw
Route:	intravenous, single application
Study conducted:	2004
GLP:	in compliance

2 groups were used, one (n=4) for mass balance and one (n=6) for toxicokinetics. The animals were dosed intravenously with 2.5 mg/kg bw [ring-U-^{14}C]-toluene-2,5-diamine sulfate. In the mass balance groups urine and faeces were collected for 0-8, 8-24, 24-48, 48-72, 72-96 h intervals and several tissues were collected. In the toxicokinetic groups blood was sampled at the time points 5, 15, and 30 min and 1, 2, 4, 6, 24, and 48 h after dosing.

Results
Urinary excretion accounted for 54% and excretion via faeces for 27% of the applied dose. 81 - 87% of the administered dose was excreted during the study period while 4.2 - 9.5%

remained in the carcass. In urine 2 major metabolites were observed and the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern was similar to that after oral administration (see Ref. 58).

Ref.: 59

Comment
The bioavailability in Kyoto rats (derived from comparison to iv administration) after oral dosing was > 90% while 2% bioavailability was found after dermal application.

Study 3, pharmacokinetics in Sprague-Dawley rats after a single oral or dermal dose

Guideline:	OECD 417 (1984) OECD 427 (draft 2000)
Species/strain:	Sprague-Dawley rats, Crl:CD (outbred)
Group size:	6 females
Test substance:	toluene-2,5-diamine sulfate oral groups 1 and 2: in water at pH 7 dermal group 3: in water:acetone 1:1 at pH 7 dermal group 4: vehicle 81905108B
Batch:	3362-259 [ring-U-^{14}C]-toluene-2,5-diamine sulfate CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate 2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	99.3% (HPLC) (radiochemical purity) 98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	oral: 2.5, 25 mg/kg bw dermal: 0.5 mg/cm^2 equal to 33.3 mg/kg bw
Route:	oral, gavage or dermal (30 min)
Study conducted:	2004
GLP:	in compliance

Rats were dosed orally with 2.5 or 25 mg/kg bw and dermally with 33.3 mg/kg bw (corresponding to 0.5 mg/cm^2). Blood was sampled at the time points 0.25, 0.5, 1, 2, 4, 6, 24, 48 and 72 h after dosing.

Results
Oral absorption of toluene-2,5-diamine sulfate was rapid with T_{max} values of 1 h. AUCs were 8.53 (low dose) and 112 mg_{eq} x h/l (high dose). After dermal application T_{max} was 2 h and the AUCs were 5 (vehicle water/acetone) and 2.27 mg_{eq} x h /l (vehicle formulation).

Ref.: 60

Study 4, toxicokinetics in Sprague-Dawley rats after a single intravenous dose

Guideline:	OECD 417 (1984)
Species/strain:	Sprague-Dawley rats, Crl:CD (outbred)
Group size:	6 females
Test substance:	toluene-2,5-diamine sulfate in water at pH 7
Batch:	CFQ13783, batch 1 [ring-U-^{14}C]-toluene-2,5-diamine sulfate 2346 (non-labelled toluene-2,5-diamine sulfate)
Purity:	98.2% (HPLC) (radiochemical purity) 98.3% (NMR) (non-labelled toluene-2,5-diamine sulfate)
Dose levels:	2.5 mg/kg bw
Route:	intravenous, single application
Study conducted:	2004
GLP:	in compliance

A single intravenous dose (2.5 mg/kg bw) of [ring-U-^{14}C]-toluene-2,5-diamine sulfate was administered to 6 animals. Blood was sampled at the time points 5, 15, and 30 min and 1, 2, 4, 6, 24, and 48 h after dosing.

Results
The radioactivity in plasma was eliminated with a half-life of 28.3 h.

Ref.: 61

Comment
The bioavailability in Sprague-Dawley rats (derived from comparison to i.v. administration) after oral dosing was 69% while 2% bioavailability was found after dermal administration in a formulation.

Study 5, absorption, disposition and elimination in humans following application to scalp

Guideline:	/
Species:	human
Group size:	5 adult males per formulation
Test substance:	[^{14}C-CH_3]-toluene-2,5-diamine sulfate in a hair dye formulation
Batch:	/
Purity:	/
Radioactivity:	19.98 µCi [^{14}C-CH_3]-toluene-2,5-diamine sulfate diluted with 1.65 g non-labelled toluene-2,5-diamine sulfate in 100 g hair dye formulation
Treatment:	A total of 45 g of a formulation containing 0.825% resorcinol and 0.825% [^{14}C-CH_3]-toluene-2,5-diamine sulfate mixed with water (formulation I) or 6% hydrogen peroxide (formulation II) corresponding to 180 mg/cm^2 formulation (1.48 mg/cm^2 [^{14}C-CH_3]-toluene-2,5-diamine sulfate, 17.98 nCi/cm^2)
Exposed area:	250 cm²
Route:	dermal on scalp for 30 minutes
Year of report:	1981
GLP:	/

The absorption and elimination of [^{14}C-CH_3]-toluene-2,5-diamine sulfate following topical application of 2 formulations to hair-bearing scalp was studied using 5 human male adults per group. 45 g of a formulation containing 0.825% resorcinol and 0.825% [^{14}C-CH_3]-toluene-2,5-diamine sulfate mixed with water (formulation I, non-oxidative conditions) or 6% hydrogen peroxide (formulation II, oxidative conditions) corresponding to 1.48 mg/cm^2 [^{14}C-CH_3]-toluene-2,5-diamine sulfate was applied on the scalp for 30 minutes. The formulation was rinsed off and the hair was shampooed. Radioactivity in urine and faeces as well as in in blood samples taken at certain time periods was measured.

Results
Following application of formulation I (non-oxidative conditions), the sum of excreted radioactivity in urine (0-48 h) was 2.31% (8.55 mg of 370 mg applied) whereas the respective figure for formulation II (oxidative conditions) was 0.78% (2.89 mg of 370 mg applied). The mean total elimination rate of radioactivity in urine and faeces was 4.81 ± 0.62% (formulation I, non-oxidative conditions) and 1.31 ± 0.14% (formulation II, oxidative conditions) of the applied dose. The absorption rates of toluene-2,5-diamine sulfate were 71 ± 9.26 $\mu g_{eq}/cm^2$ (formulation I, non-oxidative conditions) and 20 ± 2.02 $\mu g_{eq}/cm^2$ (formulation II, oxidative conditions).

Half life in blood ranged from 1.2 – 2.7 h and AUCs were calculated to be 41.6 ± 1.7 ng_{eq} x h/ml (formulation I, non-oxidative conditions) and 9.2 ± 3.1 ng_{eq} x h/ml (formulation II, oxidative conditions).

Ref.: 62

Comment of the SCCS
This study was performed using only 5 persons per formulation and a concentration of 0.825% on the scalp. In contrast, the applicant's dossier intends the use of an on-head concentration of 3.6% toluene-2,5-diamine sulfate. The mean recovery was only 86 and 47% under non-oxidative and oxidative conditions, respectively. Study reporting was not according to modern standards.

Study 6, *In Vivo* Toxicokinetic Study with Dermal Application in the Sprague Dawley rat (Submission III, 2010)

Guideline:	OECD 417/427 (1984/2004)
Species/strain:	Rat, strain Sprague Dawley, Crl;CD (outbred, SPF quality), females
Group size:	4 animals per dose group
Test substance:	toluene-2,5-diamine sulfate
Batch:	CFQ40199 batch 1 [ring-^{14}C(U)]-toluene-2,5-diamine sulfate 2346 (non-radiolabelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity: 99.0% by HPLC - [ring-^{14}C(U)]-toluene-2,5-diamine sulfate, Batch CFQ40199, batch 1 Non-radiolabelled toluene 2,5-diamine sulfate: >99% at 254 nm
Dose levels (actual):	2.1 mg/mL; 21 µg/cm^2 - dosing surface area 20 cm^2; 1.4 mg/kg bw 7.7 mg/mL; 75 µg/cm^2 - dosing surface area 20 cm^2; 6.0 mg/kg bw 24 mg/mL; 224 µg/cm^2 - dosing surface area 20 cm^2; 19.5 mg/kg bw 72 mg/mL; 734 µg/cm^2 - dosing surface area 20 cm^2; 63.2 mg/kg bw 7.3 mg/mL; 80 µg/cm^2 - dosing surface area 5 cm^2; 1.5 mg/kg bw 72 mg/mL; 677 µg/cm^2 - dosing surface area 5 cm^2; 13.3 mg/kg bw
Route:	Dermal
Dose volume:	0.2 ml for 20 cm^2 surface area; 0.05 ml for 5 cm^2 surface area
Vehicle:	Ammonia (25%)/Milli-Q water/hair dye formulation 81905108B (5/15/80); final concentration of ammonia was 1%
Dosing Schedule:	Single application for 30 minutes
Study date:	7/2008 – 8/2008
GLP:	In compliance

A single dose of [^{14}C]-toluene-2,5-diamine sulfate was administered by dermal application to groups of 4 female Sprague Dawley rats. Approximately 24 h prior to treatment, the fur was shaved on the back of the animals. For dose groups involving a total dosing surface area of 20 cm^2, two rubber "O"-rings with an internal surface area of 10 cm^2 were glued to the skin with Histoacryl Tissue Adhesive (B. Braun, Meslungen, Germany). For dose groups involving a total dosing surface area of 5 cm^2, one rubber "O"-ring with an internal surface area of 5 cm^2 was glued onto the skin. Animals wore a collar to prevent grooming of the treated area. Exposure was terminated 30 minutes post-application by cleaning the application site with cotton swabs moistened with a 10% aqueous shampoo solution or water.

