



December 22, 2014

Office of Environmental Health Hazard Assessment
P.O. Box 4010, MS-19B
Sacramento, California 95812-4010
P65Public.comments@oehha.ca.gov

Re: Request for Relevant Information on the Carcinogenic Hazards of Diaminotoluenes

To Whom It May Concern:

The Personal Care Products Council¹ (the Council) appreciates the opportunity to provide comments on the above referenced topic. The Office of Health Hazard Assessment (OEHHA) has requested scientific information on diaminotoluene isomers, in response to a petition for reconsideration of the listing of diaminotoluene (mixed). One of the individual isomers listed in the notice is 2,5-diaminotoluene. The 2,5- isomer and its sulfate salt are used within the personal care product industry as hair dyes, and therefore consideration by the Carcinogen Identification Committee for listing under Proposition 65 is of significant interest to Council members.

The toxicology data which has been developed for 2,5-diaminotoluene shows conclusively that there is no basis for listing 2,5-diaminotoluene as a carcinogen under Proposition 65. Data addressing genotoxicity, carcinogenicity and toxicokinetics are summarized in this submission. Further, 2,5-diaminotoluene is not a component of the chemical group diaminotoluene (mixed). The synthetic pathway used in the manufacture of this isomer is distinctly different from the pathway used in the manufacture of diaminotoluene (mixed). Diaminotoluene (mixed) is defined by its CAS number (25376-45-8). The CAS number should be added to the Proposition 65 listing for diaminotoluene (mixed) to provide clarity to the listing.

The comments here will address data relevant to the lack of carcinogenicity of 2,5-diaminotoluene as well as information relating to the absence of 2,5-diaminotoluene in diaminotoluene (mixed). An overview is provided on the following pages. A summary of individual relevant toxicology studies is provided in Appendix I. Information on the synthesis of the diaminotoluenes, explaining why 2,5-diaminotoluene is not included in the definition of diaminotoluene (mixed), is provided in Appendix II.

¹Founded in 1894, the Council is the national trade association representing the personal care products industry. Our membership includes approximately 300 active member companies that manufacture or distribute personal care products and approximately 300 additional associate members who provide goods and services to manufacturers and distributors of personal care products.

2,5-Diaminotoluene does not meet the Proposition 65 standard for listing “known to the State to cause cancer”

2,5-Diaminotoluene (also known as toluene-2,5-diamine) and 2,5-diaminotoluene sulfate are used as oxidative hair dye precursors in hair coloring products. Their use in hair dye underwent review by the Scientific Advisory Board of the European Commission in 2012. The outcome of the review by the Scientific Committee on Consumer Safety (SCCS) is publicly available as “Opinion on Toluene-2,5-diamine and its sulfate” [Toluene-2,5-diamine and its sulfate 17 COLIPA n°A5](#)  (794 KB).² Its use was concluded to be ‘safe with regard to systemic toxicity’. The data reviewed by the SCCS relevant to the issue of carcinogenicity is briefly summarized here, and individual study summaries are in Appendix I.

Genotoxicity

Genotoxicity data covering all of the relevant genetic endpoints have been developed for 2,5-diaminotoluene (free base, sulfate and/or dihydrochloride), both *in vitro* and *in vivo*, and the weight of the evidence indicates that 2,5-diaminotoluene does not pose a genotoxic hazard. While 2,5-diaminotoluene is considered to be genotoxic *in vitro*, the *in vivo* data are negative with one exception, as discussed below. Overall, the weight of the evidence indicates 2,5-diaminotoluene does not pose a genotoxic hazard *in vivo* and therefore does not pose a risk to humans with regards to mutagenicity/genotoxicity.

In *in vitro* studies, 2,5-diaminotoluene induced gene mutations, chromosomal aberrations, and unscheduled DNA-repair synthesis in primary hepatocytes *in vitro*. In *in vivo* studies, 2,5-diaminotoluene was negative in two mouse bone marrow micronucleus tests, following oral and i.p. administration, an *in vivo/in vitro* UDS test following oral administration, and an *in vivo* Comet assay (oral gavage) in mice and rats in all organs evaluated except for the rat stomach. Issues with regard to interpretation and validity of the *in vivo* Comet assay result have been discussed, i.e. that effects observed only in the stomach may be due to localized irritation/toxicity, and the validity of this study has been questioned in the scientific community for several reasons (*see corresponding references on page 12 in Appendix I: Hartmann et al., 2003; Brendler-Schwaab et al., 2005*). 2,5-Diaminotoluene was negative in two *in vivo* mouse spot tests following dermal and intraperitoneal administration indicating lack of mutagenic activity *in vivo*. In addition, 2,5-diaminotoluene was negative in two dominant lethal assays indicating lack of clastogenic activity in germ cells *in vivo*.

