



Scientific Committee on Consumer Safety
SCCS

OPINION ON
3-amino-2,6-dimethylphenol
COLIPA n° A162

The SCCS adopted this opinion at its 5th plenary meeting
of 27 March 2014

Revision of the opinion on 3-amino-2,6-dimethylphenol (A162)

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Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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SCCS

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

Revision of the opinion on 3-amino-2,6-dimethylphenol (A162)

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

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Revision of the opinion on 3-amino-2,6-dimethylphenol (A162)

1. BACKGROUND

Submission I for the new hair dye substance 3-amino-2,6-dimethylphenol (CAS 6994-64-5) (EC 230-268-6) A162 was received in July 2013 by Cosmetics Europe.

The present submission I includes a complete dossier according to the hair dye strategy and the applicant asks for the safety evaluation of 3-amino-2,6-dimethylphenol (A162), when used in hair dye formulations at an on-head concentration up to 2.0%.

2. TERMS OF REFERENCE

1. *Does the SCCS consider 3-amino-2,6-dimethylphenol safe for use as oxidative hair dye formulations with an on-head concentration of maximum 2.0% taken into account the scientific data provided?*

2. *And/or does the SCCS recommend any restrictions with regard to the use of 3-amino-2,6-dimethylphenol in oxidative hair dye formulations (e.g. max conc. in the finished cosmetic product, dilution ratio with hydrogen peroxide, warning)?*

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3. OPINION

3.1. Chemical and physical specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

3-Amino-2,6-dimethylphenol (INCI)

3.1.1.2. Chemical names

- Phenol, 3-amino-2,6-dimethyl (CA INDEX NAME, 9CI)
- 3-Amino-2,6-dimethylphenol (IUPAC)
- 3-Amino-2,6-xylenol
- 3-Amino-xylenol

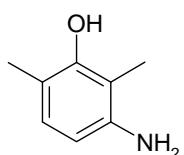
3.1.1.3. Trade names and abbreviations

Trade names: Mepur, Methylpurpur, Me-AHT, 3-amino-2,6-dimethylphenol
COLIPA n°: A162

3.1.1.4. CAS / EC number

CAS: 6994-64-5
EC: 230-268-6

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

C₈H₁₁NO

3.1.2. Physical form

White to beige powder

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3.1.3. Molecular weight

137.18 g/mol

3.1.4. Purity, composition and substance codes

Two batches of 3-amino-2,6-dimethylphenol (RD-CRU095-04/10-04 and GST083-04/70-07) were used in the toxicological testing reported in this dossier. Summary of the analytical data is described in the table below.

	RD-CRU095-04/10-04		Batch GST083-04/70-07	
	Result	Ref.	Result	Ref.
Purity [%area]	210 nm 254 nm 280 nm	98.6 98.9 97.7	2* 2* 2*	99.7 99.2 99.0
Assay [% w/w] by NMR	Not determined		97.8	6
Water [% w/w]	0.15	2*	0.85	5
Ash [% w/w]	Not determined		0.27	5
Elemental screening [ppm]*	Na (43) Si (29) P (44) K (74) Ca (65) Sb, Cd, Hg, Pb, As, Ni, Cu not detected (LOD=10 ppm)	3	Na (62) P (89) K (122) Sb, Cd, Hg, Pb, As, Ni, Cu not detected (LOD=10 ppm)	7
4-Amino-2,6-dimethyl-phenol-hydrochloride monohydrate	< LOD: 20 ppm	2*	< LOD: 50 ppm	5
4-[(2-Amino-4-hydroxy-3,5-methylphenyl)imino]-2,6-dimethyl-2,5-cyclohexadien-1-one	1 ppm	2*	< LOD: 20 ppm	5
3,5-Diamino-2,6-dimethylphenol diphosphate	Not determined		< LOD: 50 ppm	5
2,2'-Diamino-3,3',5,5'-tetramethylbiphenyl-4,4'-diol	Not determined		0.1% (w/w)	5
2,6-Dimethylphenol	Not determined		< LOD: 20 ppm	
Unknown impurity**	0.38% at 210 nm 0.35% at 254 nm 1.69% at 280 nm	2*, 4	0.04% at 210 nm 0.09% at 254 nm 0.42% at 280 nm	4, 5

* The unknown peak at tR= 17.95 min was detected with a molecule weight of 259 g/Mol performed by LC/MS after adjusting the used LC-method to MS.

** Only the results of LAN analysis were submitted. No study report on chemical analyses was submitted.

In addition, composition of three other batches of 3-amino-2,6-dimethylphenol (as certificate of analysis) was also submitted as described in the table below.

3-amino-2,6-dimethylphenol	Batch number		
HPLC purity area %	20110901	20110902	20110903
210 nm	99.74	99.71	99.71
254 nm	99.88	99.91	99.87

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280 nm	99.64	99.49	99.58
No single impurity in HPLC chromatogram greater than 0.1% area at 210nm	Conforms	Conforms	Conforms
HPLC assay vs reference standard	100.49%	100.01%	100.35%
Ash	0.02	0.04%	0.02%
Water (Karl Fischer)	0.21%	0.17%	0.16%
Toluene	0.105%	0.09%	0.109%
Iron	4 ppm	7 ppm	3 ppm
Heavy metals	<20 ppm	<20 ppm	<20 ppm

Ref.: 8

3.1.5. Impurities / accompanying contaminants

See 3.1.4.

3.1.6. Solubility

Solvent	Solubility (g/l)	Reference
Water	9.34	9 (EC Method A.6)
Acetone/water 1:1	> 200	10
DMSO	> 200	10
DMF (dimethylformamide)	> 200	10
DMSO/water 1:1	> 200	10
Acetone	> 200	10

SCCS comment

It is noted that the solubility of 3-amino-2,6-dimethylphenol in propylene glycol is not provided. Propylene glycol is the vehicle used in the studies on the acute and subchronic oral toxicity as well as the prenatal developmental toxicity. The study reports on the subchronic (90-days) oral toxicity study (see 3.3.5.2.) and the prenatal developmental toxicity study (see 3.3.8.2.) state that the formulations were suspensions. From the dose levels and the dosing volumes used in these studies, the solubility of 3-amino-2,6-dimethylphenol in propylene glycol can be estimated to be less than 12 g/l.

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}= 0.19 (pH 7.0)

EC Method A.8

Ref.: 21

3.1.8. Additional physical and chemical specifications

		Reference
Particle size distribution	mean particle diameter without fine dust (>32 - <250 µm) was calculated to be 135.9 µm	11
pH-value	7.53 (0.93% (w/w) aqueous solution, 20 °C)	9
Melting point	102.3 – 103.1 °C	12
Boiling point	not determined because decomposition starts about 250 °C	13
Density	1.2023 g/ml at 20 °C	14
Vapour pressure	0.3 x 10 ⁻⁵ hPa (20 °C, ex.-plates)	15
Surface tension	71.06 mN/m (19.9 °C, in water)	16

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Flammability (solids)	not highly flammable	17
Explosive properties	no danger of explosion	18
Relative self-ignition temperature	no self-ignition up to >403 °C	19
Oxidizing properties	not oxidizing	20
UV/Vis spectrum	/	

3.1.9. Homogeneity and stability

A 10% (w/v) solution of 3-amino-2,6-dimethylphenol in DMSO and in water/acetone (1:1 v/v) was found to be stable over a 7-day time period at room temperature (recovery > 96%).

Ref.: 22

In the dermal penetration study, the solubility of 3-amino-2,6-dimethylphenol in the receptor fluid was determined as 9.2 mg/ml. At a concentration of 1 mg/ml, 98% of 3-amino-2,6-dimethylphenol could be recovered from receptor fluid after 72 hours in the presence of 0.3% ascorbic acid and 0.4% sodium sulfite.

In a 90-day subchronic toxicity study, the concentrations of 3-amino-2,6-dimethylphenol determined by HPLC analysis in the test formulations (suspensions in propylene glycol) were between 97% and 114% of the target concentrations of 13.5 mg/g, 40.6 mg/g and 122 mg/g. The suspensions were demonstrated to be homogeneous (coefficient of variation <10%). 3-Amino-2,6-dimethylphenol in the test formulations (13.5 mg/g and 131 mg/g in propylene glycol) was demonstrated to be stable for at least 5 hours (relative difference of <5.8% of the nominal concentration), when the formulations were stored at room temperature in the dark.

In the prenatal developmental toxicity study, the dose formulations were prepared daily as homogenous suspensions. Samples of each concentration of the dosing formulations were analyzed for concentration and stability over 4 hours.

SCCS comment

Stability of 3-amino-2,6-dimethylphenol in typical hair dye formulations is not reported.

3.2. Function and uses

3-Amino-2,6-dimethylphenol is used as an oxidative hair colouring agent (coupler). The intended maximum on-head concentration is 2.0% in oxidative hair dye formulations. The oxidative colouring agent and the developer are mixed at a ratio of 1+1 to 1+3 (g dye formulation + g developer formulation).

3.3. Toxicological evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline: OECD Guideline no. 423 (2001)
Species/strain: Rat, Wistar strain CRL:WI(SPF)
Group size: 12 females, 3 per group
Test substance: 3-Amino-2,6-dimethylphenol

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Batch:	GST083-04/70-07
Purity:	99.2 area% (HPLC at 254 nm)
Vehicle:	Propylene glycol
Dosing volume:	10 ml/kg bw
Dose levels:	2000 mg/kg bw, 300 mg/kg bw (2 treatment groups for each dose)
Administration:	Oral gavage, single application
Observation period:	14 days
GLP:	In compliance
Study period:	June 2007 – July 2007

Formulations were prepared as suspensions within 4 hours prior to dosing and were visually inspected for homogeneity.

Mortality was checked twice daily and clinical signs were checked daily during the 14-day observation period. Body weights were recorded on day 1 prior to administration and on days 8 and 15. A gross necropsy was performed on all animals on the day of death or after terminal sacrifice (day 15).

Results

In the first treatment group at 2000 mg/kg bw, no animal died. The clinical signs observed were lethargy, flat and/or hunched posture, uncoordinated movements, rales and/or piloerection on the days 1 to 3. In the second treatment group at 2000 mg/kg bw lethargy, flat posture, uncoordinated movements, rales, laboured respiration and fluid discharge from the eyes were observed on day 1. One animal of this dose group was found dead on day 1, the remaining two animals were found dead on day 2.

In the first treatment group at 300 mg/kg bw clinical signs of lethargy, hunched posture, uncoordinated movements, rales, ptosis and/or piloerection were observed on the days 1 to 3. In the second treatment group at 300 mg/kg bw lethargy, hunched posture and piloerection were observed on days 1 and 2. No animal died in these treatment groups.

The mortality rates after a single oral application are summarized in the following table.

Group	Dose [mg/kg bw]	
	2000	300
First set	0/3	0/3
Second set	3/3	0/3

The mean body weight gain from the surviving animals was considered as normal for rats of this age and strain.