Blood samples (ca. 200 µl) were collected from the tail vein at 0.5, 1, 2, 3, 4, 8, 24, and 48 h after application. Within a maximum of 1 hour after sampling, blood was centrifuged to obtain plasma, which was stored at $\leq$ -75 ºC until determination of radioactivity. After the final blood sample was collected, the animals were euthanized by an O_2/CO_2 procedure. The total amount of radioactivity in plasma samples was measured by liquid scintillation counting.

Concentrations of radioactivity in plasma were calculated as mg equivalents of toluene-2,5-diamine sulfate/kg sample based on the specific activity of the radiolabeled toluene-2,5-diamine sulfate formulation. Toxicokinetic parameters were calculated from the curves constructed from the individual values at each time point using the WinNonlin 5.2 program. Parameters that were calculated included C_{max}, t_{max}, t_{last}, AUC_{last}, AUC_{∞}, λ_z, and $t_{1/2}$. C_{max} and AUC values were also dose-normalized to 1 mg/kg bw.

Results and Discussion

Dermal absorption was rapid with t_{max} reached at 0.5 - 1 h after dosing in all groups. C_{max} values of the groups 1 – 4 ranged from 0.06 ± 0.06 mg/kg plasma (group 1, 1.4 mg/kg bw) to 6.5 ± 7.0 mg/kg plasma (group 4, 63.2 mg/kg bw). Plasma AUC values for each of the dose groups are summarized in the table below. For Groups 1, 5 and 6 values for plasma $AUC_{(0-\infty)}$ were only approximations because of the lack of measurable plasma concentration values in the terminal elimination phase of the plasma concentration *versus* time profile.

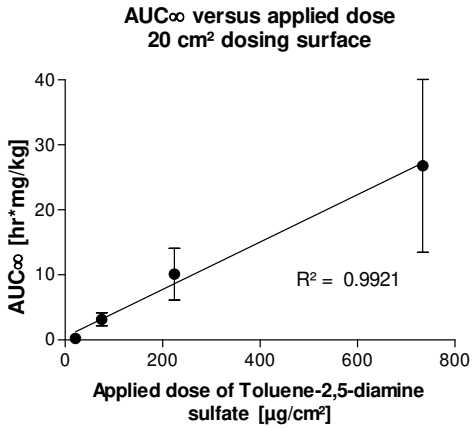
SUMMARY TABLE

Group	Dose Level	Dosing area (cm^2)	Dose level ($\mu g/cm^2$)	AUC_{∞} (hr*mg/kg)[1]	#AUC_{∞} (hr*mg/kg/(mg/kg))[1]	#AUC_{last} (hr*mg/kg/(mg/kg))[1]
1	1.4 mg/kg; 2.1 mg/mL	20	21	0.232* ± 0.184	0.153* ± 0.109	0.129 ± 0.104 (t_{last}: 4-8 h)
2	6.0 mg/kg; 7.7 mg/mL	20	75	3.16 ± 2.00	0.536 ± 0.369	0.508 ± 0.359 (t_{last}: 24 h)
3	19.5 mg/kg; 24 mg/mL	20	224	10.1 ± 7.94	0.513 ± 0.395	0.472 ± 0.399 (t_{last}: 3-24 h)
4	63.2 mg/kg; 72 mg/mL	20	734	26.8 ± 26.6	0.431 ± 0.427	0.411 ± 0.426 (t_{last}: 8-24 h)
5	1.5 mg/kg; 7.3 mg/mL	5	80	0.494* ± 0.393	0.327* ± 0.247	0.258 ± 0.244 (t_{last}: 8 h)
6	13.3 mg/kg; 72 mg/mL	5	677	2.05* ± 0.652	0.156* ± 0.0531	0.130 ± 0.0467 (t_{last}: 8 h)

* = approximation

[1] Values represent mean ± SD

Analysis of the dose groups 1 - 4 involving a 20 cm^2 dosing surface indicates that plasma $AUC_{(0-\infty)}$ increased proportionately with increasing applied dose (expressed as $\mu g/cm^2$). A linear regression analysis gave a correlation coefficient of 0.9921 (see figure below).



Plasma AUC values were greater after application to skin surface area of 20 cm^2 *versus* 5 cm^2 when comparing approximately equally applied doses (Groups 2 and 5 with applied doses of 75 and 80 $\mu g/cm^2$, respectively, and Groups 4 and 6 with applied doses of 677 and

734 µg/cm^2, respectively). However, in Groups 5 and 6 (5 cm^2 surface area of application) values for plasma $AUC_{(0-\infty)}$ were only approximations because of the lack of measurable plasma concentration values in the terminal elimination phase of the plasma concentration *versus* time profile. Therefore, a quantitative comparison of mean $AUC_{(0-\infty)}$ values for different skin surface areas with similar µg/cm^2 doses was not considered appropriate.

Ref.: 12 (subm III)

Study 7, toxicokinetics of [^{14}C] toluene-2,5-diamine sulfate following a single oral administration to Sprague-Dawley rats (Submission III, 2010)

Guideline:	OECD 417
Species/strain:	Sprague-Dawley rat
Group size:	6 per sex per group
Test substance:	toluene-2,5-diamine sulfate (non-radiolabelled) [Ring-^{14}C(U)]-toluene-2,5-diamine sulfate, specific activity 75 mCi/mmol
Batch:	CFQ40808, Batch B1- [ring-^{14}C(U)]-toluene-2,5-diamine sulfate R0022279 – (Non-radiolabelled toluene-2,5-diamine sulfate)
Purity:	Radiochemical purity: 98.9% by HPLC [ring-^{14}C(U)]-toluene-2,5-diamine sulfate Non-radiolabelled toluene-2,5-diamine sulfate: 99.5% by NMR
Vehicle:	Reverse osmosis deionized water, pH 5.0 $\pm$ 0.3
Dose levels:	5 and 10 mg/kg bw (containing approximately 100 µCi of radioactivity per dose)
Route:	Oral gavage
Dosing:	Single administration
Study date:	04/2010 – 09/2010 (performed outside the EU)
GLP:	In compliance

A single dose of [^{14}C]-toluene-2,5-diamine sulfate was administered orally by gavage at 5 or 10 mg/kg bw to fasted animals in a dose volume of 10 ml/kg bw. Dosing solutions were prepared on the day of dosing using the same vehicle used for the 91-day oral toxicity study (Charles River study VK00169, 18 August 2010). Blood (*circa* 0.3 ml) was collected via the cannulated jugular or carotid artery at 0.25, 0.5, 1, 2, 4, 8, 24, 48, and 72 h post dose and transferred to tubes containing K_2-EDTA anticoagulant and kept on wet ice until centrifugation. Within 1 hour of collection blood was centrifuged refrigerated (4 ºC) at 2700 rpm for approximately 10 min to separate plasma. Blood was also collected via the cannulated jugular or carotid from undosed control animals (Group 1, one male and one female). The plasma obtained from these animals was used to determine background levels of radioactivity. Duplicate weighed aliquots of dose formulation and plasma samples were mixed directly with liquid scintillation fluid for radioactivity measurement.

Plasma concentrations of radioactivity in dpm/g and mass eq/g were calculated based on the measured specific activity of the radiolabelled test material in the dose formulation. The toxicokinetic profile for each animal was characterized by non-compartmental analysis of the plasma radioactivity concentration data using validated computer software. The area under the radioactivity plasma concentration *versus* time curve (AUC) was calculated using the linear trapezoidal method. The parameters calculated included the following: C_{max}, T_{max}, $AUC_{(0\text{-tlast})}$, $T_{1/2}$, $AUC_{(0-\infty)}$.

Results and Discussion
Toxicokinetic parameters calculated from the concentration *versus* time profiles for each treatment group (5 and 10 mg/kg bw) are presented below:

Pharmaco-kinetic Parameter	Units	Group 2 (5 mg/kg bw) (mean[a] ± SD)			Group 3 (10 mg/kg bw) (mean[a] ± SD)		
		M	F	M and F	M	F	M and F
T_{max}	H	0.40	0.57	0.567	0.6	0.5	0.534
C_{max}	μg_{eq} / ml	6.42 ± 0.471	7.24 ± 0.446	6.83 ± 0.613	12.6 ± 1.11	13.3 ± 0.984	12.9 ± 1.07
$AUC_{(0\text{-}tlast)}$	μg_{eq} x h/ml	19.2 ± 2.68	25.1 ± 4.53	22.1 ± 4.72	40.7 ± 3.25	51.2 ± 4.04	46.0 ± 6.53
$t_{1/2}$	h	12.8 ± 6.14	12.6 ± 4.28	12.7 ± 5.05	14.2 ± 4.37	11.3 ± 5.94	12.8 ± 5.20
$AUC_{(0\text{-}\infty)}$	μg_{eq} x h/ml	19.3 ± 2.65	25.3 ± 4.51	22.3 ± 4.72	41.0 ± 3.30	51.6 ± 3.92	46.3 ± 6.51

[a] Median value reported for T_{max}

The highest mean observed concentration (C_{max}) was similar for both males and females and increased proportionally with an increase in dose. The C_{max} was observed between 0.40 and 0.60 h post dose, suggesting a rapid absorption of the test material following the oral dose. Following the C_{max}, the plasma radioactivity concentrations declined but were still detectable at 72 h post dose for both groups. Systemic exposure based on $AUC_{(0\text{-}\infty)}$ was slightly higher for females as compared to males and increased proportionally with an increase in dose. The mean terminal elimination half life ($T_{1/2}$) was similar for both sexes and ranged from 11.3 to 14.2 h.