In order to further support the absence of a genotoxicity potential under *in vivo* conditions, the genotoxicity of the mono- and diacetylated metabolites of 2,5-diaminotoluene were also investigated. These are the key metabolism products after systemic exposure (*see study summaries for toxicokinetics in vitro and toxicokinetics in rats in Appendix I*). The lack of genotoxicity observed for N-acetylated metabolites of 2,5-diaminotoluene confirms its overall favorable *in vivo* genotoxicity profile and provides an explanation as to why the *in vitro* findings from standard testing are not relevant for the *in vivo* situation.

²SCCS opinion is posted at http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_093.pdf

Carcinogenicity

Carcinogenicity studies have been conducted by the US National Cancer Institute in male and female Fischer 344 rats and B6C3F1 mice, with 2,5-diaminotoluene sulfate administered in the diet. The conclusion of these studies was that “sufficient evidence was not obtained to demonstrate the carcinogenicity of 2,5-toluenediamine sulfate in either Fischer 344 rats or B6C3F1 mice.” Although certain aspects of the study execution differed from current practice in study conduct, the study findings were designated as negative by the US National Toxicology Program. Other long term carcinogenicity studies in rats and mice after dermal application of hair dye formulations containing 2,5-diaminotoluene showed no evidence of carcinogenicity at the application site or in any other tissue.

2,5-Diaminotoluene underwent evaluation by IARC, and was classified in 1987 as Group 3, “not classifiable as to its carcinogenicity to humans.” There was concluded to be ‘inadequate evidence’ in animals, and ‘no adequate data’ in humans. There are no data developed since the time of the IARC review that would bring that conclusion into question.

Toxicokinetics

In vitro metabolism studies in human, mouse, and rat cryopreserved hepatocytes indicate that 2,5-diaminotoluene sulfate is extensively metabolised by all three species. *N*-acetylation was the major metabolic pathway in all three species. The metabolite profile differed between species with evidence of hydroxylation occurring only in mouse hepatocytes. There was no evidence of hydroxylation in human hepatocytes. Further studies with human hepatic microsomes and human recombinant cytochrome P450 isozymes also showed no evidence of oxidative metabolism of 2,5-diaminotoluene sulfate.

The most recent human study indicates that the systemic exposure to 2,5-diaminotoluene sulfate following a typical hair dyeing process is in the range of $0.86 \pm 0.27\%$ and $0.87 \pm 0.45\%$ of the applied radioactivity for the concentrations of 1.5% and 4%, respectively, with nearly all of this found in urine (0.83% of the applied radioactivity for both concentrations).

Absorption, distribution, metabolism and excretion were investigated in the female Wistar Kyoto rat, a strain with a slow acetylator phenotype, and in Sprague-Dawley rats. The results indicate extensive metabolism in both strains after both oral and dermal administration, with reasonably high to high bioavailability after oral administration (53-95 %) and relatively low bioavailability after dermal application (2-3 %).

2,5 Diaminotoluene is not present in Diaminotoluene (mixed)

2,5-Diaminotoluene and diaminotoluene (mixed) are synthesized via distinctly separate pathways. The end result is that diaminotoluene (mixed) does not contain 2,5-diaminotoluene.

Briefly, diaminotoluene (mixed) is made by nitrosation of toluene. The end result is a discrete set of products that is a mixture primarily of 2,4 and 2,6 diaminotoluene. The synthesis of 2,5-diaminotoluene must use different starting materials because of the impossibility of generating any amount of this product from a pathway that starts with toluene. An explanation of the synthetic pathways is provided in Appendix II.

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Diaminotoluene (mixed) is defined by its CAS number (25376-45-8), as listed in the Code of Federal Regulations, 40 CFR §372.65. This CAS number brings back a list of synonyms, none of which are 2,5-Diaminotoluene, and refers to the 2,4- and 2,6- isomers.

Summary

The information provided within this submission supports the lack of carcinogenicity of 2,5-diaminotoluene and its sulfate salt. Listing of this isomer under Proposition 65 is not warranted. 2,5-diaminotoluene is not a component of diaminotoluene (mixed). Addition of the CAS number for diaminotoluene (mixed) would clarify the identity of the listed material.

Thank you for your attention to these issues.

Sincerely,

A handwritten signature in cursive script that reads "Linda Loretz". The signature is written in dark ink on a light-colored background.