No abnormalities were found during the post mortem macroscopic examination of the animals

Conclusion

The oral LD₅₀ value of 3-amino-2,6-dimethylphenol in Wistar rats was established to be within the range of 300-2000 mg/kg bw. According to the OECD 423 test guideline, the LD₅₀ cut-off value for 3-amino-2,6-dimethylphenol was set at 500 mg/kg bw.

Ref.: 45

3.3.1.2. Acute dermal toxicity

No data submitted.

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3.3.1.3. Acute inhalation toxicity

No data submitted.

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

In vitro skin corrosion: Transcutaneous electrical resistance (TER) assay

Guideline:	OECD Test Guideline 430 (adopted in April 2004)
Test system:	<i>Ex vivo</i> skin discs from rat (1 male; strain: HsdRccHan ^{®TM} :WIST ^{®TM}) dorsal skin
Test substance:	3-Amino-2,6-dimethylphenol
Batch:	GST083-04/70-07
Purity:	99.2 area % (at 254 nm, HPLC)
Test concentration:	Neat (100%; powder)
Dose volume:	Sufficient test item was applied evenly to the skin discs to ensure that the whole surface of the epidermis was covered
Contact period:	24 hours
Positive control:	Hydrochloric acid 10 M (~36%)
Negative control:	Sterile distilled water
GLP:	In compliance
Study period:	December 2008 - February 2009

One male rat was used for the study. After sacrifice, the dorsal skin was removed from the rat as a single pelt and two skin discs of approximately 0.79 cm² were taken from the pelt and the TER measured as a quality control procedure. Each disc had to give a resistance value of greater than 10 kΩ in order for the remainder of the pelt to be used in the assay. 3-Amino-2,6-dimethylphenol was applied neat to the epidermal surface of three skin discs for a contact period of 24 hours. Sufficient test item was applied evenly to the skin discs to ensure that the whole surface of the epidermis was covered. 150 µL distilled water was applied to ensure good contact with the skin. At the end of the exposure period, the test item was removed by washing the skin disc with a jet of warm tap water until no further test item could be removed. Three positive (hydrochloric acid 10 M (~36%)) and negative control (sterile distilled water) skin discs were also assayed for a contact period of 24 hours. The TER was measured using a Wheatstone Bridge with a low voltage alternating current and the value expressed as in Ω/kΩ per skin disc was determined. The mean TER for the skin discs was calculated.

Results are accepted if the mean positive and negative control results for the assay fall within the accepted ranges of 0.5 to 1.0 kΩ for the positive control (hydrochloric acid 10 M (~36%)) and 10 to 25 kΩ for the negative control (sterile distilled water).

The test item is classified as 'Non-Corrosive' if the mean TER value recorded for the 24-hour contact period is greater than 5 kΩ. The test substance will be classified as 'Corrosive' if the mean TER value recorded for the 24-hour contact period is 5 kΩ or lower.

Results

The mean TER after a contact period of 24 hours with the test item 3-amino-2,6-dimethylphenol was 19.4 kΩ (\pm 4.1 SD). The quality criteria for acceptance of the results were satisfied based on a TER for the positive control (hydrochloric acid 10 M (~36%)) of 871 Ω (\pm 49.6 SD) and 18.6 kΩ (\pm 3.2 SD) for the negative control (sterile distilled water).

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Conclusion

In conclusion, 3-amino-2,6-dimethylphenol as neat substance is classified as 'non-corrosive'.

Ref.: 24

SCCS comment

In contrast to OECD 430 the animal delivering the skin discs was 21-23 days old while OECD demands 28-30 days.

In vitro skin irritation: EpiSkin™ reconstructed human epidermis (RHE) test

Guideline:	Not available at time of study conduct but performed using EpiSkin™ RHE test method validated by ECVAM (April 2007). OECD Test Guideline 439 adopted in July 2010.
Test system:	<i>In vitro</i> EpiSkin™ RHE model consisting of adult human-derived epidermal keratinocytes seeded on a dermal substitute consisting of a collagen type I matrix coated with type IV collagen
Endpoints:	MTT cytotoxicity determination; morphology changes by histology
Treatment period:	15 minutes + 42 ± h post-treatment incubation period
Test substance:	3-amino-2,6-dimethylphenol
Batch:	GST083-04/70-07
Purity:	99.2 area % (at 254 nm, HPLC)
Concentrations:	Neat (100%; powder) and 1% (w/v) in 5% dimethylsulfoxide (DMSO)
Exposure conditions:	Single application of 10 mg (neat) or 10 µL of 1.0% (w/v) test item solubilised in 5% DMSO for 15 minutes at room temperature, followed by a rinsing step and a 42-hour post-treatment incubation.
Positive control:	Sodium dodecyl sulfate 5% (w/v) applied in sterile water
Negative control:	Sterile water
GLP:	In compliance
Study period:	December 2008 - October 2010

3-Amino-2,6-dimethylphenol was applied as neat test item and at a concentration of 1.0% (w/v) in 5% DMSO in sterile water to triplicate tissues of EpiSkin™ RHE model. Amount of test item administered was 10 mg ± 2 mg (following wetting of the tissue surface with 5 µL of sterile water to improve contact) for neat and 10 µL for diluted test item. The treatment period was 15 minutes followed by a rinsing step and a 42 ± h post-treatment incubation period. Two endpoints, namely cytotoxicity in the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay, expressed as percent viability of treated cultures in comparison to negative controls, as well as morphological changes identified by histology, were evaluated.

Study controls were sodium dodecyl sulfate 5% (w/v) applied in sterile water (positive control), sterile water (negative control) and 5% DMSO (v/v) in sterile water (vehicle control). Additional controls were included to correct for possible interference of test item still present within/on tissue at the time of the MTT test. These controls were: 1) colour correction tissues (treated with test item but not stained with MTT) to correct for non-specific absorbance due to intrinsic colouring properties of the test item and 2) killed tissues (unable to metabolically reduce MTT) to correct for possible direct reduction of MTT by the test item.

The test item was classified based on tissue viability analysis according to the prediction model: non-irritant to skin if tissue viability is > 50% (Not Classified) and irritant to skin if tissue viability is ≤ 50% (Category 2). Histological evaluation of the tissues at the end of the treatment was performed as an additional measure to correlate with cytotoxicity to evaluate irritation for test items which may interfere with MTT. A decrease in MTT reduction capacity and changes in tissue morphology were used as indicators of potential irritancy.

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Results

3-Amino-2,6-dimethylphenol did not induce a significant decrease in cell viability in the MTT assay for either of the concentrations tested with viability measurements of $92.9 \pm 5.7\%$ (neat) and $87.2 \pm 5.7\%$ (1.0% (w/v) solubilised in 5% DMSO). The colour of the test item did not interfere with the MTT test at either of the concentrations tested. Furthermore, histological evaluation of the treated tissues showed no marked epidermal effects in the treated cultures in comparison to the negative control cultures for 3-amino-2,6-dimethylphenol tested neat and at 1.0% (w/v) in 5% DMSO.

Conclusion

In conclusion, 3-amino-2,6-dimethylphenol is classified as non-irritant (MTT viability > 50%), when tested neat and at 1.0% (w/v) in 5% DMSO. The histological examinations confirmed the absence of cytotoxicity for 3-amino-2,6-dimethylphenol when tested both neat and at 1.0% (w/v) in 5% DMSO.

Ref.: 25

SCCS comment on skin irritation

The EpiSkinTM RHE test method only allows identification of CLP Category 2 (UN GHS Category 2) irritants, but does not allow the identification of mild skin irritants (UN GHS Category 3; no CLP category). Therefore, mild skin irritancy cannot be excluded from the submitted data.

3.3.2.2. Mucous membrane irritation

Isolated chicken eye test (ICE): Study 1

Guideline:	Not available at time of study conduct but performed in accordance with the test method validated by ECVAM (May 2007) for identification of severe ocular irritants. OECD Test Guideline 438 (Isolated Chicken Eye Test Method for Identifying Ocular Corrosives and Severe Irritants) adopted in September 2009.
Test system:	Isolated chicken eyes (ROSS, spring chickens)
Parameters assessed:	Corneal thickness (expressed as corneal swelling), corneal opacity and fluorescein retention.
Number of replicates:	Three eyes per test concentration, three eyes for solvent control, one eye for positive control and one eye for negative control
Test substance:	3-Amino-2,6-dimethylphenol
Batch:	GST083-04/70-07
Purity:	99.2 area % (at 254 nm, HPLC), for details 2.4.
Concentrations:	Neat and 2.0% (w/w) in 10% dimethylsulfoxide (DMSO)
Exposure conditions:	Single application of 30 µL (equivalent) of neat and 30 µL of 2.0% (w/w) test item for 10 seconds
Positive control:	Benzalkonium chloride 5% (w/w) aqueous solution
Negative control:	Physiological saline
GLP:	Not in compliance
Study period:	February 2008- December 2008

Approximately 7-week old chickens were used as eye donors. Within 2 hours after sacrifice, eyes were carefully dissected and placed in a superfusion apparatus. Eyes with a corneal thickness deviating more than 10% of the average corneal thickness of the eyes, eyes that showed opacity (score higher than 0.5) or were unacceptably stained with fluorescein (score

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higher than 0.5) were rejected and were replaced. A total of eleven eyes were selected for testing: three for neat test item, three for 2.0% (w/w) test item in 10% DMSO, three for the solvent control DMSO 10% (v/v) aqueous, one for the positive control (benzalkonium chloride 5% (w/w) aqueous solution) and one for the negative control (physiological saline). After an equilibration period of 45-60 minutes, the corneal thickness of the eyes was measured once more to determine the zero reference value for corneal swelling calculations. At time $t = 0$, corneas were treated with 30 μL equivalent of neat 3-amino-2,6-dimethylphenol and three corneas were treated with 30 μL of 2.0% (w/w) 3-amino-2,6-dimethylphenol in 10% aqueous DMSO. After an exposure period of 10 seconds, the corneal surface was rinsed thoroughly with 20 mL of isotonic saline of ambient temperature. After rinsing, each eye in the holder was returned to its chamber. The negative control eye was treated with physiological saline only, the positive control eye with benzalkonium chloride 5% (w/w) aqueous solution and the three solvent controls eyes with DMSO 10% (v/v) aqueous solution. The eyes were examined at 0, 30, 75, 120, 180 and 240 minutes after treatment. All examinations were performed with a slit-lamp microscope. Fluorescein retention was scored only at 30 minutes after treatment. After the final examination, the test and control eyes were preserved in a neutral aqueous phosphate-buffered solution of 4% formaldehyde. The tissues selected were embedded in paraffin wax, sectioned at 5 μM and stained with PAS for histopathological examination. Ocular effects were evaluated using the endpoints of corneal thickness (swelling), corneal opacity and fluorescein retention.

Defined scoring scales are used for each endpoint to define the severity of effects into four categories (I-IV). Four classes of eye irritancy (not irritating; slightly irritating; moderately irritating; severely irritating) can be identified in the ICE test by combination of the categories defined for each of the evaluation parameters.