Ref.: 14 (subm III)

Exposure to 2 commercial hair dye products (brown-red colour shade and black-brown colour shade) was investigated in 2 females in Germany (Schettgen *et al.*, 2011). Both were genotyped as slow acetylators (NAT2). Urinary samples were collected for a time period of 48 h after personal application of the hair dye creams which were labelled to contain toluene-2,5-diamine. The concentrations of the indicated ingredient were unknown. The urine samples were heated with 37% hydrochloric acid for 1 h at 80 °C in order to hydrolyse the amine conjugates and then analysed for aromatic diamines as well as for o-toluidine and 4-aminobiphenyl (as possible contaminants) using a GC-MS method. The cumulative excreted amounts of toluene-2,5-diamine were 700 µg and 1500 µg, respectively. The kinetics of urinary toluene-2,5-diamine after dermal application showed a phase of absorption and distribution within the body of about 12 h. Excretion mainly followed a first-order kinetics with a urinary half-life of about 8 hours.

Ref.:AR3

Study 8, consumer exposure to an oxidative hair dye. A [^{14}C]-labelled toluene-2,5-diamine mass balance study (Submission IV, 2012)

Guideline:	/
Species:	Human
Group Size:	16 Healthy adult male and female subjects per group
Methodology:	Open study with a single application of an oxidative hair dye mixture onto the hair
Test Substance:	2,5-Diamino[ring-U-^{14}C]-toluene sulfate - 80 mCi/mmol; 2.96 GBq/mmol; radiochemical purity 97.8% (HPLC)
Batch:	CFQ41174
Test Formulations:	Isotopic dilution of 2,5-Diamino[ring-U-14C]-toluene sulfate in a dark shade oxidative hair dye formulation with a final on-head concentration of either 1.5% or 4.0% toluene-2,5-diamine (expressed as free base)

	plus couplers, following mixing with hydrogen peroxide-containing developer (1:1, w/w).
Batch:	DTF 0938002 BF0 2 (1.5% toluene-2,5-diamine formulation); DTF 0938002 AF0 3 (4% toluene-2,5-diamine formulation)
Study period:	Aug-Sep 2011
GCP Status:	In compliance; ICH Guideline for Good Clinical Practice (ICH topic E6, adopted 01-05 1996 and implemented 17-01 1997)

In this study, two groups of human subjects (N=16 per group, 27 males and 5 females, age range 18-45 years) received a single application of an oxidative hair dye mixture by professional hairdressers. The final on-head concentration of [^{14}C]-toluene-2,5-diamine in the hair dye mixture applied was either 1.5% or 4.0% (expressed as free base). Both of the oxidative hair dye mixtures also contained m-aminophenol (A015), 4-amino-2-hydroxytoluene (A027), and 2,4-diaminophenoxyethanol dihydrochloride (A042) as couplers. The duration of the hair colouring exposure was 30 minutes. Following hair colouring, the dye was rinsed off, and the hair was shampooed, dried and clipped. Urine and faeces were collected quantitatively for 48 hours, and blood samples were taken prior to hair dyeing and at 2, 4, 6, 8, 10, 24, and 48 hours after hair colouring was begun. A protective cap was worn after clipping of the hair until the scalp was washed the following morning in order to collect radiolabelled substance exfoliated from the scalp during that time. Urine and faeces samples, as well as clipped hair, protective caps, wash water, and other materials (combs, brushes, towels, and gloves) were analysed for [^{14}C] radioactivity by liquid scintillation counting to calculate an overall mass balance. Plasma samples were analysed for [^{14}C] radioactivity to evaluate plasma kinetics of [^{14}C]-toluene-2,5-diamine equivalents. Calculated plasma kinetic parameters included T_{max}, C_{max}, $AUC_{(0-48hr)}$, $AUC_{(0-\infty)}$, and $T_{1/2}$.

Results

The overall mean mass balance obtained in this study was 98.85 $\pm$ 2.70% and 98.49 $\pm$ 2.97% for the low (1.5%) and high (4.0%) concentrations, respectively. The bulk of radioactivity was recovered in washing water and coloured hair. The washing water contained a mean of 56.28 $\pm$ 6.34% and 66.48 $\pm$ 5.40% of the applied radioactivity for the low and high concentration mixtures, respectively. The coloured hair contained a mean of 41.55 $\pm$ 6.09% and 31.16 $\pm$ 5.18% of the applied radioactivity for the low and high concentration mixtures, respectively.

Mean urinary excretion represented 0.83 $\pm$ 0.26% and 0.83 $\pm$ 0.42% of the applied radioactivity while mean faecal excretion represented 0.035 $\pm$ 0.02% and 0.042 $\pm$ 0.04% for the low and high concentration mixtures, respectively. Taken together, excretion in the 48 hour urine and faeces accounted for 0.86 $\pm$ 0.27% and 0.87 $\pm$ 0.45% of the applied radioactivity which would correspond to 6.28 $\pm$ 1.72 mg and 17.99 $\pm$ 8.76 mg toluene-2,5-diamine equivalents for the low and high concentration hair dye mixtures, respectively.

Plasma kinetic results are summarised in the following table.

Parameter	Units	Mean	SD	Min	Max
		1.5% toluene-2,5-diamine			
T_{max}	hr	2.4	0.8	2.0	4.0
C_{max}	ng eq/mL	99.1	38.4	46	180
$AUC_{(0-48h)}$	ng eq*hr/mL	1189	390	543	2128
$AUC_{(0-\infty)}$	ng eq*hr/mL	1241	402	583	2175
$T_{1/2}$	hr	10.1	1.3	8.5	12.4
Excretion $urine_{(0-48h)}$	% of dose	0.826	0.25	0.40	1.31
Excretion $faeces_{(0-48h)}$	% of dose	0.035	0.024	0.005	0.096
Excretion $total_{(0-48h)}$	% of dose	0.86	0.27	0.41	1.41
		4.0% toluene-2,5-diamine			
T_{max}	hr	2.6	1.0	2.0	4.0
C_{max}	ng eq/mL	266	145	111	599
$AUC_{(0-48h)}$	ng eq-hr/mL	3341	1474	1785	7340
$AUC_{(0-\infty)}$	ng eq-hr/mL	3353	1519	1894	7796
$T_{1/2}$	hr	11.3	2.6	7.7	17.5
Excretion $urine_{(0-48h)}$	% of dose	0.83	0.42	0.35	2.00
Excretion $faeces_{(0-48h)}$	% of dose	0.042	0.04	0.003	0.163
Excretion $total_{(0-48h)}$	% of dose	0.87	0.46	0.35	2.16

These results indicate a C_{max} of 99.1 $\pm$ 38.4 ng_{eq}/mL and 266 $\pm$ 145 ng_{eq}/mL for the low and high concentration mixtures, respectively. $T_{1/2}$ was similar in both treatment groups (10.1 $\pm$ 1.3 hr and 11.3 $\pm$ 2.6 hr for the low and high concentration mixtures, respectively). The mean $AUC_{(0-\infty)}$ was 1241 $\pm$ 402 ng_{eq} x hr/mL and 3553 $\pm$ 1519 ng_{eq} x hr/mL for the low and high concentration mixtures, respectively, and these values were used in the MoS calculation.

Ref.: 15

Comment

The parent compound and its metabolites were not analyzed in urinary samples. Plasma half-lives of the test substance in the high dose group differed by 2.3-fold, in the low dose group by 1.5-fold suggesting that individual differences in the rate-limiting step(s) of elimination (metabolism and/or excretion) apparently do not play a major role. However, data on the N-acetylator status (NAT1, NAT2) of the study participants was not reported. Furthermore, in the high dose group, excretion in the 48 hour urine and faeces (combined) and C_{max} values differed by about 6-fold. In the low dose group, the differences were less marked (about 3-fold). In both dose groups, the AUC values differed by about 4-fold. The high dermal absorption in some cases (both high C_{max} and AUC values in 2 individuals in the high dose group and in 1 individual in the low dose group) were explained as follows: "The variability observed in the individual results is likely due to the variation in plasma levels that is frequently observed in studies performed via topical application." However, such differences may become crucial when the calculation of a toxicokinetic MoS gives a borderline result.

Conclusion on toxicokinetics

In an *in vitro* metabolism study with primary hepatocytes of human, rat and mouse toluene-2,5-diamine sulfate was extensively metabolized by all hepatocytes (order: rat ≈ mouse > human). With human hepatocytes the substance was completely metabolized after 4 h. For all three species N-acetylation was the major metabolic reaction and the results indicate that toluene-2,5-diamine sulfate is substrate for both types on N-acetyltransferases NAT1 and NAT2. Only a mono-N-acetylated metabolite was detected under the conditions of the study. No conclusions can be drawn from the study results with regards to the relative

activity or preference of human NAT1 and NAT2 towards the substrate toluene-2,5-diamine. Mouse hepatocytes additionally showed extensive hydroxylation of toluene-2,5-diamine sulfate.

The human hepatic metabolism *in vitro* of toluene-2,5-diamine sulfate was investigated in hepatocytes from human donors, pooled human liver microsomes and bacterially expressed human CYP isozymes. With microsomes there was no evidence for the production of oxidative metabolites. Incubation with recombinant CYP isozymes yielded no oxidative metabolites. Following incubation with human hepatocytes an indication was found by MS for the formation of a mono-acetylated derivative. No glucuronides, sulphate esters or mono-hydroxylated metabolites were detected.

Absorption, distribution, metabolism and excretion (ADME) after a single oral or dermal dose was studied in Kyoto rats, a strain with a slow acetylator phenotype. After oral dosing three metabolites were observed in the urine, the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern after dermal dosing was similar to that after oral dosing. The comparison of AUCs showed differences between oral (25 mg/kg bw: 174 mg_{eq} x h/l) and dermal application (33 mg/kg bw: 4.39 mg_{eq} x h/l). Following iv application, in urine 2 major metabolites were observed and the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern was similar to that after oral administration. The bioavailability (derived from comparison to iv administration) after oral dosing was > 90% while 2% bioavailability was found after dermal application.
In similar toxicokinetic studies with Sprague-Dawley rats the comparison of AUCs showed differences between oral (25 mg/kg bw: 112 mg_{eq} x h/l) and dermal application (33 mg/kg bw in formulation: 2.27 mg_{eq} x h/l). Following oral administration of 2.5 mg/kg bw (NOAEL dose) the AUC was 8.53 mg_{eq} x h/l. The bioavailability (derived from comparison to iv administration) after oral dosing was 69% while 2% bioavailability was found after dermal administration in a formulation.