Linda J. Loretz, Ph.D., DABT
Director, Safety & Regulatory Toxicology

Appendix I Relevant Information - Diaminotoluenes

Appendix I summarizes individual studies conducted on *2,5-Diaminotoluene* (free base, sulfate or dihydrochloride) that are relevant to an assessment of its carcinogenicity. Sections on Mutagenicity/Genotoxicity, Carcinogenicity, and Toxicokinetics are included.

Mutagenicity/Genotoxicity

Many *in vitro* and *in vivo* mutagenicity tests have been conducted with *2,5-diaminotoluene* (free base, sulfate or dihydrochloride). In the following sections, state-of-the-art studies are described in detail. Older studies that add to the safety assessment are described if they are guideline/scientific compliant and tested a specified material. In addition, two *in vivo* mouse spot tests and two *in vivo* mouse dominant lethal tests are briefly described even though they are non-GLP studies conducted with unspecified test material. They are included since they add to the assessment of the genotoxicity of *2,5-diaminotoluene in vivo*. Other genotoxicity studies conducted on *2,5-diaminotoluene* are available upon request.

Survey of key *in vitro* and *in vivo* genotoxicity studies for *2,5-diaminotoluene* (Sulfate, free base or dihydrochloride):

	<u>Study type</u>	<u>Genetic endpoint</u>	<u>Result</u>
<u><i>In vitro</i> studies</u>	Ames-Test	Gene mutation	Negative: – S9 Positive: + S9
	Mouse Lymphoma Forward Mutation Assay	Gene mutation	Negative: - S9 Inconclusive: + S9
	Chromosome Aberration Assay	Chromosome aberration	Positive: - S9 Positive: + S9
	UDS	DNA damage	Positive in rat hepatocytes and weakly positive in hamster hepatocytes
<u><i>In vivo</i> studies</u>	Mouse bone marrow micronucleus assay	Chromosome aberration, aneuploidy	Study 1: Negative Study 2: Negative
	In vivo/in vitro UDSTest	DNA damage	Negative
	Mouse and rat multiple organ Comet Assay	DNA damage	Negative: Mouse, Rat all organs except rat stomach Positive: Rat stomach
	Mouse Spot Test	Somatic cell mutation	Negative
	Dominant Lethal Assay	Germ cell clastogenicity assay	Negative
<u><i>In vitro</i> studies</u> with mono- and di-acetylated metabolites	Ames	Gene mutation	Negative
	<i>In vitro</i> micronucleus test	Chromosome aberration, aneuploidy	Negative

Mutagenicity/Genotoxicity *in vitro*

Ames Test

Study Design

Guideline:	According to ninth addendum to OECD guideline No. 471 (1997)
Species/strain:	Salmonella typhimurium / strains TA 1537, TA 98, TA 1535, TA 102, TA 100
Replicates:	Triplicates per test concentration; In accordance with current guidelines only one experiment was performed because a clearly positive result was obtained in experiment 1.
Assay conditions:	Plate incorporation assay with and without addition of S9 from phenobarbital/ β -naphthoflavone induced rat liver.
Test substance:	<i>2,5-diaminotoluene Sulfate</i>
Vehicle:	DMSO
Batch No.:	2346
Purity:	HPLC: 99.2 area % (254 nm), 99.8 area % (305 nm)
Content:	NMR: 98.3 weight %
Concentrations:	3; 10; 33; 100; 333; 1000; 2500; 5000 μ g/plate
GLP:	In compliance

2,5-diaminotoluene Sulfate was tested for mutagenicity in the reverse mutation assay in bacteria using the plate incorporation method both with and without addition of S9 (phenobarbital/ β -naphthoflavone induced rat liver). Salmonella typhimurium / strains TA 1537, TA 98, TA 1535, TA 102, TA 100 were exposed to the test substance at concentrations of 3; 10; 33; 100; 333; 1000; 2500; 5000 μ g/plate. For control purposes, solvent (DMSO) and positive controls (without S9 mix: 4-nitro-o-phenylene-diamine for strain TA98 and TA1537, sodium azide for strain TA100 and TA1535; methyl methane sulfonate for strain TA102; with S9 mix: 2-aminoanthracence for all tester strains) were evaluated in parallel.

Results

With metabolic activation, substantial and dose dependent increases in revertant colony numbers were observed in strains TA 1537, TA 98, TA 1535, and TA 100, but not TA102. The number of revertant colonies reached or exceeded the thresholds for a positive effect of twice (TA 98 and TA100) or thrice (TA 1535 and TA 1537) the colony count of the respective solvent control with maximum enhancement factors at a concentration of 1000 μ g/plate. Toxic effects as evidenced by reduction in the number of revertants occurred at concentrations of 2500 μ g/plate and 5000 μ g/plate. No mutagenic effect was observed without metabolic activation.