Results

3-Amino-2,6-dimethylphenol tested neat caused very slight corneal effects in that there was no swelling, very slight opacity (0.5) and very slight fluorescein retention (0.5). The Irritation Index calculated was 20. Microscopic examination of the treated corneas identified no significant corneal effects with no toxicological significance attached to the findings of an irregular epithelial surface and presence of necrosis in the epithelium of one cornea.

3-Amino-2,6-dimethylphenol tested at 2.0% (w/w) in 10% DMSO caused almost no corneal effects in that there was very slight swelling (1%), no opacity and very slight fluorescein retention (0.3). The Irritation Index calculated was 7. Microscopic examination of the treated corneas revealed no corneal effects.

Conclusion

On the basis of the results obtained in the ICE study, 3-amino-2,6-dimethylphenol tested neat and at 2.0% (w/w) in 10% aqueous DMSO is identified as not irritating to eyes.

Ref.: 30

Isolated chicken eye test (ICE): Study 2

Guideline:	OECD Test Guideline 438 (Isolated Chicken Eye Test Method for Identifying Ocular Corrosives and Severe Irritants) adopted in September 2009.
Test system:	Isolated chicken eyes (ROSS, spring chickens)
Parameters assessed:	Corneal thickness (expressed as corneal swelling), corneal opacity and fluorescein retention
Number of replicates:	Three eyes per test concentration, three eyes for solvent control, three eyes for positive control and three eyes for negative control
Test substance:	3-Amino-2,6-dimethylphenol

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Batch:	GST083-04/70-07
Purity:	99.2 area % (at 254 nm, HPLC)
Concentrations:	1.0% (w/w) in 5% aqueous dimethylsulfoxide (DMSO)
Exposure conditions:	Single application of 30 µL of 1.0% (w/w) test item for 10 seconds
Positive control:	Benzalkonium chloride 5% (w/w) aqueous solution
Negative control:	Physiological saline
GLP:	In compliance
Study period:	November 2009 - March 2009

Approximately 7-week old chickens were used as eye donors. Within 2 hours after sacrifice, eyes were carefully dissected and placed in a superfusion apparatus. Eyes with a corneal thickness deviating more than 10% of the average corneal thickness of the eyes, eyes that showed opacity (score higher than 0.5) or were unacceptably stained with fluorescein (score higher than 0.5) were rejected and were replaced. A total of twelve eyes were selected for testing: three for the test item, three for the positive control (benzalkonium chloride 5% (w/w) aqueous solution); three for the negative control (physiological saline) and three for the solvent control (DMSO 5% (v/v) aqueous solution). After an equilibration period of 45-60 minutes, the corneal thickness of the eyes was measured once more to determine the zero reference value for corneal swelling calculations. At time t = 0, three corneas were treated with 30 µL of 1.0% (w/w) 3-amino-2,6-dimethylphenol in 5% aqueous DMSO. After an exposure period of 10 seconds, the corneal surface was rinsed thoroughly with 20 mL of isotonic saline of ambient temperature. After rinsing, each eye in the holder was returned to its chamber. The three negative control eyes were treated with physiological saline only, the three positive control eyes with benzalkonium chloride 5% (w/w) aqueous solution and the three solvent controls eyes with DMSO 5% (v/v) aqueous solution. The eyes were examined at 0, 30, 75, 120, 180 and 240 minutes after treatment. All examinations were performed with a slit-lamp microscope. Fluorescein retention was scored only at 30 minutes after treatment. After the final examination the test and control eyes were preserved in a neutral aqueous phosphate-buffered solution of 4% formaldehyde. The tissues selected were embedded in paraffin wax, sectioned at 5 µM and stained with PAS for histopathological examination. Ocular effects were evaluated using the endpoints of corneal thickness (swelling), corneal opacity and fluorescein retention.

Defined scoring scales are used for each endpoint to define the severity of effects into four categories (I-IV). Four classes of eye irritancy (not irritating; slightly irritating; moderately irritating; severely irritating) can be identified in the ICE test by combination of the categories defined for each of the evaluation parameters.

Results

3-Amino-2,6-dimethylphenol tested at 1.0% (w/w) in 5% aqueous DMSO caused almost no corneal effects in that there was almost no swelling (2%), no opacity and no fluorescein retention. The Irritation Index calculated was 2. Microscopic examination of the treated corneas identified no corneal effects.

The negative, positive and vehicle controls confirmed the validity of the assay. The negative (physiological saline) and solvent (DMSO 5% (v/v) aqueous) controls caused almost no corneal effects in that there was almost no swelling (1% for both controls), no opacity and no fluorescein retention. The Irritation Index calculated was 1 for both the negative (physiological saline) and solvent (DMSO 5% (v/v) aqueous) controls. Microscopic examination of the negative (physiological saline) and solvent (DMSO 5% (v/v) aqueous) control corneas confirmed no corneal effects. The positive control (benzalkonium chloride 5% (w/w) aqueous solution) caused moderate or severe corneal effects in that moderate swelling (30%), severe opacity (3.0) and severe fluorescein retention (3.0) were observed. The calculated Irritation index was 150. Microscopic examination of the positive control (benzalkonium chloride 5% (w/w)) corneas confirmed the severity of the corneal effects observed.

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Conclusion

On the basis of the results obtained with the ICE test, 1.0% (w/w) 3-amino-2,6-dimethylphenol in 5% DMSO was determined as not irritating to eyes.

Ref.: 31

Statement of the applicant regarding eye irritation:

The applicant used a read-across approach for dyes of the aminophenols class for which *in vivo* eye irritation data that address the range of intended use concentrations are available and had been previously evaluated by the SCCS. The applicant considered this read-across approach as the primary element in a "Weight of Evidence" approach to identification of 3-amino-2,6-dimethylphenol as non-irritating to eyes at the intended in-use concentration, and concluded that:

- As a class of dye materials, aminophenols are non-irritating to eyes when tested *in vivo* at the intended use concentrations.
- 3-Amino-2,6-dimethylphenol and the dye materials [Colipa A94 (5-amino-6-chloro-o-cresol, A27 (4-amino-2-hydroxytoluene), A31 (2-methyl-5-hydroxyethylaminophenol) and A15 (m-aminophenol)] have the same chemical reactivity in the colouring system and very similar physicochemical properties.
- The m-aminophenol 3-amino-2,6-dimethylphenol is predicted to be non-irritating to eyes when used at the intended use concentration of 2.0%.

The *in vitro* data support the primary conclusion that 3-amino-2,6-dimethylphenol is not irritating to eyes at the in-use concentration (2.0%) since they demonstrate that 3-amino-2,6-dimethylphenol is not irritating to eyes neat, at 1.0% (w/w) and 2.0% (w/w) tested *in vitro*.

SCCS comment on eye irritation

Substances mildly irritating to eyes (UN GHS Category 2B) may not be detected.

3.3.3. Skin sensitisation

Local lymph node assay (LLNA)

Guideline:	According to OECD guideline no. 429 (2002)
Species/strain:	Mouse, strain CBA/J
Group size:	5 females per test concentration
Test substance:	3-Amino-2,6-dimethylphenol
Batch:	GST 083-04/70-07
Purity:	99.2 area% (HPLC, 254 nm), for details see 2.4.
Concentrations:	0.5, 1.5, 5.0, 15.0%
Vehicle:	Acetone/water (1:1) mixed with olive oil (3:1)
Dosing schedule:	Once daily on three consecutive days
Positive control:	alpha-Hexylcinnamic aldehyde (HCA) at a concentration of 25.0% in acetone/water (1:1) mixed with olive oil (3:1)
GLP:	In compliance
Study period:	February-June 2007

The skin sensitising potential of 3-amino-2,6-dimethylphenol was investigated in CBA/J mice by measuring the lymphocyte proliferation in the draining lymph nodes after topical application on the ear.

25 µl of 0 (vehicle only), 0.5, 1.5, 5.0 and 15.0% 3-amino-2,6-dimethylphenol in acetone/water (1:1) mixed with olive oil (3:1) were applied to the surface of the ear of five

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female mice per group for three consecutive days. As a positive control, alpha-hexylcinnamic aldehyde (HCA) at a concentration of 25.0% in acetone/water (1:1) mixed with olive oil (3:1) was investigated in parallel under identical test conditions.

Animals were checked for morbidity/mortality at least once daily. Observation for clinical signs was done once per day. Body weight was determined at day -1 and day 5.

At day 5, the mice received an intravenous injection of 250 µl solution containing 20.6 µCi of [³H-methyl]-thymidine. Approximately five hours later, the mice were sacrificed, and the draining auricular lymph nodes were removed and collected in PBS. After preparing a single cell suspension for each mouse, cells were precipitated by 5% trichloroacetic acid (TCA), and the radioactivity was determined (incorporation of [³H-methyl]-thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm).

Results

No animal died and no test item related clinical signs in the treated animals were observed during the study. There were no treatment-related effects on body weight or body weight gain.

The positive control alpha-hexylcinnamic aldehyde (HCA) at a concentration of 25% induced a 5.7-fold increase in the lymphocyte proliferation in the draining auricular lymph nodes relative to the vehicle.

3-Amino-2,6-dimethylphenol in acetone/water (1:1) mixed with olive oil (3:1) did not induce a relevant immune response in the local lymph node assay, since there was less than a 3-fold increase in lymphocyte proliferation in the draining auricular lymph nodes relative to the vehicle control. The mean stimulation indices were 1.3, 1.1, 1.0 and 2.0 at the concentrations of 0.5, 1.5, 5.0 and 15.0%, respectively. The EC₃ value was therefore not calculated.

Conclusion

3-Amino-2,6-dimethylphenol is not a skin sensitizer under the defined experimental conditions.

Ref.: 32

SCCS comment

A sensitisation potential cannot be excluded as the maximum test concentration was too low.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (2004)
Tissue:	Porcine back or flank skin (frozen/thawed; thickness: ≤ 1000 µm)
Method:	Diffusion Teflon-chambers
Diffusion cells:	Flow-through system with Teflon-chamber with 9.1 cm ² . in-house development, 32 ± 2 °C
Group size:	Two experiments with 6 chambers each from 3 donors
Skin integrity:	Measure of penetration using tritiated water (<2%)
Test substance:	3-Amino-2,6-dimethylphenol
Batch:	GST 083-04/70-07
Purity:	99.2 area % (at 254 nm, HPLC), for details see 2.4.
Test substance:	3-Amino-2,6-dimethyl[U- ¹⁴ C]phenol
Batch no:	CFQ15282 Batch 1
Radiochemical purity:	99.5 %
Chemical identity:	HPLC profile and retention time of the labelled product is identical to that of the standard compound
Concentration:	2.0 mg/cm ² , tested as part of an oxidative hair dye formulation

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Receptor fluid:	Physiological solution: 0.14 M NaCl, 2 mM K ₂ HPO ₄ , 0.4 mM KH ₂ PO ₄ , 100 IU penicillin/ml and 97 µg streptomycin/ml
Solubility in receptor fluid:	9.21 mg/ml
Quantification:	Scintillation counting
GLP:	In compliance
Study period:	September - November 2007

The cutaneous absorption of 2.0% 3-amino-2,6-dimethylphenol in a typical hair dye formulation in the presence of hydrogen peroxide and a reaction partner was investigated *in vitro*, using pig skin preparations, which were continuously rinsed from underneath with physiological receptor fluid (flow through cell apparatus) at a temperature of 32 ± 2 °C. The experiment was performed with 12 diffusion cells from 3 donors. The integrity of each skin preparation was determined by examination of penetration characteristics with tritiated water.