Absorption, disposition and elimination in humans was studied following application of a hair dye formulation at a concentration of 0.825% toluene-2,5-diamine sulfate to the scalp. The exposed area was 250 cm^2. Following application of formulation I (non-oxidative conditions), the sum of excreted radioactivity in urine (0-48 h) was 2.31% (8.55 mg of 370 mg applied) whereas the respective figure for formulation II (oxidative conditions) was 0.78% (2.89 mg of 370 mg applied). With a H_2O_2 containing formulation, the mean absorption rate was 20 ± 2.02 $\mu g_{eq}/cm^2$ and the AUC was 9.2 ± 3.1 ng_{eq} x h/mL. This study was performed using a concentration of 0.825% on the scalp. However, the applicant intends the use of an on-head concentration of 3.6% toluene-2,5-diamine sulfate. The value of the sum of excreted radioactivity in urine determined was 2.89 mg toluene-2,5-diamine sulfate (= 1.6 mg toluene-2,5-diamine). Similarly, in the study of Schettgen et al. (2011) which investigated exposure to 2 commercial hair dye products in two females (both genotyped as slow acetylators with regard to NAT2), the cumulative urinary excreted amounts of toluene-2,5-diamine after collection for a time period of 48 h were 700 µg and 1500 µg, respectively. The kinetics of urinary toluene-2,5-diamine after dermal application showed a phase of absorption and distribution within the body of about 12 h. Excretion mainly followed a first-order kinetics with a urinary half-life of about 8 hours.

Studies added in submission III and IV

A further percutaneous absorption study was provided in submission III of 2010. The skin absorption of toluene-2,5-diamine sulfate at concentrations of 0.25, 0.8, 2.4, or 7.2% was investigated with human skin (abdomen, breast, back, thickness ca 400 µm). An area dose of 20 mg/cm² of the final formulation was applied once to the skin (0.64 cm²) in a commercial oxidative hair dye formulation in either the presence or absence of hydrogen peroxide for 30 min. The study was well performed. Therefore, mean +1SD may be used as

the amount absorbed in calculating the MOS. The intended use of toluene-2,5-diamine is at a maximum of 4% 'on head' (7.2% as sulfate). Experiments were conducted with toluene-2,5-diamine sulfate in a range of dilutions from 0.25 to 7.2%. For 7.2% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 137.7 µg/cm². Under oxidative conditions, the amount absorbed is 69.4 µg/cm², which may be used for MOS calculation.

A further toxicokinetic study was conducted in female Sprague Dawley rats in accordance with OECD Guidelines 417 and 427 to determine plasma AUC of toluene-2,5-diamine sulfate equivalents after dermal application. The purpose of this study was to evaluate the concentration dependency of percutaneous absorption of toluene-2,5-diamine under *in vivo* conditions. The analysis of the results obtained in the four dose groups involving a 20 cm^2 dosing surface indicates that mean plasma $AUC_{(0-\infty)}$ was directly proportional to applied dose (expressed as µg/cm^2), and thus proportional to concentration used.

An additional rat oral toxicokinetic study was conducted in order to determine systemic exposure after a single dose of either 5 or 10 mg/kg bw toluene-2,5-diamine sulfate. The latter dose corresponds to the NOAEL determined in the new 91-day oral toxicity study. The $AUC_{(0-\infty)}$ value obtained in the 10 mg/kg bw dose group is considered to represent the systemic exposure at the dose corresponding to the NOAEL from the 91-day oral toxicity study.

Male and female animals (6 per sex per dose) were administered an oral gavage dose of [^{14}C]-toluene-2-5-diamine sulfate. Blood samples were collected at regular intervals over a 72-hour period, and radioactivity was measured in plasma. Toxicokinetic parameters including $AUC_{(0-\infty)}$ were calculated from the plasma concentration *versus* time profiles.

Toluene-2,5-diamine sulfate was rapidly absorbed following an oral dose, with a T_{max} of approximately 0.5 hr. C_{max} values corresponding to a dose of 10 mg/kg bw were 12.6 $\pm$ 1.11 μg_{eq} x h/ml and 13.3 $\pm$ 0.984 μg_{eq} x h/ml in males and females, respectively. Mean plasma $AUC_{(0-\infty)}$ values corresponding to a dose of 10 mg/kg bw were 41.0 $\pm$ 3.30 μg_{eq} x h/ml and 51.6 μg_{eq} x h/ml in males and females, respectively. The overall mean plasma $AUC_{(0-\infty)}$ for males and females combined was 46.3 μg_{eq} x h/ml, and this value was used by the applicant in the proposed toxicokinetics based MoS calculation.

In a further study, human systemic exposure (32 persons) was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 $\pm$ 402 ng x hr/mL and 3553 $\pm$ 1519 ng x hr/mL for the low and high concentration mixtures, respectively. These values were used for interpolation of 2% toluene-2,5-diamine in order to calculate the MoS. However, no data on the N-acetylator status (NAT1, NAT2) of the study participants were provided and, furthermore, marked individual differences in dermal absorption, C_{max} and AUC values were found.

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

See point 3.3.3. Sensitisation

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Toluene-2,5-diamine sulfate
(Oxidative conditions)

A. Conventional method

Absorption through the skin	**A (mean + 1SD)**	**=**	**40.02 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**23.21 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.39 mg/kg bw**
No observed adverse effect level (90-day, rat, oral)	**NOAEL**	**=**	**10 mg/kg bw/d**
NOAEL corrected for bio-availability (69%)		**=**	**6.9 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**18**

B. Based on toxicokinetics

Following the SCCS Notes of Guidance, available oral absorption data and the toxicokinetic properties of the substance should be taken into account. According to WHO, the 100-fold uncertainty factor can be subdivided in interspecies differences (10-fold) in toxicodynamics (2.5) and toxicokinetics (4) and inter-individual differences (10-fold) in toxicodynamics (3.2) and toxicokinetics (3.2). Given the AUC figures obtained from rats and humans the 4-fold factor for interspecies differences in toxicokinetics can be set to 1 which results in a remaining uncertainty factor of 25 which should be achieved. The applicant proposed to use the new human exposure study *in vivo* to calculate the Margin of Safety using the area under curve. This approach is accepted as being scientifically valid.

In this new study human systemic exposure was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 ng_{eq}-hr/mL and 3553 ng_{eq}-hr/mL for the low and high concentration mixtures, respectively. These values were compared to the rat mean $AUC_{(0-\infty)}$ of 46.3 μg_{eq}-hr/mL (see Ref. 14) at the NOAEL dose of 10 mg/kg bw/day established in the 91 day oral toxicity study. The toxicokinetics-based Margin of Safety was 37.3 and 13.0, respectively. By interpolation assuming direct proportionality the human plasma $AUC_{(0-\infty)}$ value corresponding to a MoS of 25 was calculated to be 1852 ng eq-hr/mL which corresponds to a concentration of 2.24% derived from the 1.5% AUC and assuming again proportionality. Therefore, the applied concentration of 2% is considered to be safe with regard to systemic toxicity (toxicokinetics based MoS >25). Furthermore, the intermittent exposure from oxidative hair dyes has to be taken into account.

3.3.14. Discussion

Physico-chemical properties
Toluene-2,5-diamine and its sulfate are used as an oxidative hair colouring agent (precursor). The intended maximum on-head concentration is 2.0% (calculated as free

base), or 3.6% (calculated as sulfate). Not all batches used were properly characterised. The stability of toluene-2,5-diamine and its sulfate in typical hair dye formulations was not reported. The impurity o-toluidine is classified by the EU as carcinogenic category 1B. No documentation was provided to support the reported data on the free base. Solubility of toluene-2,5-diamine has not been determined according to standard methods (e.g. EU - A.6).

Irritation / sensitisation
In an *in vivo* study in rabbits a 50.6% toluene-2,5-diamine applied under semi-occlusive conditions did not produce evidence of oedema and could not be evaluated for erythema due to black colouration of the skin. In the second experiment, which did not conform to guidelines or GLP, it is unclear whether the test concentration of toluene-2,5-diamine was 2.5% w/v or 25% w/v. However, in this experiment the test substance was irritant to rabbit skin under occlusive conditions.
Eye irritation studies have demonstrated that 50.6% toluene-2,5-diamine is irritant to the rabbit eye. Some irritant effects were also seen with 2.5% toluene-2,5-diamine.

The animal data indicate that toluene-2,5-diamine is an extreme skin sensitiser.
Human data: the results from several diagnostic patch studies in dermatitis patients show a high rate of contact allergy to toluene-2,5-diamine and to toluene-2,5-diamine sulfate. Due to different selection criteria and different patch test substances used, conclusions cannot be drawn concerning the trend over time of contact allergy to toluene-2,5-diamine and toluene-2,5-diamine sulfate. The results indicate that patch test reactivity is higher to toluene-2,5-diamine than toluene-2,5-diamine sulfate.

Dermal absorption
The data obtained in the different percutaneous absorption studies *in vitro* vary in the range of approximately 10 to 70 µg/cm^2. Such a variation is expected in view of the differences in design and data evaluation across studies. Factors which influenced the results were related to the test formulation (test substance concentration, presence of hydrogen peroxide and reaction partner, vehicle) to test model details (species/source and skin type) and to the number and type of compartments included in the calculation of systemic exposure.