The mutagenic response to appropriate positive controls demonstrated the sensitivity of the test system. The historical range of positive controls was exceeded in strain TA1535 without metabolic activation and in strain TA1537 with metabolic activation. This does not compromise the assay, but instead indicates the sensitivity of the strains.

Conclusion

2,5-diaminotoluene Sulfate in the presence of S9 induced gene mutations under the experimental conditions reported.

Reference: Sokolowski, A.; *Salmonella Typhimurium* reverse mutation assay with INCI: 2,5-diaminotoluene sulfate; RCC-CCR; 2003 34)

Mouse lymphoma assay

Study design

Guideline:	According to second addendum to OECD guideline No 476 (1984)
Species/strain:	Mouse lymphoma TK ^{+/-} L5178Y cells
Replicates:	Single cultures per concentration
Test substance:	2,5-diaminotoluene Sulfate
Vehicle:	1 % v/v ammonium hydroxide in media
Batch No.:	EHF 290394
Purity:	HPLC: 99.3 area % (210 nm), 99.0 area % (254 nm), 99.6 area % (303 nm)
Content:	NMR: 97.3 weight %
Concentrations:	1, 3, 6, 10, 15 µg/ml (experiment 1 without S9) 3, 10, 30, 60, 100 µg/ml (experiment 1 with S9) 1, 6, 10, 15, µg/ml (experiment 2 without S9) 3, 10, 30, 100 µg/ml (experiment 2 with S9)
Dosing schedule:	Exposure period 4 h (with and without S9)
GLP:	In compliance

2,5-diaminotoluene Sulfate was examined for its mutagenic activity in the L5178Y TK^{+/-} mouse lymphoma assay with and without metabolic activation (S9 mix from the liver of Aroclor 1254 induced male Wistar rats). A range-finding test measuring relative suspension growth and two independent mutagenicity experiments were carried out. In experiments 1 and 2, concentrations tested ranged from 1– 15 µg/ml without and 3-100 µg/ml with metabolic activation. Culture medium containing 1 % v/v ammonium hydroxide was used as the solvent control. Ethylmethanesulfonate (EMS, 0.8 mg/ml) and benzo[a]pyrene (B[a]P 3 µg/ml) were used as positive controls without and with metabolic activation system, respectively. In experiments 1 and 2, the incubation time was 4 h in the presence and absence of S9 mix. Single cultures were investigated for each concentration and test group. Mutant frequency and cell survival (measured as total suspension growth) were determined.

Results

In the range-finding assay, toxic effects were observed at concentrations higher than 3 µg/ml (without S9) and 10 µg/ml (with S9). In experiments 1 and 2 without S9, toxic effects determined by the relative total growth, were observed at concentrations of 6 µg/ml and above. In cultures with S9 no toxicity was observed even at the highest concentrations. No biological sensitivity of the test system was demonstrated by the response to the positive control substances ethylmethanesulfonate and 3-methylcholanthrene.

Conclusion

Under the conditions of these experiments 2,5-diaminotoluene Sulfate did not induce mutations at the thymidine kinase locus in mouse lymphoma TK^{+/-} L5178Y cells in two independent experiments. However the concentrations of 2,5-diaminotoluene Sulfate tested with metabolic activation were non-toxic so this portion of the assay is not definitive. Based on genotoxic effects induced in the other *in vitro* genotoxicity assays, there is no need to further evaluate 2,5-diaminotoluene Sulfate in the mouse lymphoma TK^{+/-} L5178Y assay with metabolic activation.

(Reference: Wollny, H.-E.; *Cell mutation assay at the thymidine kinase (TK +/-) locus in mouse lymphoma L5178Y cells with A5; RCC-CCR; 1995*)

Chromosome aberration test *in vitro*

Study design

Guideline:	According to Ninth addendum to OECD Guideline No. 473 (1997)
Species/strain:	Chinese Hamster / V79 cell line
Replicates:	2
Test substance:	<i>2,5-diaminotoluene Sulfate</i>
Vehicle:	Minimum Essential Medium
Batch No.:	46847
Purity:	99.9 area % (HPLC)
Concentrations:	0.6; 1.3; 2.5; 5; 10; 20 µg/ml (without S9) 100; 200; 300; 400; 500; 600 µg/ml (with S9)
Dosing schedule:	Exposure period of 4h, 14 h recovery before harvest
GLP:	In compliance

2,5-diaminotoluene Sulfate was examined for its clastogenic activity in V79 Chinese hamster ovary cells in the absence and presence of metabolic activation (S9 was from the liver from phenobarbital/β-naphthoflavone-induced male Wistar rats). A range-finding test measuring growth inhibition with 4 h (20 h recovery) with and without metabolic activation, and 24 h continuous treatment without metabolic activation. Cells were exposed to the test article for 4 h or 24 h. Based on the results of the range-finding test, 0.6; 1.3; 2.5; 5; 10; 20 µg/ml (without S9) and 100; 200; 300; 400; 500; 600 µg/ml (with S9) were evaluated for induction of chromosome aberrations.