After checking the skin integrity, 400 mg of the formulation (= 100 mg/cm²), containing 2.0% of 3-amino-2,6-dimethylphenol, was applied to the skin samples (= 2.0 mg of test item/cm²) for 30 minutes and subsequently washed off with water and shampoo. The determination of the amount of 3-amino-2,6-dimethylphenol in the washings (= amount dislodgeable from the skin surface) was performed by measuring the radioactivity by means of liquid scintillation counting. At 16, 24, 40, 48, 64 and 72 hours, the content of 3-amino-2,6-dimethylphenol was determined in the receptor fluid by the same method. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the radioactivity was quantified by means of liquid scintillation counting.

Results

The majority of the test substance was found in the rinsing solutions (1852.05 ± 22.10 µg/cm²). Small amounts of 3-amino-2,6-dimethylphenol were found in the upper skin (4.688 ± 0.871 µg/cm²), in the lower skin (0.645 ± 0.339 µg/cm²) and in the fractions of the receptor fluid collected within 72 hours (5.941 ± 0.857 µg/cm²). The mass balance of the test substance resulted in values of 97.3 to 100.8% recovery for all (12) skin samples taken into consideration for the calculation of the mean values.

The results of the cutaneous absorption experiment are summarised in the following table.

Summary of the cutaneous absorption of 2.0% of 3-amino-2,6-dimethylphenol

Amount of 3-amino-2,6-dimethylphenol in:	µg/cm ² (mean ± S.D, n=12)			%* (mean ± S.D, n=12)		
Receptor fluid (72 hours)	5.941	±	0.857	0.306	±	0.043
Lower skin (72 hours)	0.645	±	0.339	0.033	±	0.017
Upper skin (72 hours)	4.688	±	0.871	0.241	±	0.044
Rinsing solution (after 60 min.)	1852.05	±	22.10	95.39	±	0.97
Total balance (recovery)**	1921.72	±	19.07	98.98	±	1.08

* Corrected for individual applied dose

** Total is corrected for losses on pipette tips

With respect to the receptor fluid samples, 3-amino-2,6-dimethylphenol was detectable predominantly within the first fractions collected during 72 hours (fractions 0-16 hours). Smaller amounts of 3-amino-2,6-dimethylphenol were detectable in the subsequent fractions. At the end of the experiment (after 72 hours) the amounts of 3-amino-2,6-dimethylphenol detectable in the receptor fluid declined towards amounts in the range of

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the limit of quantification, thus indicating that no further 3-amino-2,6-dimethylphenol remaining on or in the skin after 72 hours migrates to deeper layers. With such an absorption profile, the amounts of 3-amino-2,6-dimethylphenol found in the upper skin are not considered as biologically available (no depot effect).

Conclusion

Under the assumption that a depot effect is absent, the amount of 3-amino-2,6-dimethylphenol considered as biologically available was $6.586 \pm 0.922 \mu\text{g}/\text{cm}^2$ ($n=12$, three donors; receptor fluid + lower skin; $5.941 \mu\text{g}/\text{cm}^2 + 0.645 \mu\text{g}/\text{cm}^2$). Thus, as a conservative assumption, an amount of 3-amino-2,6-dimethylphenol of $7.508 \mu\text{g}/\text{cm}^2$ (maximum absorption + 1 SD) was considered as bioavailable for the final risk assessment. One SD was added to the mean, because three donors instead of 4 were used.

Ref.: 33

SCCS comment

The amount of formulation applied was higher than recommended ($100 \mu\text{g}/\text{cm}^2$ instead of $20 \mu\text{g}/\text{cm}^2$) and three donors instead of four were used. The amount of 3-amino-2,6-dimethylphenol considered as biologically available was $6.59 \pm 0.92 \mu\text{g}/\text{cm}^2$.

The amount considered absorbed for calculating the MoS is Mean + 2 SD = $8.43 \mu\text{g}/\text{cm}^2$.

3.3.5. Repeated dose toxicity

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

No data submitted.

3.3.5.2. Sub-chronic (90 days) oral toxicity

Guideline:	OECD 408 (1998)
Species/strain:	Rat, Wistar strain Crl:WI BR (outbred, SPF quality)
Group size:	10 per sex and dose group
Test substance:	3-Amino-2,6-dimethylphenol
Batch:	GST 083-04/70-07
Purity:	99.2 area % (at 254 nm, HPLC)
Vehicle:	Propylene glycol
Dose levels:	70, 210 and 630 mg/kg bw/day
Dose volume:	5 ml/kg bw
Route:	Oral gavage
Administration:	Daily for 90 days
Control:	Vehicle only
GLP:	In compliance
Study period:	August 2007 – November 2007

In the subchronic oral toxicity study, formulations were prepared daily and placed on a magnetic stirrer during dosing in order to achieve homogeneous suspensions.

Clinical signs were recorded daily; outside cage observation, food consumption and body weights were recorded weekly during the acclimatisation and the treatment periods. Water consumption was recorded on days 78-91. Ophthalmoscopic examinations were performed prior to and at the end of the treatment. Functional observational battery, locomotor activity and grip strength were performed during week 12-13. At the end of the dosing, blood samples were withdrawn for haematology and plasma chemistry analyses. T3, T4 and TSH levels were determined in serum from all males and females. Urine samples were collected

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for urine analyses. All animals were killed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all animals.

Results

Formulation analyses confirmed that formulations of test substance in propylene glycol were prepared accurately and homogenously, and were stable over at least 5 hours.

No mortality occurred during the study period. Yellow urine was noted in both sexes at 210 and 630 mg/kg bw/day. Hunched posture was observed among all animals of both sexes at 630 mg/kg bw/day between weeks 5-9, but it was not considered toxicologically significant since it was temporary and slight in nature. Salivation was noted among all animals at 630 mg/kg bw/day, and incidentally at lower dosages. One male at 70 mg/kg bw/day incidentally showed clonic spasms, opisthotonus and cramped posture during the treatment period which was considered also an incidental finding as well as alopecia of various body parts, scabs, a wound on the tail and rales. No clinical signs were noted among control females and females at 70 mg/kg bw/day.

An increase in water consumption was noted for males at 210 and 630 mg/kg bw/day and females at 630 mg/kg bw/day between days 78-91. Water consumption was not quantitatively evaluated during earlier time periods of the study, but was subjectively assessed as being increased in these groups. This higher water consumption was accompanied by an increase in urinary volume in males at 210 and 630 mg/kg bw/day, and in females at 70 mg/kg bw/day and higher (no clear dose-related response in females).

As a consequence, secondary urinary changes related to the higher urinary volume consisted of reduced specific gravity in males at 210 and 630 mg/kg bw/day and in females at 70 mg/kg bw/day, and increased clarity in males at 210 and 630 mg/kg bw/day. Reduced urinary potassium concentration in males at 210 and 630 mg/kg bw/day, and in females at 70 mg/kg bw/day and higher was considered related to the higher urinary volume since potassium excretion (i.e. corrected for urinary volume) remained similar to control values.

Histopathological lesions in kidneys included tubular dilatation in both sexes at 630 mg/kg bw/day and in females at 210 mg/kg bw/day, increased incidence and/or severity of tubular basophilia (corticomedullary) in both sexes at 210 and 630 mg/kg bw/day, slightly increased incidence and/or severity of hyaline casts in both sexes at 630 and in males at 210 mg/kg bw/day, increased incidence and/or severity of interstitial inflammation (lymphocytic) in both sexes at 210 and 630 mg/kg bw/day, pelvic inflammation in females at 210 and 630 mg/kg bw/day and urothelial hyperplasia in females at 210 mg/kg bw/day. These renal histopathological changes occurred in conjunction with increased kidney weights and kidney to body weight ratios in both sexes at 630 mg/kg bw/day, and in females also at 210 mg/kg bw/day. One female at both 210 and 630 mg/kg bw/day exhibited macroscopically visible enlargement of the kidneys. In addition, a few females at 210 and/or 630 mg/kg bw/day exhibited pelvic inflammation and renal urothelial hyperplasia, and lesions in the urinary bladder that were comprised of lymphocytic inflammation and urothelial hyperplasia. In addition, perturbations in urinary electrolyte status and pH consisted of a reduced pH in males at 630 mg/kg bw/day, increased sodium excretion in males at 210 and 630 mg/kg bw/day, and in females at 70 mg/kg bw/day and higher, and increased calcium excretion in males at 630 mg/kg bw/day, and in females at 210 and 630 mg/kg bw/day. The marginally increased sodium excretion in the urine in females at 70 mg/kg bw/day was not statistically significant and not accompanied by histopathological changes in the kidneys. Furthermore, the values at 70 mg/kg bw/day were within the historical control range of the test laboratory for this rat strain and sex and no changes in the blood plasma electrolyte status could be observed. Therefore this change was concluded as of no toxicological concern (no adversity).

Increased liver weight and liver to body weight ratios were noted in both sexes at 630 mg/kg bw/day and in females at 210 mg/kg bw/day. Histologically, hypertrophy of hepatocytes was noted in both sexes at 210 and 630 mg/kg bw/day, and microvesicular vacuolation of hepatocytes was noted in males at 210 and 630 mg/kg bw/day. Increased alanine aminotransferase activity (ALAT) in both sexes at 630 mg/kg bw/day and in males at 210 mg/kg bw/day, as well as an increased bilirubin level in males at 630 mg/kg bw/day

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were observed. Slightly increased cholesterol levels were noted in both sexes at 630 mg/kg bw/day and in females at 210 mg/kg bw/day.

Reduced thymus weight and thymus to body weight ratios were noted in females at 210 and 630 mg/kg bw/day and in males at 630 mg/kg bw/day. This finding was accompanied by a slight increase in thymic lymphoid atrophy at these dosages. No correlated changes in white blood cell counts were noted, so the lymphoid atrophy was considered to be a secondary non-specific response to stress associated with treatment.