A further percutaneous absorption study *in vitro* was provided in submission III of 2010. The skin absorption of toluene-2,5-diamine sulfate was investigated with human skin (abdomen, breast, back, thickness ca 400 µm). An area dose of 20 mg/cm² of the final formulation was applied once to the skin (0.64 cm²) in a commercial oxidative hair dye formulation in either the presence or absence of hydrogen peroxide for 30 min. The study was well performed. Therefore, mean +1SD may be used for MOS calculation. The intended use of toluene-2,5-diamine is at a maximum of 4% (calculated as free base) or 7.2% (calculated as sulfate) 'on head'. Experiments were conducted in a range of dilutions from 0.25 to 7.2% toluene-2,5-diamine sulfate. For 2.4% toluene-2,5-diamine sulfate, the amount absorbed under non-oxidative conditions (mean + 1SD) is 9.25 ± 2.54 = 11.79 µg/cm². Under oxidative conditions, the amount absorbed is 21.81 ± 4.87 = 26.68 µg/cm². The latter value may be used for the conventional MOS calculation after correction (26.68 x 3.6 / 2.4 = 40.02 µg/cm²) since the intended on head concentration is 3.6%.

General toxicity
The acute median lethal oral dose was calculated to be 102 mg/kg bw. In a 90-day study, the NOAEL is considered to be 10 mg/kg bw/d based on an increase in AST levels and myocyte degeneration observed at 20 mg/kg bw/d.

The NOAEL for reproductive toxicity was 45 mg/kg bw/d. The NOAEL of embryotoxicity and teratogenicity of toluene-2,5-diamine sulfate in rabbits is 50 mg/kg bw/d. The NOAEL of

toluene-2,5-diamine sulfate in rats for maternal toxicity is 50 mg/kg bw/d, the NOAEL of embryotoxicity and teratogenicity 80 mg/kg bw/d.

Toxicokinetics
In an *in vitro* metabolism study with primary hepatocytes of human, rat and mouse toluene-2,5-diamine sulfate was extensively metabolized by the hepatocytes of all species investigated (order: rat ≈ mouse > human). With human hepatocytes the substance was completely metabolized after 4 h. For all three species N-acetylation was the major metabolic reaction and the results indicate that toluene-2,5-diamine sulfate is substrate for both types on N-acetyltransferases NAT1 and NAT2. Only the mono-N-acetylated metabolite was detected under the conditions of the study. No firm conclusions can be drawn from the study results with regards to the relative activity or preference of human NAT1 and NAT2 towards the substrate toluene-2,5-diamine. Mouse hepatocytes additionally showed extensive hydroxylation of toluene-2,5-diamine sulfate.

The human hepatic metabolism *in vitro* of toluene-2,5-diamine sulfate was investigated in hepatocytes from human donors, pooled human liver microsomes and bacterially expressed human CYP isozymes. With microsomes there was no evidence for the production of oxidative metabolites. Incubation with recombinant CYP isozymes yielded no oxidative metabolites. Following incubation with human hepatocytes, an indication was found for the formation of a mono-acetylated derivative using mass spectrometry. No glucuronides, sulphate esters or mono-hydroxylated metabolites were detected.

Absorption, distribution, metabolism and excretion after a single oral or dermal dose was studied in Kyoto rats, a strain with a slow acetylator phenotype. After oral dosing three metabolites were observed in the urine, the largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern after dermal dosing was similar to that after oral dosing. The comparison of AUCs showed differences between oral (25 mg/kg bw: 174 mg_{eq} x h/l) and dermal application (33 mg/kg bw: 4.39 mg_{eq} x h/l). Following iv administration, 2 major metabolites in urine were observed. The largest peak was identified as N,N-diacetyl-toluene-2,5-diamine which was also found in faeces. The metabolite pattern was similar to that after oral administration. The bioavailability (derived from comparison to iv administration) after oral dosing was > 90% while 2% bioavailability was found after dermal application.
In similar toxicokinetic studies with Sprague-Dawley rats the comparison of AUCs showed differences between oral (25 mg/kg bw: 112 mg_{eq} x h/l) and dermal application (33 mg/kg bw in formulation: 2.27 mg_{eq} x h/l). Following 2.5 mg/kg bw the AUC was 8.53 mg_{eq} x h/l. The bioavailability (derived from comparison to iv administration) after oral dosing of 10 mg/kg bw was 69% while 2% bioavailability was found after dermal administration in a formulation.

Absorption, disposition and elimination in humans was studied following application of a hair dye formulation at a concentration of 0.825% toluene-2,5-diamine sulfate to the scalp. The exposed area was 250 cm^2. Following application of a non-oxidative formulation, the sum of excreted radioactivity in urine (0-48 h) was 2.31% (8.55 mg of 370 mg applied) whereas the respective figure for an oxidative formulation was 0.78% (2.89 mg of 370 mg applied). With the H_2O_2 containing formulation, the mean absorption rate was 20 ± 2.02 $\mu g_{eq}/cm^2$ and the AUC was 9.2 ± 3.1 ng_{eq} x h/mL. This study was performed using a concentration of 0.825% on the scalp, while the applicant intends the use of an on-head concentration of 3.6% toluene-2,5-diamine sulfate.
In a recent study in in two females (both genotyped as slow acetylators with regard to NAT2), (Schettgen et al., 2011) a cumulative excretion of 700 to 1500 µg toluene-2,5-diamine after exposure to two different commercial hair dye products with unknown toluene-2,5-diamine content was found. The kinetics of urinary toluene-2,5-diamine after dermal application showed a phase of absorption and distribution within the body of about

12 h. Excretion mainly followed a first-order kinetics with a urinary half-life of about 8 hours.

A further toxicokinetic study was conducted in female Sprague Dawley rats in accordance with OECD Guidelines 417 and 427 to determine plasma AUC of toluene-2,5-diamine sulfate equivalents after dermal application. The purpose of this study was to evaluate the concentration dependency of percutaneous absorption of toluene-2,5-diamine under *in vivo* conditions. The analysis of the results obtained in the four dose groups involving a 20 cm^2 dosing surface indicates that mean plasma $AUC_{(0-\infty)}$ was directly proportional to applied dose (expressed as $\mu g/cm^2$), and thus proportional to concentration used.

An additional rat oral toxicokinetic study was conducted in order to determine systemic exposure after a single dose of either 5 or 10 mg/kg bw toluene-2,5-diamine sulfate. The latter dose corresponds to the NOAEL determined in the new 91-day oral toxicity study. The $AUC_{(0-\infty)}$ value obtained in the 10 mg/kg bw dose group is considered to represent the systemic exposure at the dose corresponding to the NOAEL from the 91-day oral toxicity study. Male and female animals (6 per sex per dose) were administered an oral gavage dose of [^{14}C]-toluene-2-5-diamine sulfate. Blood samples were collected at regular intervals over a 72-hour period, and radioactivity was measured in plasma. Toxicokinetic parameters including $AUC_{(0-\infty)}$ were calculated from the plasma concentration *versus* time profiles. Toluene-2,5-diamine sulfate was rapidly absorbed following an oral dose, with a T_{max} of approximately 0.5 hr. C_{max} values corresponding to a dose of 10 mg/kg bw were 12.6 $\pm$ 1.11 μg_{eq} x h/ml and 13.3 $\pm$ 0.984 μg_{eq} x h/ml in males and females, respectively. Mean plasma $AUC_{(0-\infty)}$ values corresponding to a dose of 10 mg/kg bw were 41.0 $\pm$ 3.30 μg_{eq} x h/ml and 51.6 μg_{eq} x h/ml in males and females, respectively. The overall mean plasma $AUC_{(0-\infty)}$ for males and females combined was 46.3 μg_{eq} x h/ml, and this value was used in the toxicokinetics-based MoS calculation.

In a further study human systemic exposure was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 ± 402 ng_{eq}-hr/mL and 3553 $\pm$ 1519 ng_{eq}-hr/mL for the low and high concentration mixtures, respectively. These values were used in the toxicokinetics-based MoS calculation.

The knowledge on the metabolism of toluene-2,5-diamine in human skin and in systemic circulation is limited. From an *in vitro* study using human hepatocytes it was assumed by the study authors that NAT1 and NAT2 share the N-acetylation of toluene-2,5-diamine but the evidence is considered weak. Depending on the model system used, mono- and di-N-acetylated conjugates of toluene-2,5-diamine were detected, the di-conjugate mostly as the major metabolite. In human *in vivo* studies, the parent compound and its metabolites were not analyzed in urinary samples. From a scientific point of view, the role of N-acetylation of toluene-2,5-diamine by human NAT1 in human skin as a possible major metabolic inactivation step should be further investigated as well as polymorphisms of human NAT1.

Mutagenicity / genotoxicity

Overall, the genotoxicity of toluene-2,5-diamine sulfate is sufficiently investigated for the three types of mutation: gene mutation, structural chromosome mutation and aneuploidy.

Toluene-2,5-diamine sulfate is genotoxic *in vitro* inducing gene mutations in bacteria but not in mammalian cells, chromosomal aberrations, and unscheduled DNA-repair synthesis in primary hepatocytes *in vitro*.