Culture medium was used as the solvent control. Ethylmethane sulfonate (200 µg/ml) without metabolic activation and cyclophosphamide (0.7 µg/ml) with metabolic activation were used as positive controls.

In the main experiment, 2 cultures per experimental group were set up for cytogenetic analysis, and 2 additional cultures per group for the determination of the cell number. A 4 h treatment (14 h recovery) in the presence and absence of metabolic activation was used. Cytogenetic analysis for chromosome aberrations was done on 100 metaphases/culture, 200 metaphases total. Screening for polyploidy in 500 metaphases/culture, 1000 cells total.

Results

In the range-finding study, there was toxicity at all concentrations after a 4h exposure (20h recovery) and a 24 h exposure in the absence of S9. In the presence of S9, toxicity was observed at 276.3 µg/ml and above after a 4 h exposure. Based on this, 20 µg/ml was selected as the top concentration in the absence of S9, and 600 µg/ml was selected as the top concentration with S9. In the main experiment, cells were exposed to the test article for 4h with and without S9 and allowed to recover for 14 h before harvesting. Chromosomes were prepared for analyses 18 h after the start of treatment. In the absence of S9, at 10 µg/ml and above, toxicity was induced as evidenced by reduction in cell number compared to control. With S9, toxicity was observed at 400 µg/ml and above. In the absence of S9, a dose-related increase in cells with chromosome aberrations was observed at concentrations of 2.5, 5, and 10 µg/ml where 14.5 % aberrant cells were induced (gaps excluded; solvent control induced 1 % aberrant cells).

In the presence of S9 a dose-related increase in the percentage of aberrant cells with 5.5 %, 7.5 % and 18.5 % aberrant cells (gaps excluded; solvent control induced 2 % aberrant cells) was observed at concentrations of 200, 300, and 400 µg/ml, respectively. No increase in polyploid

cells compared to the control values was observed. The sensitivity of the test system was demonstrated by the positive results obtained with the positive controls, ethyl methanesulfonate and cyclophosphamide.

Because a clear positive result with and without S9 was observed in the main experiment with a 4 h exposure, a repeat study was not conducted.

Conclusion

2,5-diaminotoluene Sulfate was clastogenic in V79 cells both in the presence and absence of metabolic activation under the experimental conditions of this test.

(Reference: Schulz, M.; *In vitro chromosome aberration test in Chinese hamster V79 cells with SAT 010935; RCC-CCR; 2002*)

Unscheduled DNA synthesis *in vitro*

Study design

Guideline:	Not indicated but similar to OECD 482 (1986)
Species/strain:	Hepatocytes from Sprague-Dawley rats and Golden Syrian hamsters
Test substance:	<i>2,5-diaminotoluene Sulfate</i>
Vehicle:	DMSO
Batch No.:	not indicated
Purity:	95 %
Dose levels:	10^{-4} ; 10^{-5} ; 10^{-6} ; 2×10^{-7} molar
Dosing schedule:	Exposure period 4h
Replicates:	2 independent experiments
GLP:	Not indicated

2,5-diaminotoluene Sulfate was assessed for its potential to induce DNA damage and repair in the *in vitro* UDS test using hepatocytes obtained from male Sprague-Dawley rats and male Golden Syrian hamsters by *in situ* liver perfusion. *2,5-diaminotoluene Sulfate* was solubilised in DMSO and added to freshly prepared cultures of hepatocytes at concentrations of 10^{-4} ; 10^{-5} ; 10^{-6} ; 2×10^{-7} molar. The final DMSO concentration was 1 % in the culture medium. DMSO was analyzed as the concurrent solvent control. There was no positive control, though many chemicals in this evaluation produced a positive response demonstrating the sensitivity of the test system.