Haematological changes consisted of reduced red blood cell counts (both sexes at 630 mg/kg bw/day and females at 210 mg/kg bw/day), haemoglobin (both sexes at 210 and 630 mg/kg bw/day), haematocrit (females at 630 mg/kg bw/day), mean corpuscular haemoglobin concentration (both sexes at 630 mg/kg bw/day), and increased mean corpuscular haemoglobin and mean corpuscular volume (both sexes at 630 mg/kg bw/day). Haematology also revealed an increased number of reticulocytes (young, immature red blood cells) in both sexes at 630 mg/kg bw/day, and an increased red blood cell distribution width in males at 630 mg/kg bw/day. Slightly increased incidence and/or severity of extramedullary haematopoiesis in the form of haemopoietic foci (primarily erythropoiesis) in both sexes at 210 and 630 mg/kg bw/day and slightly increased severity of hemosiderin pigments in the spleen in both sexes at 630 mg/kg bw/day and in males at 210 mg/kg bw/day indicates that breakdown of red blood cells likely occurred along with a regenerative response to a lower number of circulating red blood cells. The higher potassium level in males at 210 and 630 mg/kg bw/day and increased total bilirubin levels in males at 630 mg/kg bw/day were also considered to be the result of red blood cell breakdown.

No effect on the morphology of thyroid follicular cells and no biologically relevant effect on thyroid hormones were observed in either male or female rats.

No test item-related adverse effects were observed in the animals treated with 70 mg/kg bw/day of 3-amino-2,6-dimethylphenol.

Conclusion

Treatment with 3-amino-2,6-dimethylphenol at doses of 630 and 210 mg/kg bw/day resulted in adverse effects in liver, kidney, and the haematopoietic system. Since no treatment related changes of toxicological concern were noted at 70 mg/kg bw/day, the No Observed Adverse Effect Level (NOAEL) for 3-amino-2,6-dimethylphenol was concluded to be 70 mg/kg bw/day.

Ref.: 46, 47

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted.

3.3.6. Mutagenicity / genotoxicity

3.3.6.1 Mutagenicity / genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline: OECD 471 (1997)

Test system: *Salmonella typhimurium* TA 98, TA 100, TA 102 TA 1535, TA 1537

Replicates: triplicates per test concentration

Test substance: 3-Amino-2,6-dimethylphenol (WR803169)

Solvent: DMSO

Batch: GST 083-04/70-07

Purity: 99.7 area % (HPLC at 254 nm)

Concentrations: Experiment I: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate

Experiment II: 33, 100, 333, 1000, 2500 and 5000 µg/plate

Treatment: the standard plate incorporation and pre-incubation assay

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GLP: In compliance
Study period: May 16-August 23 2007

with and without Phenobarbital/β-Naphthoflavone induced S9-mix

3-Amino-2,6-dimethylphenol was assessed in two experiments for its potential to induce gene mutations in *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 with and without exogenous metabolic activation S9-mix. Test concentrations were based on the results of a pre-experiment for toxicity and mutation induction with all strains used 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. In the direct plate incorporation test (experiment I) 8 concentrations from 3-5000 µg/plate and in the pre-incubation test (experiment II) 6 concentrations from 33-5000 µg/plate were used. Negative and positive controls were in accordance with the OECD guideline.

Results

No toxic effects, evident as a reduction in the number of revertants in the test groups either with or without S9-mix, were detected. No biologically relevant increase in revertant colony numbers of any of the five tested strains was observed at any concentration, either in the presence or in the absence of S9-mix.

Conclusion

Under the experimental conditions used, 3-amino-2,6-dimethylphenol is considered to be non mutagenic in *Salmonella typhimurium* reverse mutation assay.

Ref.: 36

In vitro Gene Mutation Assay (*hprt*-locus)

Guideline: OECD 476 (1997)
Species/strain: Chinese Hamster Lung Cell Line V79
Replicates: Duplicate cultures per concentration, two independent experiments
Test substance: 3-Amino-2,6-dimethylphenol (WR803169)
Solvent: DMSO
Batch No.: GST 083-04/70-07
Purity: 99.7 area% (HPLC at 254 nm)
Concentrations: Experiment I: 172.5; 345.0; 690.0; 1035.0; and 1380.0 µg/ml
with and without Phenobarbital/β-Naphthoflavone induced S9-mix
Experiment II:
Treatment 43.1; 86.3; 172.5; 345.0; and 517.5 µg/ml without S9-mix
Experiment I: 4 h treatment with and without S9-mix
Experiment I: 24 h treatment without S9-mix
GLP: In compliance
Study period: November 30 2007 – April 8 2008

3-Amino-2,6-dimethylphenol was assessed for its potential to induce gene mutations at the *hprt* locus in Chinese hamster V79 cells after 4 hours treatment with and without exogenous metabolic activation S9-mix and 24 hours treatment without S9-mix. A pre-test for toxicity determined the concentration range for the mutagenicity experiments measuring the colony forming ability using 8 concentrations between 10.1 and 1380 µg/ml. The highest concentration used in the pre-test was chosen with regard to the purity and the molecular weight of the test item. In the main tests, cells were treated for 4 h without and with S9-mix and for 24 h without S9-mix followed by an expression period of 7 days to fix the DNA damage into stable *hprt* mutations. Positive and negative controls were according to OECD guideline.

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Results

Toxic effects indicated by a relative cloning efficiency of less than 50% were observed after 4 h treatment at 1035 and 1380 µg/ml without metabolic activation and at 172.5 µg/mL and above after 24 h treatment. No toxic effects were found with metabolic activation. No precipitation was observed up to the highest concentration with and without metabolic activation.

No biologically relevant increase of the mutant frequency was observed in both main experiments up to the maximum concentration with and without metabolic activation. Some single cultures exceeded the mutant frequency of the corresponding control after 4 and 24h treatment. However, the increases were not dose dependent, did not occur in the duplicate cultures and the mean values of both parallel cultures remained within the range of the historical controls of the negative and solvent controls. Therefore, these isolated effects were considered not biologically relevant.

Conclusion

Under the experimental conditions used, 3-amino-2,6-dimethylphenol did not induce gene mutations at the *hprt* locus in V79 cells and is not considered to be mutagenic in this assay.

Ref.: 38

Micronucleus assay *in vitro*

Guideline:	Draft OECD 487 (2004)
Species/strain:	Human peripheral blood lymphocytes
Replicates:	Duplicate cultures in two independent experiments; 4 cultures for the solvent control,
Test substance:	3-Amino-2,6-dimethylphenol
Solvent:	DMSO (sterile anhydrous analytical grade)
Batch.:	GST 083-04/70-07
Purity:	99.7 area% (HPLC at 254 nm)
Concentrations:	Experiment I: without S9-mix: 400, 800, 950 µg/ml with S9-mix: 800, 1200 and 1372 µg/ml Experiment II: without S9-mix: 400, 900 and 1000 µg/ml with S9-mix: 600, 1000 and 1372 µg/ml
Treatment:	Experiment I: 24 h PHA: 20 h and 28 h recovery without S9-mix 3 h and 45 h recovery with S9-mix (Aroclor 1254) Experiment II: 48h PHA: 20 h treatment and 28 h recovery without S9-mix, 3 h treatment and 45 h recovery with S9-mix (Aroclor 1254)
GLP:	In compliance
Study period:	May 1 2007 - February 6 2008

3-Amino-2,6-dimethylphenol was examined for its clastogenicity and aneugenicity in two independent experiments and two duplicates in the absence and presence of exogenous metabolic activation in the *in vitro* micronucleus assay using human lymphocyte cultures from pooled blood of two healthy female donors. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a cytotoxicity range-finder; cultures of human peripheral blood lymphocytes were treated with a range of 12 and 16 increasing concentrations, respectively, with the highest dose 1372 µg/mL (equivalent to 10 mM). Both in the cytotoxicity range-finder and the micronucleus test, treatment of lymphocytes commenced approximately 24 or 48 h after mitogen stimulation by phytohaemagglutinin (PHA). Cells were treated for 3 h in the presence of S9-mix and 20 h in the absence of S9-mix; cells were harvested 48 h after the beginning of treatment. The final 28 h of incubation was in the presence of

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cytochalasin B (final concentration 6 µg/ml). Toxicity was determined by measuring the reduction in replication index (RI). The top concentration for analysis was to be the one with approximately 50-60% reduction in RI. The lower concentrations were chosen such that a range from maximum to little or none cytotoxicity is covered. Micronucleus preparations were stained with Giemsa and examined microscopically for RI and micronuclei. Negative and positive controls were included.

Results

In the first experiment, in the absence and presence of metabolic activation the reduction in the replication index was approximately 52% and 30%, respectively. In the experiment II without and with metabolic activation, the reduction in the replication index was approximately 54% and 19%, respectively. The highest concentrations 1372 µg/mL (equivalent to 10 mM) marginally reached recommended 55 ± 5% cytotoxicity.

In both experiments (24 h and 48 h PHA) in the absence of metabolic activation 3-amino-2,6-dimethylphenol induced statistically significant increase in frequencies of micronucleated binucleate cells compared to vehicle controls. However, in condition of 24 h mitogen stimulation (experiment I) the level of cells with micronuclei did not exceed historical vehicle control (95% reference range) values. Thus the data of the first experiment have no biological relevance. In the experiment 2 (48 h PHA) 20 h treatment of cells in the absence of metabolic activation induced statistically significant and dose dependent increase in cells with micronuclei in all three concentrations used compared to vehicle control as well as historical control.

Treatment of cells in the presence of metabolic activation (S9-mix) did not result in frequencies of micronucleated binucleate cells different from concurrent vehicle controls for the majority of concentrations analysed. The two exceptions to this were Experiment 1 treatments at 1372 µg/ml and Experiment 2 treatments at 1000 µg/ml where small, statistically significant increases in cells with micronuclei were observed. However, these levels were in the same range as historical vehicle control (95% reference ranges) values, thus can be considered to be of no biological relevance.

Conclusion

3-Amino-2,6-dimethylphenol induced biologically relevant increase in cells with micronuclei in cultured human peripheral blood lymphocytes after 20 h treatment in the absence of S9-mix and consequently is clastogenic and/or aneugenic in this test.

Ref.: 37

SCCS comment

In experiment II the cytotoxicity observed at the highest concentration was too high (81%).

3.3.6.2. Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474 (1997)
Species/strain:	Mouse, strain NMRI
Group size:	6 females per dose group
Test substance:	3-Amino-2,6-dimethylphenol
Batch No.:	GST 083-04/70-07
Purity:	99.7 area% (HPLC at 254 nm), for details see 2.4.
Dose levels:	312.5, 625 and 1250 mg/kg bw (24 h) and 1250 mg/kg bw (48 h),
Route:	oral, gavage
Vehicle:	30% DMSO + 70% PEG 400
Sacrifice times:	24 and 48 h (high dose only)
GLP:	In compliance

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Study period: November 30, 2007 - March 11, 2008

3-Amino-2,6-dimethylphenol was investigated for the induction of chromosomal damage in the bone marrow cells of mice. The acute toxicity symptoms and signs (reduction of spontaneous activity, abdominal position, ruffled fur, apathy, urine colour, death) were assessed in 6 pre-experiments at the doses 100, 1000, 1250, 1750, and 2000 mg/kg bw; 2 male and female animals were treated with the test substance and observed at intervals around of 1h, 2-4h, 6h, 24h, 30h and 48h. From these results 1250 mg/kg bw was chosen as maximum dose level for the main experiment.