The positive *in vitro* results could not be confirmed in *in vivo* experiments covering the same endpoints. Toluene-2,5-diamine sulfate was negative in two mouse bone marrow micronucleus tests, following oral and i.p. administration and in an *in vivo* UDS test following oral administration. The results of the *in vivo* Comet assay (oral gavage) in mice and rats in all organs evaluated except for the rat stomach may confirm the lack of genotoxic activity of toluene-2,5-diamine sulfate *in vivo*. However, issues with regard to interpretation and validity of the *in vivo* Comet assay in general and of the positive result in

the rat stomach in particular remain. In addition, toluene-2,5-diamine sulfate was negative in two dominant lethal assays indicating lack of genotoxic activity in germ cells *in vivo*. The negative results in two *in vivo* mouse spot tests following dermal and *ip* administration may confirm the lack of potential of toluene-2,5-diamine sulfate to induce gene mutations.
As the clastogenic effects found *in vitro* were not confirmed in *in vivo* tests, toluene-2,5-diamine sulfate can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
Toluene-2,5-diamine sulfate has been studied for carcinogenicity after oral administration by US National Cancer Institute. The conclusion drawn in the NCI report for this study was: *"under the conditions of this bioassay, sufficient evidence was not provided to conclusively demonstrate the carcinogenicity of 2,5-toluenediamine sulfate in either Fischer 344 rats or B6C3F1 mice".* Hair dye formulations of toluene-2,5-diamine together with hydrogen peroxide have been tested in three experimental studies after topical application to mice or rats. The sensitivity of these studies may have been low as no response to hair dye formulations containing known carcinogens was observed. Thus, no conclusions with regard to carcinogenicity can be drawn from the skin painting studies.

Margin of Safety
Toxicokinetic data for Sprague-Dawley rats indicate that oral availability of toluene-2,5-diamine at 10 mg/kg bw is 69%. The NOAEL from a 90-day study (10 mg/kg bw/d) was corrected accordingly to 6.9 resulting in a MOS of 18.

The applicant proposed to use the new human exposure study *in vivo* to calculate the Margin of Safety using the area under curve. This approach is accepted as being scientifically valid. The SCCS favours a toxicokinetics-based Margin of Safety. In a new study human systemic exposure was determined following application of an oxidative hair colouring formulation containing 1.5 and 4.0% toluene-2,5-diamine, respectively. The mean plasma $AUC_{(0-\infty)}$ values were 1241 ng eq-hr/mL and 3553 ng eq-hr/mL for the low and high concentration mixtures, respectively. These values were compared to the rat mean $AUC_{(0-\infty)}$ of 46.3 µg eq-hr/mL (see Ref. 14) at the NOAEL dose of 10 mg/kg bw/day established in the 91 day oral toxicity study. The toxicokinetics-based Margin of Safety was 37.3 and 13.0, respectively. By interpolation assuming direct proportionality the human plasma $AUC_{(0-\infty)}$ value corresponding to a MoS of 25 was calculated to be 1852 ng eq-hr/mL which corresponds to a concentration of 2.24% derived from the 1.5% AUC and assuming again proportionality. Therefore, the applied concentration of 2% is considered to be safe with regard to systemic toxicity (toxicokinetics based MoS >25).

4. CONCLUSION

Using the conventional risk assessment approach, a Margin of Safety of 18 was calculated.

However, using a toxicokinetics-based approach based on a new human exposure study *in vivo* the Margin of Safety was calculated using the area under the curve. The applied concentration on-head of maximum 2% (calculated as free base) or 3.6% (calculated as sulfate salt) is considered to be safe with regard to systemic toxicity (toxicokinetics based MoS >25).

Toluene-2,5-diamine is an extreme skin sensitiser. The frequency of allergic reactions in hairdressers and consumers remains a considerable concern for consumer safety.

5. MINORITY OPINION

Not applicable

6. REFERENCES

1. First report on the harmonisation of risk assessment procedures (Chapter 9 - Risk Characterisation); EUROPEAN COMMISSION; 95-108; 2000
2. Contrera, J. F.; Jacobs, A. C.; Prasanna, H. R.; Mehta, M.; Schmidt, W. J.; De George, J.; A systemic exposure-based alternative to the maximum tolerated dose for carcinogenicity studies of human therapeutics; J. AM. COLL. TOXICOL.; 14, 1-10; 1995
3. Damkröger, G.; Identity, stability and purity test of 1,4-Diamino-2-methyl-benzene-sulphate (1:1); WELLA AG; 2000
4. Lange, J.; Sieve analysis; NOACK; 2004
5. Franke, J.; Particle size distribution; SIEMENS AXIVA GMBH & CO. KG; 2004
6. Lange, J.; Water solubility (Flask Method); NOACK; 2004
7. Lange, J.; Prediction of the pKa-value; NOACK; 2004
8. Smeykal, H.; Melting point / Boiling point; SIEMENS AXIVA GMBH & CO. KG; 2004
9. Lange, J.; Determination of the density; NOACK; 2004
10. Franke, J.; Vapour pressure; SIEMENS AXIVA GMBH & CO. KG; 2004
11. Lange, J.; Surface tension; NOACK; 2004
12. Lange, J.; Flammability of solids; NOACK; 2004
13. Smeykal, H.; Auto-Flammability (Solids - Determination of relative self-ignition temperature); SIEMENS AXIVA GMBH & CO. KG; 2004
14. Opinion of A005; SCCNFP; 1985
15. Final report on the safety assessment of Toluene-2,5-diamine, Toluene-2,5-diamine sulfate, and Toluene-3,4-diamine; J. AM. COLL. TOXICOL.; 11, 423-445; 1992
16. Kynoch, S. R.; Lloyd, G. K.; Acute oral toxicity to rats of Toluene-2,5-diamine; HUNTINGDON; 1975
17. de Jouffrey, S.; Acute dermal irritation in rabbits; CENTRE INTERNATIONAL DE TOXICOLOGIE; 1994
18. Kynoch, S. R.; Liggett, M. P.; Irritant effects of Toluene-2,5-diamine on rabbit skin; HUNTINGDON; 1975
19. Clouzeau, J.; Acute eye irritation in rabbits; CENTRE INTERNATIONAL DE TOXICOLOGIE; 1994
20. Kynoch, S. R.; Liggett, M. P.; Irritant effects of Toluene-2,5-diamine on rabbit eye mucosa; HUNTINGDON; 1975
21. Schröder, K.; In-vitro-test: HET-CAM [HCI]- End-Point assessment - „p-Toluylendiamin sulfat - Colipa A5 - pTD"; HENKEL; 2002
22. Aeby, P.; Wyss, C.; Beck, H.; Griem, P.; Scheffler, H.; Goebel, C.; Characterization of the sensitizing potential of chemicals by in vitro analysis of dendritic cell activation and skin penetration; J. INVEST. DERMATOL.; 122, 1154-1164; 2004
23. Contact sensitisation: Classification according to potency; ECETOC; 2003
24. Albrecht, A.; Test for Sensitization (Local Lymph Node Assay - LLNA) with Haarbraun; BIOSERVICE; 2001, No. 000322D
25. Albrecht, A.; Test for sensitization (Local Lymph Node Assay - LLNA) with Haarbraun; BIOSERVICE; 2001, No. 000322H
26. Xie, Z.; Hayakawa, R.; Sugiura, M.; Kojima, H.; Konishi, H.; Ichihara, G.; Takeuchi, Y.; Experimental study on skin sensitization potencies and cross-reactivities of hair-dye-related chemicals in guinea pigs; CONTACT DERMATITIS; 42, 270-275; 2000
27. Ishihara, M.; Nogami, T.; Itoh, M.; Nishimura, M.; Sensitization potency of dye intermediates and modifiers in guinea pigs; SKIN RES.; 27, 585-590; 1985
28. Leclerc, C.; In vitro transcutaneous absorption of Toluene 2,5-diamine sulfate; L'OREAL; 1997

29. Bornatowicz, N.; Dermal absorption / percutaneous penetration of "pTD-sulfate" in vitro; SEIBERSDORF; 2002
30. Wyss, C.; Cutaneous absorption of Toluene-2,5-diamine sulfate in formulation through pig skin in vitro; COSMITAL; 2004
31. Wyss, C.; Cutaneous absorption of 2-Methyl-1,4-benzenediamine sulphate (Haarbraun) in Aceton/Aqua/Olive Oil (2:2:1) through pig skin in vitro; COSMITAL; 2001
32. Hill, R. E.; Paratoluenediamine 14 day oral (gavage) rangefinding study in the rat; TOXICOL LAB. LTD.; 1994
33. Hill, R. E.; Paratoluenediamine 13 week oral (gavage) toxicity study in the rat; QUINTILES ENGLAND LTD; 1997
34. Sokolowski, A.; Salmonella Typhimurium reverse mutation assay with INCI: Toluene-2,5-diamine sulfate; RCC-CCR; 2003
35. Wollny, H.-E.; Cell mutation assay at the thymidine kinase (TK +/-) locus in mouse lymphoma L5178Y cells with A5; RCC-CCR; 1995
36. Schulz, M.; In vitro chromosome aberration test in Chinese hamster V79 cells with SAT 010935; RCC-CCR; 2002
37. Kornbrust, D. J.; Barfknecht, T. R.; Comparison of 7 azo dyes and their azo reduction products in the rat and hamster hepatocyte primary culture/DNA-repair assays; MUTATION RESEARCH; 136, 255-266; 1984
38. Bornatowicz, N.; "SAT 010935": Micronucleus test with mice; SEIBERSDORF; 2002
39. Völkner, W.; Micronucleus assay in bone marrow cells of the mouse with A 5; RCC-CCR; 1995
40. Getuli, C.; SAT 010935 Unscheduled DNA synthesis (UDS) in primary rat hepatocytes after in vivo treatment; RTC; 2002
41. Cinelli, S.; SAT 010935 Unscheduled DNA synthesis (UDS) in primary rat hepatocytes after in vivo treatment; RTC; 2004
42. Hartmann, A.; Agurell, E.; Beevers, C.; Brendler-Schwaab, S.; Burlinson, B.; Clay, P.; Collins, A.; Smith, A.; Speit, G.; Thybaud, V.; Tice, R. R.; Recommendations for conducting the in vivo alkaline comet assay; MUTAGENESIS; 18, 45-51; 2003
43. Brendler-Schwaab, S.; Hartmann, A.; Pfuhler, S.; Speit, G.; The in vivo comet assay: use and status in genotoxicity testing; MUTAGENESIS; 20, 245-254; 2005
44. Sekihashi, K.; Yamamoto, A.; Matsumura, Y.; Ueno, S.; Watanabe-Akanuma, M.; Kassie, F.; Knasmüller, S.; Tsuda, S.; Sasaki, Y. F.; Comparative investigation of multiple organs of mice and rats in the comet assay; MUTATION RESEARCH; 517, 53-75; 2002
45. Sasaki, Y. F.; Sekihashi, K.; Izumiyama, F.; Nishidate, E.; Saga, A.; Ishida, K.; Tsuda, S.; The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database; CRIT. REV. TOXICOL.; 30, 629-799; 2000
46. Soares, E. R.; Lock; L. F.; Lack of an indication of mutagenic effects of dinitrotoluenes and diaminotoluenes in mice; ENVIRON. MUTAGENESIS; 2, 111-124; 1980
47. Matheson, D. W.; Mutagenicity evaluation of Orex 111 in the somatic mutation assay; LITTON BIONETICS; 1978
48. Burnett, C.; Loehr, R.; Corbett, J.; Dominant Lethal mutagenicity study on hair dyes; J. TOXICOL. ENVIRON. HEALTH; 2, 657-662; 1977
49. Matheson, D. W.; Mutagenicity evaluation of 2,5-Diaminotoluene, dihydrochloride in the mouse dominant lethal assay; LITTON BIONETICS; 1978
50. Compendium of abstracts from long-term cancer studies reported by the National Toxicology Program from 1976 to 1992; NATIONAL INSTITUTE OF HEALTH; 101, 72-74; 1993
51. Bioassay of 2,5-Toluenediamine sulfate for possible carcinogenicity; NATIONAL INSTITUTE OF HEALTH; 1978
52. Bornatowicz, N.; Two-generation fertility study with p-Toluylenediamine sulphate in rats; SEIBERSDORF; 1986
53. Osterburg, I.; p-Toluylenediamine sulphate oral (gavage) teratology study in the New Zealand White rabbit; HAZLETON; 1982