Six cell cultures containing 1×10^5 viable cells were used per test concentration. After a 2.5 h attachment period, ^3H -thymidine and the test substance were added and incubation continued for another 4 hours. Following the 4h exposure period, the cells were washed and incubated in media overnight. After overnight incubation, cultures were washed, and the slides processed for autoradiography. Evaluation was done by counting nuclear grains in 60 nuclei/treatment, averaging the nuclear grain count, and subtracting the average grain count from 3 adjacent cytoplasmic areas.

Results

In experiment 1, 1×10^{-4} M, *2,5-diaminotoluene Sulfate* was toxic to both rat and hamster hepatocytes (data not shown). At 1×10^{-5} M *2,5-diaminotoluene Sulfate* induced a positive response (average net nuclear grains of 5 or greater) in rat hepatocytes and a weak response in hamster hepatocytes (only 4 average net nuclear grains, but 46 % of cells had greater than or

equal to 5 net nuclear grains). At lower concentrations the response was negative. In experiment 2, a positive effect was again observed in rat hepatocytes and a weak effect in hamster hepatocytes after exposure to 1×10^{-5} M 2,5-diaminotoluene Sulfate.

Conclusion

2,5-diaminotoluene Sulfate induces DNA damage leading to increased repair synthesis in rat hepatocytes and a weak effect in hamster hepatocytes.

(Reference: 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)

Summary in vitro mutagenicity/genotoxicity

The mutagenic potential of 2,5-diaminotoluene Sulfate was investigated in bacterial and mammalian cells *in vitro*. The Ames test, the in vitro chromosome aberration test, and the in vitro UDS test were positive. The mouse lymphoma assay was negative in the absence of metabolic activation but was inadequate in the presence of S9. Based on the positive results in the other in vitro genotoxicity tests, it is not considered necessary to retest 2,5-diaminotoluene Sulfate in the mouse lymphoma assay in the presence of S9.

2,5-diaminotoluene Sulfate is concluded to be genotoxic *in vitro*.

Mutagenicity/Genotoxicity in vivo

Mouse bone marrow micronucleus test, study 1

Study Design

Guideline:	OECD guideline No.: 474 (1997)
Species/strain:	Mouse / Crl: NMRI BR
Group size:	5 Males and 5 females per treatment group
Test substance:	2,5-diaminotoluene Sulfate (code: SAT 010935)
Batch No.:	46847
Purity:	99.9 area % (HPLC)
Dose levels:	25, 50, 90 mg/kg bw
Route:	Intraperitoneal
Vehicle:	Ethanol/deionised water (20:80 v/v)
Dosing schedule:	Single administration
Sacrifice times:	24 and 48 hours (high dose only)
GLP:	In compliance

Two range-finding studies were conducted. In the first, mice were administered 200, 500 and 900 mg/kg bw of 2,5-diaminotoluene Sulfate by i.p. injection. In the second range-finding study, animals were administered 25 and 75 mg/kg bw of 2,5-diaminotoluene Sulfate by i.p. injection. Dose selection was based on the results of the range-finding studies.

In the main study, the test substance and vehicle (ethanol/deionized water 20:80 v/v) were administered by i.p. injection, cyclophosphamide (40 mg/kg bw) was administered by gavage.

The dose groups 25, 50 and 90 mg/kg bw were scheduled for sacrifice at 24h, an additional group dosed with 90 mg/kg bw was planned to be sacrificed after 48h.

Bone marrow was sampled at 24 h after dosing for all groups and additionally after 48 h (high dose). Slide preparation was performed according to accepted methods. Slides were analysed without knowledge of the dose group. For each animal, 2000 polychromatic erythrocytes (PCE) were examined for the presence of micronuclei. Micronucleated normochromatic erythrocytes (NCE) were recorded, too. The ratio of nucleated cells to erythrocytes was measured in at least 400 cells/animal and the PCE/NCE ratio was measured in 1000 erythrocytes were determined as indicators for bone marrow toxicity. Five male and five female animals per test group were evaluated.

Results

Stability and homogeneity data are not provided in the study. Dosing solutions were prepared fresh before administration and stirred during the dosing procedure.

In the first range-finding study, mice were administered 200, 500 and 900 mg/kg bw 2,5-*diaminotoluene Sulfate*. Deaths occurred within thirty minutes after administration of 900 mg/kg bw. In a second range-finding study, animals were administered 25 and 75 mg/kg bw 2,5-*diaminotoluene Sulfate* and survived 48 h after administration. At 75 mg/kg bw, clinical signs of toxicity were noted. The LD50 was estimated to be about 123 mg/kg bw and 90 mg/kg bw was selected as the high dose (75 % of the estimated LD50). At 24 h, the dose of 90 mg/kg bw had marked toxic effects, and none of animals in the 48 h group survived to the harvest time. Due to high mortality at 90 mg/kg bw, an additional group of animals was administered 50 mg/kg bw and sacrificed 48 h after administration of the test item.