In the main experiment female mice were exposed orally with single doses of 0, 312.5, 625 and 1250 mg/kg bw and sacrificed 24 h after administration and with 1250 mg/kg bw sacrificed 48 h after administration. Bone marrow preparations were stained with Giemsa and examined microscopically for chromosomal aberrations. At least 2000 polychromatic erythrocytes (PCE) per animal were analysed for presence of cells with micronuclei and the ratio between polychromatic and normochromatic erythrocytes were determined in the same sample. Five females per test group were evaluated. Negative and positive controls were included.

Results

The number of PCEs after the treatment with 3-amino-2,6-dimethylphenol was not decreased compared to mean value of PCEs of the vehicle control indicating no cytotoxicity in the bone marrow. The urine of the animals treated with the high dose of 3-amino-2,6-dimethylphenol was coloured as sign of systemic distribution and thus its bioavailability. The mean values of cells with micronuclei observed after the treatment with 3-amino-2,6-dimethylphenol were not significantly different from the controls and were within the historical control range. 3-amino-2,6-dimethylphenol was not mutagenic in mouse after a single oral administration of doses of up to 1250 mg/kg bw.

Conclusion

Under the test conditions used, 3-amino-2,6-dimethylphenol did not induce cells with micronuclei in the bone marrow cells of mice.

Ref.: 39

3.3.7. Carcinogenicity

No data submitted.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted.

3.3.8.2. Teratogenicity (prenatal developmental toxicity)

Guideline: OECD 414 (2001)
Species/strain: Rat, strain HanRcc: WIST(SPF)
Group size: 22 mated females
Test substance: 3-Amino-2,6-dimethylphenol
Batch No.: GST 083-04/70-07
Purity: 99.2 area% (HPLC at 254 nm)
Vehicle: Propylene glycol
Dose level: 60, 150 and 400 mg/kg bw/day

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Dose volume:	5 ml/kg bw
Route:	Oral gavage
Dosing schedule:	Days 6 through 20 of gestation
Control:	Vehicle only
GLP:	In compliance
Study period:	October 2007 - June 2008

Homogeneity of the test item suspended in the vehicle was maintained during the daily administration period using a magnetic stirrer (see 3.1.9.).

In order to assess the effects of orally administered 3-amino-2,6-dimethylphenol on pregnant female rats and embryo-foetal development, all animals were checked at least twice daily for mortalities and daily for clinical signs. Food consumption data were calculated for the periods from day 0 to 3, 3 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18, and 18 to 21 post coitum. Body weights were recorded daily from day 0 until day 21 post coitum. At day 21 post coitum, all mated females were sacrificed. Post mortem examination, including gross macroscopic examination of all internal organs, with emphasis on the uterus, uterine contents, position of foetuses in the uterus was performed, and the data recorded. The intact uterus (prepared by Caesarean section) was removed and the percentage of resorption sites (early, late) and foetuses (live or dead) as well as their uterine position were recorded. In addition, uterine weight was determined. The number of implantation sites and corpora lutea was also determined. Each viable foetus was weighed, sexed and examined for gross external malformations and euthanized. Half of the foetuses were prepared for the micro-dissection technique with fixation in Bouin's fixative to examine the soft tissues. The remaining foetuses were eviscerated, processed for the cartilage (Alcian blue) and skeletal (Alizarin red S) staining to examine the skeleton and cartilage.

Results

Dosing solutions were analysed twice during the duration of the dosing period, the measured individual values were all within the required range of $\pm 10\%$ of the nominal concentration.

All the animals survived until the scheduled necropsy.

Treatment at 400 mg/kg bw/day resulted in ruffled fur and sedation at the start of the treatment in all females. Discoloration of the faeces and of the bedding material was noted during the second half of the treatment due to the bioavailability of the test item. Food consumption and body weight were statistically significantly reduced for the whole treatment period. In particular, this reduction was greater on days 6 - 9 post coitum. Corrected body weight gain was also statistically significantly reduced. Mean foetal body weight was reduced. Skeletal and cartilage examination showed that there was a statistically significantly higher incidence of supernumerary and/or rudimentary ribs. Such findings in rodent studies are considered to be associated with maternal toxicity (see reference 48).

Treatment at 150 mg/kg bw/day resulted in a statistically significant reduction of mean food consumption and body weight. This was considered to be due to the treatment with the test item even though no dose dependency could be observed.

During visceral examination of foetuses, there was a dose-dependent increase in the incidence of an enlarged thyroid at 150 and 400 mg/kg bw/day.

The treatment with the test item did not affect the pre- and post-implantation, the sex ratios and the number of foetuses at any dose level.

At 60 mg/kg bw/day, no test item-related changes were observed.

Conclusion

Based on the effects in the dams observed at 400 and 150 mg/kg bw/day, the NOAEL (no observed adverse effect level) for maternal toxicity of 3-amino-2,6-dimethylphenol was considered to be 60 mg/kg bw/day. Based on the described effects in foetuses (body weight reduction and higher incidence of supernumerary and/or rudimentary ribs) at 400 mg/kg

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bw/day and increased incidence of enlarged thyroids observed at 400 and 150 mg/kg bw/day), the NOAEL for the foetuses was considered to be 60 mg/kg bw/day.

Ref.: 41

3.3.9. Toxicokinetics

3.3.9.1. Toxicokinetics *in vitro*

Bioavailability across intestinal barrier in CaCo-2 (human intestinal epithelial carcinoma) cells

Guideline:	/
Cells:	Human intestinal epithelial cell line CaCo-2 (subclone HTB 37)
Replicates:	Two independent experiments
Test substance:	3-Amino-2,6-dimethylphenol
Batch No.:	GST 083-04/70-07
Purity:	99.2 area% (HPLC at 254 nm), for details see 2.4.
Test concentration:	50 µM (equivalent to 6.86 µg/ml)
Incubation time:	60 minutes
Medium:	Hank's buffer (pH 7.4)
Solubility:	2.7% in water, >20% in DMSO
Analytical method:	HLPC-MS/MS
GLP:	Not in compliance
Study period:	May 2007 - March 2008

The absorption of 3-amino-2,6-dimethylphenol across the intestinal barrier was investigated in human intestinal epithelial (CaCo-2, subclone HTB 37) cells *in vitro*. The permeability from the apical (pH 6.5) to the basolateral (pH 7.4) side was investigated at 37 C in 96-well transwell plates with shaking for a 60-min contact time. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of HLPC-MS/MS and the apparent permeability coefficient (P_{app}) was calculated for two independent experiments. Only monolayers revealing a transendothelial or transepithelial electrical resistance (TEER) $> 280 \Omega \times \text{cm}^2$ were used in the assay. Propranolol and ranitidine were analysed concurrently to demonstrate the validity of the test system.

According to the laboratories classification system (regarding the average of the controls), a low permeability is considered for test items revealing a $P_{app} < 2 \times 10^{-6} \text{ cm/sec}$. A P_{app} of $2 - 20 \times 10^{-6} \text{ cm/sec}$ and a $P_{app} \geq 20 \times 10^{-6} \text{ cm/sec}$ classify a substance to have a moderate and a high permeability, respectively. Ranitidine, which has a 50% absorption in humans, was used as low permeability reference compound, as recommended by FDA.

The permeability coefficient as velocity for the uptake [cm/s] of the test item across the intestinal barrier (CaCo-2) was determined and compared to the reference substances propranolol (high permeability reference) and ranitidine (low permeability reference).

Results

The P_{app} [cm/s] for the uptake of 3-amino-2,6-dimethylphenol across the epithelial cells for the apical to basolateral transport was determined to be $29.2 \times 10^{-6} \text{ cm/s}$ (Experiment HV1) and $P_{app} = 39.1 \times 10^{-6} \text{ cm/s}$ (Experiment HV2).

The total recovery for the reference substances and 3-amino-2,6-dimethylphenol ranged from 68.9% (reference substance) to 181% (test compound).

The figures for the reference substance ranitidine, the low permeability reference compound, was $P_{app} = 2.29 \times 10^{-6} \text{ cm/sec}$ and $0.580 \times 10^{-6} \text{ cm/sec}$. The figures for the reference substance propranolol, the high permeability reference compound with 90% absorption in humans, was $P_{app} = 37.1 \times 10^{-6} \text{ cm/sec}$ and $39.5 \times 10^{-6} \text{ cm/sec}$.

The high permeability values for 3-amino-2,6-dimethylphenol of $P_{app} = 29.2 \times 10^{-6} \text{ cm/sec}$ (Experiment 1) and $39.1 \times 10^{-6} \text{ cm/sec}$ (Experiment 2) for the apical to basolateral

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transport indicate that the uptake across the intestinal barrier is not anticipated to limit gastrointestinal absorption.

Conclusion

With 3-amino-2,6-dimethylphenol, a mean permeability in human intestinal epithelial cells of 34.1×10^{-6} cm/sec was obtained, which classifies the test item to be of high permeability. As the absorption from the gastro-intestinal tract is likely to be permeability limited, the high permeability observed in this assay indicates a good absorption of 3-amino-2,6-dimethylphenol after oral administration.

Ref.: 42

SCCS comment

The study was not performed under GLP conditions. There is no official guideline for this assay. The generated data is considered to provide an estimation on gastrointestinal absorption of 3-amino-2,6-dimethylphenol after oral administration. The substance is expected to be highly absorbed after oral administration.

3.3.9.2. Toxicokinetics *in vivo*

Absorption, distribution, metabolism and excretion of ^{14}C -3-amino-2,6-dimethylphenol in the Wistar rat

Guideline:	OECD 417 and 427
Species/strain:	Rat, Wistar Han, Crl:WI(Han) (outbred, SPF quality)
Group size:	4 females per group in the mass balance groups (1, 2, 3, 4) 6 females per group in the toxicokinetic groups (5, 6, 7, 8)
Test substance:	3-Amino-2,6-dimethylphenol
Batch:	GST083-04/70-07
Purity:	99.2 area % (HPLC, 254 nm)
Test substance:	3-Amino-2,6-dimethyl[U- ^{14}C]phenol
Batch:	CFQ15282 Batch 1
Purity:	Radiochemical purity: 99.5% (HPLC)
Vehicle:	Intravenous administration (group 1 + 5): Ethanol/saline (20/80 w/w) Oral administration (group 2 + 6): Propylene glycol Dermal administration: 50% DMSO in milli-Q water (group 3 + 7), DMSO (groups 4, 8)
Dose levels:	Intravenous administration: 16 mg/kg bw (containing approximately 10 MBq of radioactivity) Oral administration: 70 mg/kg bw (containing approximately 10 MBq of radioactivity) Dermal administration: 20 / 87.5 mg/ml containing approximately 3 / 3 MBq of radioactivity (equal to 16 / 70 mg/kg bw and 0.2 / 0.875 mg/cm ² skin)
Dose volume:	Intravenous administration: 1 ml/kg Oral administration: 5 ml/kg Dermal administration: 0.2 ml/animal
Route:	Intravenous, oral (gavage), dermal
Dosing schedule:	Oral: Single administration Dermal: Single application for 0.5 h (low dose) and 24 h (high dose)
GLP:	In compliance
Study period:	Jan 2008 - Dec 2010

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The design of the study is summarized in the following table. Four groups of rats (groups 1-4) were used for the mass balance and four groups (5-8) for the toxicokinetic study. Rats were administered a single dose intravenously (i.v.), orally, or dermally. The dermal high dose was chosen to achieve a relatively high bioavailability for comparison of the metabolite profiles. The dermal low dose was chosen to be more representative of hair dye use conditions (e.g., 30 min exposure).