54. Osterburg, I.; p-Toluylenediamine sulphate oral (gavage) teratology study in the rat; HAZLETON; 1982
55. Marks, T. A.; Gupta, B. N.; Ledoux, T. A.; Staples, R. E.; Teratogenic evaluation of 2-Nitro-p-phenylenediamine, 4-Nitro-o-phenylenediamine, and 2,5-Toluenediamine sulfate in the mouse; TERATOLOGY; 24, 253-265; 1981
56. Krebsfänger, N.; 2,4-Diaminotoluene sulfate (23005): Metabolic stability, metabolite profile, and species comparison in primary hepatocytes of human, rat, and mouse; GENPHARMTOX; 2003
57. Powrie, R.; Human hepatic metabolism of Toluene-2,5-diamine: in vitro analysis; CXR BIOSCIENCES LTD.; 2005
58. Wenker, M. A. M.; Absorption, distribution, metabolism and excretion of 14C-Toluene-2,5-diamine sulphate in the female Wistar Kyoto rat after a single oral or dermal dose; NOTOX; 2005
59. Wenker, M. A. M.; Absorption, distribution, metabolism and excretion of 14C-Toluene-2,5-diamine sulphate in the female Wistar Kyoto rat after a single intravenous dose; NOTOX; 2005
60. Wenker, M. A. M.; Pharmacokinetics of 14C-Toluene-2,5-diamine sulphate in the female Sprague-Dawley rat after a single oral or dermal dose; NOTOX; 2005
61. Wenker, M. A. M.; Pharmacokinetics of 14C-Toluene-2,5-diamine sulphate in the female Sprague-Dawley rat after a single intravenous dose; NOTOX; 2005
62. Aylward, M.; p-(Me-14C) Toluenediamine absorption through hair-bearing scalp, disposition & elimination in adult healthy subjects following application of two formulations containing p-Toluenediamine; SIMBEC RESEARCH LABORATORIES; 1981
63. Lynde, C. W.; Mitchell, J. C.; Patch test results in 66 hairdressers 1973-81; CONTACT DERMATITIS; 8, 302-307; 1982
64. Uter, W.; Geier, J.; Schnuch, A.; Current pattern and trends in sensitization to hairdressers allergens in Germany; OCCUP. ENVIRON. DERMATOL.; 48, 55-59; 2000
65. Katsarou, A.; Koufou, B.; Takou, K.; Kalogeromitros, D.; Papanayiotou, G.; Vareltzidis, A.; Patch test results in hairdressers with contact dermatitis in Greece (1985-1994); CONTACT DERMATITIS; 33, 347-348; 1995
66. Van der Walle, H. B.; Brunsveld, V. M.; Dermatitis in hairdressers (I). The experience of the past 4 years; CONTACT DERMATITIS; 30, 217-221; 1994
67. Frosch, P. J.; Burrows, D.; Camarasa, J. G.; Dooms-Goossens, A.; Ducombs, G.; Lahti, A.; Menne, T.; Rycroft, R. J. G.; Shaw, S.; White, I.; Wilkinson, J. D.; Allergic reactions to hairdresser`s series - results of nine European centers; EUROPEAN ENVIRONMENTAL AND CONTACT DERMATITIS RESE
68. Guerra, L.; Tosti, A.; Bardazzi, F.; Pigatto, P.; List, P.; Santucci, B.; Valsecchi, R.; Schena, D.; Angelini, G.; Sertoli, A.; Ayala, F.; Kokeli, F.; Contact dermatitis in hairdressers: the Italian experience; CONTACT DERMATITIS; 26, 101-107; 1992
69. Frosch, P. J.; Aktuelle Kontaktallergene; DER HAUTARZT; 41, 129-133; 1990
70. Toxicity profile Toluene-2,5-diamine (and its salts); TNO BIBRA INTERNATIONAL LTD; 1995
71. Holm, J. O.; Veierod, M. B.; An epidemiological study of hand eczema. V. Prevalence among hairdresser trainees, compared with a general population of hairdressers; ACTA DERM. VENEREOL.; 187, 23-25; 1994
72. Uter, W.; Lessmann, H.; Geier, J.; Schnuch, A.; Contact allergy to ingredients of hair cosmetics in female hairdressers and clients - an 8-year analysis of IVDK data; CONTACT DERMATITIS; 49, 236-240; 2003
73. Iorizzo, M.; Parente, G.; Vincenzi, C.; Pazzaglia, M.; Tosti, A.; Allergic contact dermatitis in hairdressers: frequency and source of sensitisation; EUR. J. DERMATOL.; 12, 179-182; 2002
74. Schwarz, A.; Gottmann-Lückerath, J.; Allergenhäufigkeit bei Kontaktallergien in der Universitäts-Hautklinik Köln (1970-1971 und 1976-1979); Z. HAUTKR.; 57, 951-960; 1981
75. Kuhlwein, A.; Hausen, B. M.; Standardepikutantestung: Statistische Auswertung aus fünf Jahren; Z. HAUTKR.; 57, 1179-1186; 1982

76. Broeckx, W.; Blondeel, A.; Dooms-Goossens, A.; Achten, G.; Cosmetic intolerance; CONTACT DERMATITIS; 16, 189-194; 1987
77. Jarisch, R.; Sandor, I.; Epikutanstandardtestung: Ergebnisse aus fünf Jahren und ihre Auswirkungen auf zukünftige Untersuchungen; Z. HAUTKR.; 53, 462-470; 1978
78. Guerra, L.; Bardazzi, F.; Tosti, A.; Contact dermatitis in hairdressers´ clients; CONTACT DERMATITIS; 26, 108-111; 1992
79. Koopmans, A. K.; Bruynzeel, D. P.; Is PPD a useful screening agent?; CONTACT DERMATITIS; 48, 89-92; 2003
80. Goossens, A.; Beck, M. H.; Haneke, E.; McFadden, J. P.; Nolting, S.; Durupt, G.; Ries, G.; Adverse cutaneous reactions to cosmetic allergens; CONTACT DERMATITIS; 40, 112-113; 1999
81. Uter, W.; Lessmann, H.; Geier, J.; Becker, D.; Fuchs, T.; Richter, G.; The spectrum of allergic (cross-)sensitivity in clinical patch testing with 'para amino' compounds; ALLERGY; 57, 319-322; 2002
82. Kohl, L.; Blondeel, A.; Song, M.; Allergic contact dermatitis from cosmetics. Retrospective analysis of 819 patch-tested patients; DERMATOLOGY; 204, 334-337; 2002
83. Uter, W.; Lessmann, H.; Geier, J.; Becker, D.; Fuchs, Th.; Richter, G.; Die Epikutantestung mit "Parastoffen"; OCCUP. ENVIRON. DERMATOL.; 50, 97-104; 2002
84. Hamilton, S.; Sheridan, J.; Aplastic anaemia and hair dye; BR. MED. J.; 1, 834; 1976
85. Toghill, J.; Wilcox, G.; Aplastic anaemia and hair dye; BR. MED. J.; 1, 502-503; 1976
86. Hopkins, J. E.; Manoharan, A.; Severe aplastic anaemia following the use of hair dye: report of two cases and review of literature; POSTGRAD. MED. J.; 61, 1003-1005; 1985
87. König, P.; Data base search for references for A005 - Toluene-2,5-Diamine sulfate: 615-50-9 (sulfate[1:1]), 6369-59-1 (sulfate) parent substance: 2,5-Diaminotoluene: 95-70-5; WELLA AG; 2005
88. Burnett, C. M.; Goldenthal, E. I.; Multigeneration reproduction and carcinogenicity studies in Sprague-Dawley rats exposed topically to oxidative hair-colouring formulations containing p-phenylenediamine and other aromatic amines; *FD. CHEM. TOXICOL.*; *26*, *467-474*; 1988
89. Kinkel, H. J.; Holzmann, S.; Study of Long-term Percutaneous Toxicity and Carcinogenicity of Hair Dyes (Oxidizing Dyes) in Rats; *FD. COSMET. TOXICOL.*; *11*, *641-648*; 1973
90. Burnett, C.; Lanman, B.; Giovacchini, R.; Wolcott, G.; Scala, R.; Keplinger, M.; Long-term toxicity studies on oxidation hair dyes; *FD. COSMET. TOXICOL.*; *13*, *353 -357*; 1975
91. Giles, A. L.; Chung, Ch. W.; Dermal carcinogenicity study by mouse-skin painting with 2,4-toluenediamine alone or in representative hair dye formulations; *J. TOXICOL. ENVIRON. HEALTH*; *1*, *433-440*; 1976
92. Burnett, C.; Jacobs, M. M.; Seppala, A.; Shubik, P.; Evaluation of the toxicity and carcinogenicity of hair dyes; *J. TOXICOL. ENVIRON. HEALTH*; *6*, *247-257*; 1980
93. Potokar, M.; Pittermann, W.; Subakute orale Toxizität an Ratten bei einer Versuchsdauer von 12 Wochen; *HENKEL*; 1979
94. Mosesso, P.; Assessment of clastogenic action on bone marrow erythroblasts in the Chromosome Aberration test; *LSR*; 1979
95. Fautz, R.; In vivo/in vitro UDS Synthesis in rat hepatocytes with A 5; *CCR*; 1994
96. Chung, K.-T.; Murdock, C. A.; Stevens, S. E. Jr.; Li Y.-S.; Wei, C.-I.; Huang, T.-S.; Chou, M. W.; Mutagenicity and toxicity studies of p-phenylenediamine and its derivatives; *TOXICOL. LETT.*; *81*, *23-32*; 1995
97. Sasaki, Y. F.; Fujikawa, K. et. al.; The alkaline single cell gel electrophoresis assay with mouse multiple organs: results with 30 aromatic amines evaluated by the IARC and U.S. NTP.; *MUTATION RESEARCH*; *440*, *1-18*; 1999
98. Lloyd, G. K.; Liggett, M. P.; Kynoch, S. R.; Davies, R. E.; Assessment of the acute toxicity and potential irritancy of hair dye constituents; *FD. COSMET. TOXICOL.*; *15*, *607-610*; 1977