At 24 h, males administered 90 mg/kg bw had significant decreases in the numbers of nucleated cells, PCE and the PCE/NCE ratio indicating bone marrow toxicity. Systemic toxicity and slight bone marrow toxicity at 24 h were also detectable in the males and females in the 50 mg/kg bw group. The rate of micronucleated PCE was not increased compared to the vehicle control group in any of the test groups and was within the range of historical control data. There was no influence on the frequency of micronucleated NCE.

The sensitivity of the test system was demonstrated by the positive results obtained in the cyclophosphamide-treated group.

Conclusion

Under the conditions of this study 2,5-*diaminotoluene Sulfate* administered to an MTD did not induce the formation of micronuclei in polychromatic erythrocytes in the bone marrow of mice. The bioavailability of 2,5-*diaminotoluene Sulfate* was clearly demonstrated by bone marrow toxicity.

(Reference: Bornatowicz, N.; "SAT 010935": *Micronucleus test with mice*; SEIBERSDORF; 2002)

Mouse bone marrow micronucleus test, study 2

Study Design

Guideline:	According to first addendum to OECD guideline No. 474 (1983)
Species/strain:	Mouse / NMRI
Group size:	5 Males and 5 females per treatment group

Test substance:	<i>2,5-diaminotoluene Sulfate</i>
Batch No.:	EFH 290394
Purity:	>98 % HPLC: 99.3 area % (210 nm), 99 area % (254 nm), 99.6 area % (303 nm)
Content:	NMR: 97.3 weight %
Dose levels:	15, 50, 150 mg/kg bw
Route:	Oral, gavage
Vehicle:	Polyethylene glycol (PEG) 400.
Dosing Schedule	Single administration,
Sacrifice times:	24 h, 48 h (high dose only)
GLP:	In compliance

Two range finding studies were conducted. In the first study, mice were administered 200, 300 and 400 mg/kg bw *2,5-diaminotoluene Sulfate*. In a second range-finding study, animals were administered 50, 100, 150 mg/kg bw *2,5-diaminotoluene Sulfate*. Dose selection was based on the results of these range-finding studies.

The vehicle control was PEG 400 and the positive control was cyclophosphamide (30 mg/kg bw).

Bone marrow was sampled at 24 h after dosing for all groups and additionally after 48 for the high dose. Slide preparation was performed according to accepted methods. Slides were analysed without knowledge of the dose group. For each animal, 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronuclei. The ratio of PCE:NCE measured in 1000 erythrocytes was determined as an indicator for bone marrow toxicity. Five male and five female animals per test group were evaluated.

Results

Stability and homogeneity data were not provided in the study report however dosing solutions were prepared the day of the experiment.

In the first range finding study, mice were administered 200, 300 and 400 mg/kg bw *2,5-diaminotoluene Sulfate*. Deaths were observed at all dosages. In a second range-finding study, animals were administered 50, 100, 150 mg/kg bw *2,5-diaminotoluene Sulfate*. Clinical signs of toxicity were observed (eyelid closure) and all animals survived to 48 h. Dosages selected for the main study were 15, 50, and 150 mg/kg bw. The vehicle control was PEG 400 and the positive control was cyclophosphamide (30 mg/kg bw).

In the main study, animals expressed toxic responses, but no details were provided in the study report. The PCE/NCE ratio was not affected in the test groups. The observed toxicity, along with deaths occurring at higher doses in the preliminary studies supports the conclusion that an MTD was achieved in this assay.

There were no significant increases in the number of micronucleated PCE compared to the negative control in any of the test groups at 24 or 48 h. A significant increase in micronucleated PCE was recorded in the cyclophosphamide control group demonstrating the validity of the test system.

Conclusion

Under the conditions of this study *2,5-diaminotoluene Sulfate* administered to an MTD did not induce the formation of micronuclei in polychromatic erythrocytes in mouse bone marrow.