Group No.	No. of rats	Dose level and vehicle	Dosing route	Time of sacrifice of rats	Samples collected (time after dosing)
1	4	16 mg/kg, ethanol/saline (20/80)	i.v.	48 h	Urine and faeces (0-8, 8-24, 24-48 h), plasma and various organs (48 h)
2	4	70 mg/kg, propylene glycol	oral	48 h	Urine and faeces (0-8, 8-24, 24-48 h), plasma and various organs (48 h)
3	4	16 mg/kg, DMSO/water 1/1 (0.2 mg/cm ² for 0.5 h)	dermal	72 h	Urine and faeces (0-8, 8-24, 24-48, 48-72 h), plasma and various organs (72 h)
4	4	70 mg/kg, DMSO (0.87 mg/cm ² for 24 h)	dermal	72 h	Urine and faeces (0-8, 8-24, 24-48, 48-72 h), various organs (72 h)
5	6	16 mg/kg, ethanol/saline (20/80)	i.v.	48 h	Blood (0.25, 0.5, 0.75, 1, 2, 4, 8, 24, 48 h)
6	6	70 mg/kg, propylene glycol	oral	48 h	Blood (0.25, 0.5, 0.75, 1, 2, 4, 8, 24, 48 h)
7	6	16 mg/kg, DMSO/water 1/1 (0.2 mg/cm ² for 0.5 h)	dermal	48 h	Blood (0.25, 0.5, 1, 2, 4, 8, 24, 48 h)
8	6	70 mg/kg, DMSO (0.87 mg/cm ² for 24 h)	dermal	48 h	Blood (0.25, 0.5, 1, 2, 4, 8, 24, 48 h)

* Percentage of dose recovered from: excretion + cage wash + carcass + unexposed skin. Calculated without skin residue.

Animals in the mass-balance groups 1-4 were housed in metabolism cages, and radioactivity in urine, faeces, plasma and organs was determined in order to obtain a total ¹⁴C-radioactivity balance. Selected urine, faeces and plasma samples were pooled per group and the metabolite profile in these pooled samples was investigated. In the toxicokinetic groups 5-8, blood was sampled from 3 rats per time point, and total radioactivity and 3-amino-2,6-dimethylphenol equivalent concentrations were determined.

Results

No mortality was observed in the study. After i.v. dosing, piloerection was incidentally observed. After oral administration, piloerection, ptosis and lethargy were observed in several animals. After dermal dosing, red discharge from the nose and eyes was observed. The discharge is related to the collar used to prevent grooming behaviour. In addition, white spots/blisters were seen on the dosed surface shortly after dosing of the low and high dermal dose. At Day 1, dark spots (orange/brown) were observed on dosed surface area after low and high dermal dosing.

Mass balance and toxicokinetic data

Oral absorption was calculated in two ways, with the urine data and with the plasma data from group 1 and 2. With the urine data, the absorption was calculated by dividing the percentage of radioactivity recovered in the urine after oral administration by the

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percentage of radioactivity recovered in the urine after i.v. administration. The value for oral absorption calculated from the urine data was 108%. It was greater than 100%, most likely because the total recovery of radioactivity was higher in the oral group compared to the i.v. group. When corrected for the difference in total recovery, the oral absorption was 100%. With the plasma data, absorption was calculated by dividing the dose-normalized area under the curve (AUC) after oral administration by the AUC after i.v. administration, resulting in 63% oral absorption. This indicates that the oral absorption of the test substance is high.

When calculated using urine data, the average dermal absorption was low in the low dermal dose group, i.e., 1.0% of the applied dose, or $2 \mu\text{g}/\text{cm}^2$ (group 3) and higher in the high dermal dose group, i.e., 72% of the applied dose, or $680 \mu\text{g}/\text{cm}^2$ (group 4). As the amount retained in the skin of the application site skin may eventually become systemically available, the skin residue dose was added to the absorbed fraction to yield the "total potentially absorbed fraction". This was 1.1% of the applied dose, or $2 \mu\text{g}/\text{cm}^2$ in the low dermal dose group 3 and 73% of the applied dose, or $690 \mu\text{g}/\text{cm}^2$ in the high dermal dose group 4. The difference between the low and high dose in percentage absorbed can be explained by the difference in concentration dosed, the duration of exposure (24 versus 0.5 h) and the vehicles (50% DMSO versus 100% DMSO). When calculated with the plasma data, the absorption was 14% in the low and 70% in the high dose group, respectively.

From the plasma data, it was evident that absorption was fast with t_{\max} reached at 0.25 h after oral dosing and at 0.5 h after dermal dosing.

Inter-individual variation was reasonably low. In the low dermal group plasma concentrations were below the limit of quantification from 4 h onwards. Dose normalized C_{\max} values and AUC values were calculated, but should be interpreted with caution for the low dermal dosing group, since part of the data was below the limit of quantitation. It was clear that the dose-normalized exposure was not equal between the low and high dermal groups. This is considered to be related to the longer exposure duration for the high dermal group which results in a more than dose proportional increase in internal exposure.

The AUC after high dermal administration was higher than after oral administration, likely because the concentrations at later time points (e.g. 1, 2 h) are higher after dermal administration than oral, which impacts the AUC. Terminal half-lives ranged from 9.65 to 41.6 hours, but could not be reliably determined.

Urine was the most important route of excretion of 3-amino-2,6-dimethylphenol after intravenous, oral and dermal dosing. Urinary excretion accounted for 83% of the administered dose after i.v. dosing, 89% after oral dosing, 0.8% after low dermal dosing and 67% after high dermal dosing. In all groups, the highest rate of excretion was observed during the first 24 h.

Excretion via faeces was only a minor route, accounting for 2.5% of the dose after i.v., 6.5% after oral, 0.06% after low dermal (0.5 h) and 1.3% after high dermal dosing (24 h).

At termination of the study, the average total remaining radioactivity in blood, carcass and tissues was between 0.14 and 2.15% of the administered dose in the i.v., oral and dermal groups, indicating no major accumulation of radioactivity after 48-72 h. In the i.v. group, concentration equivalents slightly higher than in blood were observed in adrenals and thyroid. In the oral group, concentration equivalents higher than in blood were observed in adrenals, liver and thyroid. In the low dermal group, concentrations above the limit of quantitation were only detected in treated skin and the GI tract and its contents. In the high dermal group, concentration equivalents higher than in blood were observed in liver, carcass, untreated skin, treated skin and GI tract and its contents. The percentage of radioactivity associated with the treated skin was 0.09% in the low dermal group and 0.84% in the high dermal group. In groups 1, 2 and 4, blood concentrations at termination were 3-8 times higher than plasma concentrations, suggesting distribution of the test substance into red blood cells. Plasma concentrations were in the same order of magnitude for the i.v., oral and high dermal groups. Plasma concentrations at termination were below

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the limit of quantification for one animal after oral dosing and for all animals after low dermal dosing. Average total recovery of radioactivity in all dose groups was between 88 and 99% of the applied dose.

Summary of mass balance data

Group No.	Dose Level / Concentration	Dosing route	Absorption (%)	Excretion via urine/faeces (%)
1	16 mg/kg	i.v.	100	83 / 2.5
2	70 mg/kg	oral	108	89 / 6.5
3	16 mg/kg; 0.2 mg/cm ² for 0.5 h	dermal	1.0*	0.8 / 0.1
4	70 mg/kg; 0.875 mg/cm ² for 24 h	dermal	72*	67 / 1.3

* Percentage of dose recovered from: excretion + cage wash + carcass + unexposed skin. Calculated without skin residue.

Summary of toxicokinetic data

Group No.	Dose Level / Concentration	Dosing route	F _{abs} (%)	C _{max} (mg/kg)	AUC _{last} (hr*mg/kg)
5	16 mg/kg	i.v.	n.a.	n.a.	31.9
6	70 mg/kg	oral	63	32.4	98.2
7	16 mg/kg; 0.2 mg/cm ² for 0.5 h	dermal	14	3.87	5.76
8	70 mg/kg; 0.875 mg/cm ² for 24 h	dermal	70	34.0	130

F_{abs} : Absolute oral / dermal bioavailability, calculated as ((AUC_{last} p.o. or dermal/AUC_{last} i.v.) * (dose_{i.v.}/dose_{p.o. or dermal})* 100%; n.a.: not applicable

Identification of metabolites

Three metabolites of 3-amino-2,6-dimethylphenol were detected as radioactive peaks in the radio liquid chromatograms of the urine, faeces and plasma samples of the Wistar rats and identified as products of O-sulfation, O-glucuronidation and N-acetylation by mass spectrometry.

In urine (see table below), an O-sulfate conjugate was the main metabolite (52.8-67.1% of total radioactivity in the chromatogram) upon administration of i.v., oral and high dermal dose. In addition, a glucuronide metabolite accounted for 5.0-6.4%. The N-acetylated metabolite was present but was detectable only in MS chromatograms; the amount formed was below the limit of detection of the radioactivity detector. A metabolite resulting from a combination of O-sulfation and N-acetylation was the main metabolic reaction upon low dermal dose (84.9% of radioactivity) and was also present to a less extent (27.9-38.8% of total radioactivity) after i.v., oral or high dermal dose. In the low dermal dose group, the O-sulfate conjugate accounted for 15% of total radioactivity. No significant differences in the metabolite pattern were observed between i.v., oral or high dermal administration.