99. Burnett, C.; Goldenthal, E. I.; Harris, S. B.; Wazeter, F. X.; Strausburg, J.; Kapp, R.; Voelker, R.; Teratology and percutaneous toxicity studies on hair dyes; *J. TOXICOL. ENVIRON. HEALTH*; *1*, *1027-1040*; 1976
100. Kiese, M.; Rachor; Rauscher, E.; The absorption of some Phenylenediamines through the skin of dogs; *TOXICOL. APPL. PHARMACOL.; 12*, *495-507*; 1968
101. Hruby, R.; The absorption of p-Toluenediamine by the skin of rats and dogs; *FD. COSMET. TOXICOL.*; *15*, *595-599*; 1977
102. Kiese, M.; Rauscher, E.; The Absorption of p-Toluenediamine through human skin in hair dyeing; *TOXICOL. APPL. PHARMACOL.*; *13*, *325-331*; 1968
103. Ames, B. N.; Kammen, H. O.; Yamasaki, E.; Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients; *PROC. NATL. ACAD. SCI.*; *72*, *2423-2427*; 1975
104. Hossack, D. J. N.; Richardson, J. C.; Examination of the potential mutagenicity of hair dye constituents using the micronucleus test ; *EXPERIENTIA*; *33*, *377-378*; 1977
105. Inouye, M.; Murakami, U.; Teratogenicity of 2,5-Diaminotoluene, a hair-dye constituent, in mice; *FD. COSMET. TOXICOL.*; *15*, *447-451*; 1977
106. Burnett, C.; Fuchs, C.; Corbett, J.; Menkart, J.; The effect of Dimethylsulfoxide on the mutagenicity of the hair dye p-Phenylenediamine; *MUTATION RESEARCH*; *103*, *1-4*; 1982
107. Spengler, J.; Osterburg, I.; Korte, R.; Teratogenic evaluation of p-Toluenediamine sulphates, Resorcinol and p-Aminophenol in rats and rabbits; *TERATOLOGY*; *33*, *1-4*; 1986
108. Hofer, H.; Bornatowicz, N.; Reindl, E.; Analysis of human chromosomes after repeated hair dyeing; *FD. CHEM. TOXICOL.*; *21*, *785-789*; 1983
109. L'Oreal, Aulnay-sous-bois, France; Etude comparative de la reactivité in vitro de cinq colorants capillaries vis-à-vis de mitochondrie isolees de muscles de rat; Report No: TM 96/001; 1996
110. Büsching, J.; Subakute Toxizitätsprüfung mit p-Toluylendiaminsulfat, p-Phenylendiamin und Resorcin nach oraler Applikation an Ratten; *WELLA AG*; 1981
111. Cheung, Y. L.; Snelling, J.; Mohammed. N. N.; Gray. T. J.; Ioannides. C.; Interaction with the aromatic hydrocarbon receptor, CYP1A induction, and mutagenicity of a series of diaminotoluenes: implications for their carcinogenicity; *TOXICOL. APPL. PHARMACOL.*; *139*, *203-211*; 1996
112. National Toxicology Program; Salmonella assay; Study ID 578137; 1985
113. National Toxicology Program; Mouse lymphoma assay; Aliquot No. 821795
114. National Toxicology Program; Chromosome aberration assay and sister chromatid exchange assay in vitro; Study No. 951026
115. TOLUENE-2,5-DIAMINE, 50% Aqueous solution: Evaluation of solubility at 1 g/100ml and 10 g/100ml, 2007

Submission III, October 2010

1. Weber, K. Peer Review to: Para-toluenediamine 13 Week Oral (Gavage) Toxicity Study in the Rat (LRL/42/94): Peer Review on Histopathology Examinations from Selected Organs and on Values Recorded for Hematology and Clinical Biochemistry. 12 Oct 2008
2. Prentice D.E. A Review of the Clinical Pathology of Paratoluenediamine (PTD) in a 13 Week Oral (Gavage) Study in the Rat (LRL/42/94). 7 November 2008
3. Prentice D.E.: Paratoluenediamine (PTD) 13 Week Oral (Gavage) Study in the Rat (LRL/42/94) – Histopathology Peer Review. 6 November 2008
4. Weber, K. Peer Review to: Para-toluenediamine 14 Day Oral (Gavage) Range-finding Study in the Rat (LRL/41/93): Peer Review on Histopathology Examinations from Selected Organs. 12 Oct 2008
5. Prentice D.E. Paratoluenediamine (PTD) 14 Day Oral (Gavage) Study in the Rat (LRL/41/93) – Histopathology Peer Review. 6 November 2008
6. Aylward, M. p-(Me-14C) Toluenediamine absorption through hair-bearing scalp, disposition & elimination in adult healthy subjects following application of two

formulations containing p-Toluenediamine; Simbec Research Laboratories (1981) [report previously provided in Submission II, 14 July 2005]
7. Hill, R.E. Paratoluenediamine 13 week oral (gavage) toxicity study in the rat: LRL/42/94 QUINTILES ENGLAND LTD; 1997 [report previously provided in Submission II, 14, July 2005]
8. Schettgen T, Heinrich K, Kraus T, and Gube M. Determination of 2,5-toluylenediamine (2,5-TDA) and aromatic amines in urine after personal application of hair dyes: kinetics and doses. Arch. Toxicol. In press. Published on line: 15 June 2010
9. IPCS. International Program on Chemical Safety. Chemical-specific adjustment factors for interspecies differences and human variability: guidance document for the use of data in dose/concentration-response assessment. Harmonization Project Document No. 2. World Health Organization, Geneva, Switzerland (2005)
10. WHO. Assessing human health risks of chemicals; derivation of guidance values for health-based exposure limits. Environmental Health Criteria, 170, WHO, Geneva (1994)
11. In vitro percutaneous absorption of Toluene-2,5-diamine sulfate through human skin membranes using flow-through diffusion cells. Study Number V8184, TNO Quality of Life, The Netherlands, June 2009.
12. Pharmacokinetics of 14C-toluene-2,5-diamine sulfate in the Sprague-Dawley rat after a single dermal dose at different concentrations. Project Number 488796, NOTOX, The Netherlands, June 2009
13. A 91-day toxicity study of toluene-2,5-diamine sulfate (WR 23005, A005) administered by oral gavage to rats with a 28-day recovery period. Study Number VKA00169, Charles River, Spencerville, Ohio, USA, August 2010
14. Toxicokinetics of [14C] toluene-2,5-diamine sulfate following a single oral administration to Sprague-Dawley rats. Study Number 420782, Charles River, Senneville, Quebec, Canada, September 2010
15. Consumer exposure to an oxidative hair dye. A [14C]-labelled Toluene-2,5-diamine mass balance study. Study Number V9317, TNO, Zeist, The Netherlands, January 2012

Additional references

AR1. Munday R, Manns E, Fowke EA, Hoggard GK (1990) Structure-activity relationships in the myotoxicity of ring-methylated p-phenylenediamins in rats and correlation with autoxidation rates in vitro. Chemical-Biological Interactions 76: 31-45
AR2. Munday R, Manns E (1999) Muscle necrosis in rats induced by 2-methoxy-p-phenylenediamine. Food and Chemical Toxicology 37: 561-564
AR3. Schettgen T, Heinrich K, Kraus T, Gube M (2011) Determination of 2,5-toluylenediamine (2,5-TDA) and aromatic amines in urine after personal application of hair dyes: kinetics and doses. Archives of Toxicology 85: 127-133