Summary of metabolites of 3-amino-2,6-dimethylphenol detected in urine

RT ¹⁾ (min)	MS ²⁾ (m/z)	Possible metabolic reaction	Pattern of urinary metabolites ³⁾			
			Group 1 (i.v.)	Group 2 (oral)	Group 3 (low dermal)	Group 4 (high dermal)
4.7	-312/314	Glucuronic acid conjugation ⁴⁾	6.1 (MS+) ⁵⁾	6.4 (MS+)	- (MS-)	5.0 (MS+)
10.5	138	Parent compound ⁶⁾	3.3 (MS+)	2.1 (MS+)	- (MS-)	- (MS+)

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11.6	-216	O-sulfate conjugation ⁶⁾	58.6 (MS+)	52.8 (MS+)	15.1 (MS+)	67.1 (MS+)
12.6	-258/ 277	O-sulfate and N-acetyl conjugation	32.0 (MS+)	38.8 (MS+)	84.9 (MS+)	27.9 (MS+)

¹⁾ retention time (RT) in LC-MS

²⁾ molecular ion in negative or positive electrospray ionisation mass spectrometry (MS)

³⁾ % of total radioactivity in radio liquid chromatogram of the sample (- : not present in chromatogram)

⁴⁾ the position of the glucuronide group could not be verified, but biologically it is likely to be located at the OH-group

⁵⁾ MS+: present in MS chromatogram, MS-: not present in MS chromatogram

⁶⁾ identified by comparison to available synthetic standards; the position of the sulfate group could not be verified, but biologically it is likely to be located at the OH-group

In faeces samples, parent compound and metabolites could not be detected by radio liquid chromatography due to small amounts and low sensitivity. However, the O-sulfate and the combined O-sulfate/N-acetate were detectable by LC-MS in the faeces of the i.v., oral, and high dermal dose group.

In plasma samples (see table below), an O-sulfate was the main metabolite present (59.8-71.6% of total radioactivity) in all dose groups. In addition, a glucuronide metabolite (3.4-9.2% of total radioactivity) was found. A metabolite resulting from a combination of O-sulfation and N-acetylation (19.4-28.4% of total radioactivity) was also observed. In MS chromatograms, the N-acetylated metabolite could be detected but it was present below the limit of detection of the radioactivity detector. No significant differences were observed between the different routes of administration: intravenous, oral or high dermal.

Summary of metabolites of 3-amino-2,6-dimethylphenol detected in plasma

RT ¹⁾ (min)	MS ²⁾ (m/z)	Possible metabolic reaction	Pattern of plasma metabolites ³⁾			
			Group 5 (i.v.)	Group 6 (oral)	Group 7 (low dermal)	Group 8 (high dermal)
4.7	-312/ 314	Glucuronic acid conjugation ⁴⁾	3.4 (MS+) ⁵⁾	- (MS+)	- (MS+)	9.2 (MS+)
10.5	138	Parent compound ⁶⁾	11.5 (MS+)	34.0 (MS+)	- (MS-)	14.7 (MS+)
11.6	-216	O-sulfate conjugation ⁶⁾	63.6 (MS+)	63.0 (MS+)	71.6 (MS+)	59.8 (MS+)
12.6	-258/ 277	O-sulfate and N-acetyl conjugation	22.7 (MS+)	25.6 (MS+)	28.4 (MS+)	19.4 (MS+)

¹⁾ retention time (RT) in LC-MS

²⁾ molecular ion in negative or positive electrospray ionisation mass spectrometry (MS)

³⁾ % of total radioactivity in radio liquid chromatogram of the sample (- : not present in chromatogram); % of radioactivity is the average of three plasma samples of three time points

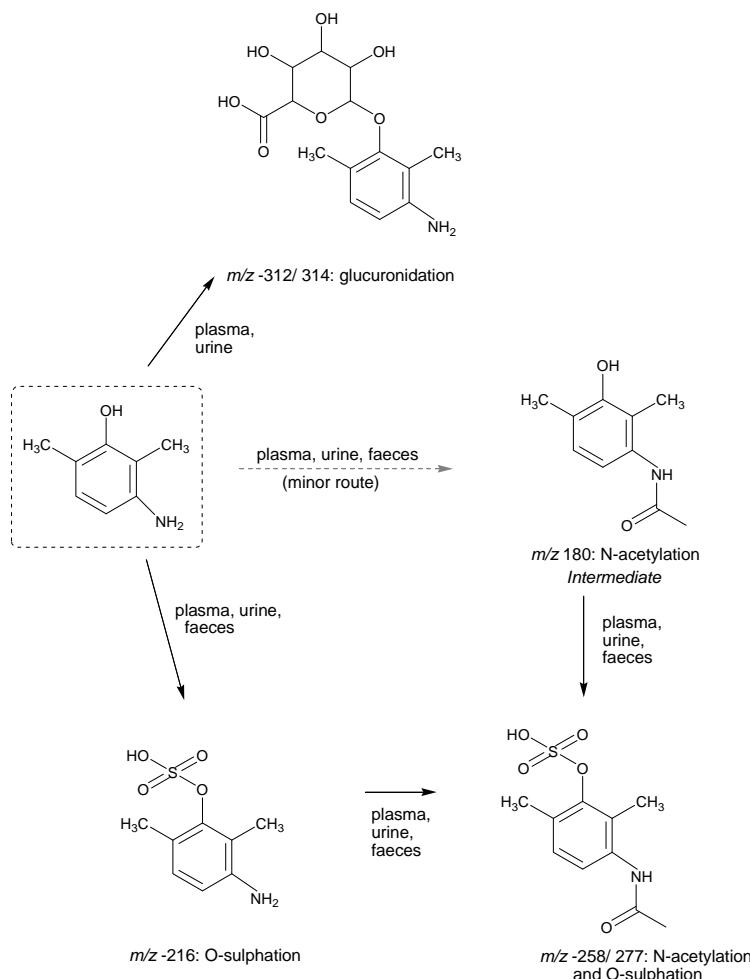
⁴⁾ the position of the glucuronide group could not be verified, but biologically it is likely to be located at the OH-group

⁵⁾ MS+: present in MS chromatogram, MS-: not present in MS chromatogram

⁶⁾ identified by comparison to available synthetic standards; the position of the sulfate group could not be verified, but biologically it is likely to be located at the OH-group

The suggested metabolic pathways of 3-amino-2,6-dimethylphenol in rats following i.v., oral and dermal exposure are presented in the following figure:

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Conclusion

After oral administration, 3-amino-2,6-dimethylphenol is extensively absorbed, readily distributed into all organs, extensively metabolized and predominantly excreted via the urine. Dermal absorption of 3-amino-2,6-dimethylphenol is high after a 24-h exposure period from a DMSO vehicle, and low after a 30-min exposure period. The dermally absorbed portion is mainly excreted via the urine. After all routes of administration, O-sulphation of the parent compound represents the major metabolic reaction. In addition, O-glucuronidation and N-acetylation are relevant metabolic pathways. No major qualitative differences were observed in the metabolite profile between intravenous, oral and dermal routes of administration.

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3.3.10. Photo-induced toxicity

No data submitted.

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted.

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted.

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3.3.11. Human data

No data submitted.

3.3.12. Special investigations

No data submitted.

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(3-amino-2,6-dimethylphenol)

(under oxidative conditions)

Absorption through the skin (pig <i>in vitro</i>)	A	=	8.43 µg/cm ²
(maximum absorption 6.59 + 2 x 0.92 (2 SD))			
Skin Area surface	SAS	=	580 cm ²
Dermal absorption per treatment	SAS x A x 0.001	=	4.89 mg
Typical body weight of human		=	60 kg
Systemic exposure dose (SED)	SAS x A x 0.001/60	=	0.08 mg/kg bw
No observed adverse effect level	NOAEL	=	60 mg/kg bw/d
(rat, prenatal developmental toxicity study)			

Margin of Safety	NOAEL/SED	=	740
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3.3.14. Discussion

Physico-chemical properties

3-Amino-2,6-dimethylphenol is used as an oxidative hair colouring agent (coupler). The intended maximum on-head concentration is 2.0% in oxidative hair dye formulations. The oxidative colouring agent and the developer are mixed at a ratio of 1+1 to 1+3 (g dye formulation + g developer formulation).

Stability of 3-amino-2,6-dimethylphenol in typical hair dye formulations is not reported.

General toxicity

After 14 days observation, the LD₅₀ after oral administration to female rats was reported to be between 300 and 2000 mg/kg bw.

In a 90-day study, the No Observed Adverse Effect Level (NOAEL) in rats after oral gavage was 70 mg/kg bw/day.

Based on a prenatal developmental toxicity study in pregnant rats, the NOAEL after oral administration was 60 mg/kg bw/day both for maternal and for foetal toxicity. This NOAEL should be used for the calculation of MoS.

No study on reproductive toxicity was provided.

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Irritation / sensitisation

Under the conditions of the studies (TER and RHE), 3-amino-2,6-dimethylphenol is classified as non-corrosive (neat substance) and non-irritant (1% in DMSO) to skin. However, the EpiSkinTM RHE test method only allows identification of CLP Category 2 (UN GHS Category 2) irritants, but does not allow the identification of mild skin irritants (UN GHS Category 3). therefore, mild skin irritancy cannot be excluded from the submitted data.

No eye irritation was found in the isolated chicken eye test using neat 3-amino-2,6-dimethylphenol, as well as 1% and 2% solutions in 5% DMSO. However, substances mildly irritating to eyes (UN GHS Category 2B) may not be detected.

3-Amino-2,6-dimethylphenol is not a skin sensitizer under the study conditions. However, a sensitisation potential cannot be excluded as the maximum test concentration was too low.

Dermal absorption

The amount of 3-amino-2,6-dimethylphenol biologically available was 6.59 µg/cm². The mean + 2 SD (6.59 ± 2 x 0.92) = 8.43 µg/cm² should be used to calculate the MoS.

Mutagenicity / genotoxicity

Overall, the genotoxicity of 3-amino-2,6-dimethylphenol was tested in three *in vitro* genotoxicity tests covering all three endpoints of genotoxicity: gene mutations, and both structural (clastogenicity) and numerical (aneugenicity) chromosome aberrations and one *in vivo* genotoxicity test. 3-Amino-2,6-dimethylphenol did not induce gene mutations in five bacteria strains and one mammalian cell system *in vitro* in the absence or presence of metabolic activation.

A significant and biologically relevant increase in cells with micronuclei was induced by 3-amino-2,6-dimethylphenol in cultured human peripheral blood lymphocytes in the absence of a metabolic activation system. While in the *in vitro* micronucleus assay the 3-amino-2,6-dimethylphenol induced clastogenic and/or aneugenic effect, the *in vivo* micronucleus in bone marrow was negative indicating no genotoxic potential *in vivo*. No further testing is necessary.

Carcinogenicity

No data submitted.

Toxicokinetics

Based on the results in an *in vitro* model for human intestinal uptake, a high absorption must be expected after oral exposure.

An *in vivo* toxicokinetic study with ¹⁴C-labeled 3-amino-2,6-dimethylphenol in rats showed a rapid and extensive intestinal absorption and a slow dermal absorption. The preferential route of excretion (e.g., > 80% of the oral dose) was the urine. The major metabolite in urine and plasma was a sulfate conjugate, together with smaller amounts of a mixed sulfate/acetyl conjugate and a glucuronide.

4. CONCLUSION

The SCCS is of the opinion that 3-amino-2,6-dimethylphenol is safe for use in oxidative hair dye formulations with an on-head concentration of maximum 2.0% taken into account the scientific data provided.

A sensitisation potential cannot be excluded.

5. MINORITY OPINION

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