(Reference: Völkner, W.; *Micronucleus assay in bone marrow cells of the mouse with A 5; RCC-CCR; 1995*)

Unscheduled DNA synthesis *in vivo/in vitro*

Study design

Guideline:	According to OECD guideline No. 486 (1997)
Species/strain:	Rat / Sprague Dawley SD
Group size:	3 Males per treatment group
Test substance:	<i>2,5-diaminotoluene Sulfate</i> (Code: SAT 010935) in sterile water
Batch No.:	46847
Purity:	> 99.9 % (HPLC)
Dose levels:	20; 40; 80 mg/kg bw
Route:	Oral, gavage
Vehicle:	sterile injectible water
Dosing schedule:	Single administration
Sacrifice times:	2 and 14 hours
Replicates:	2 independent experiments
GLP:	In compliance

Doses for the assay were provided by the Sponsor and were 20, 40, 80 mg/kg bw *2,5-diaminotoluene Sulfate*. Mortality occurred in animals dosed at 80 mg/kg bw so the high dose analyzed in the study was 40 mg/kg bw. For each treatment group and time point, four rats were treated and three rats were processed for the assay. The concurrent vehicle control group received 10 ml bw sterile injectible water. Positive control group animals were dosed with methylnitrosourea (80 mg/kg bw) for 2 h time point and 2-acetylaminofluorene (100 mg/kg bw).

Primary hepatocytes were isolated 2 h and 14 h after administration of the test substance. Cell viability was determined before initiation of 6 replicate cultures for each experimental group. After a 4h incubation period in the presence of tritiated thymidine the slides were incubated overnight in medium containing unlabelled thymidine, then further processed for autoradiography. Two slides per animal from the 20 and 40 mg/kg bw groups were analysed. Toxicity to the liver cells was examined by means of dye-exclusion (trypan blue). In total 100 cells/animal were scored for unscheduled DNA synthesis. Nuclear and cytoplasmic grains for the calculation of nuclear net grains were counted separately.

Results

Stability and homogeneity data were not provided in the study report however dosing solutions were prepared immediately before use.

The animals dosed with 80 mg/kg bw for the 14 h preparation interval all died. Based on this, and the fact that no cytotoxicity was observed in the hepatocyte preparations at 20 and 40 mg/kg bw, these doses were chosen for initial scoring. Animals survived administration of 80 mg/kg bw for the 2 h time point and these slides were evaluated at a later time point. The results from the combined analyses are described here. The mean net grain values of the vehicle control group were within the historical control range of the test facility. Treatment with *2,5-diaminotoluene*

Sulfate in doses of 20, 40 mg/kg bw and 80 mg/kg bw at the 2 h time point did not induce an increase in mean nuclear net grains nor did the doses of 20, 40 mg/kg bw at 14h.

Treatment with positive controls, methylnitrosurea and 2-acetylaminofluorene, resulted in significant increased in group mean net grain values and in percentages of cells in repair above 50 % demonstrating the validity of the test system.

Conclusion

Under the conditions of this experiment there was no indication that exposure of rats to 2,5-diaminotoluene *Sulfate* up to a maximum tolerated dose induces DNA-damage that causes repair synthesis detectable in the vivo/in vitro UDS test.

(References: Getuli, C.; SAT 010935 *Unscheduled DNA synthesis (UDS) in primary rat hepatocytes after in vivo treatment; RTC; 2002; Cinelli, S.; SAT 010935 Unscheduled DNA synthesis (UDS) in primary rat hepatocytes after in vivo treatment; RTC; 2004*)

Comet Assay in vivo

Study Design

Guideline:	Not applicable, many differences from current assay standards
Species/strain:	Mouse / ddY Rat / Wistar
Group size:	4 Males 12 Males (untreated and vehicle control groups)
Test substance:	<i>2,5-diaminotoluene Sulfate</i> CAS#6369-59-1
Batch No.:	Not indicated
Purity:	> 98 % (HPLC)
Dose level:	60 mg/kg bw
Route:	Oral, gavage
Vehicle:	Saline
Dosing schedule:	Single administration
Sacrifice Times:	3,8, 24 h
GLP:	Not indicated

The maximum dose was based on an acute toxicity study. A dose level of approximately 50 % of the LD₅₀ was chosen for both species and was 60 mg/kg bw. In this publication, where numerous chemicals were tested, vehicle control (olive oil) experiments were performed separately with different sampling times and the results were compared with results from untreated animals. With no obvious differences found between vehicle and untreated groups, untreated animals were used as zero-time controls for all chemicals including *2,5-diaminotoluene Sulfate*. No specific positive control was reported in the study with *2,5-diaminotoluene Sulfate*, but positive effects were observed with other chemicals in this compilation.

After intervals of 3 h, 8 h or 24 h the animals were killed and 8 organs were removed: stomach, colon, liver, kidney, urinary bladder, lung, brain and bone marrow. The organs were examined regarding size, colour and texture but no data were provided. A small part of each organ was fixed for histopathology and in the case of a positive result the respective organ a microscopic examination was examined, but no data were provided.

Slides for the comet assay were prepared from nuclei isolated by homogenisation of tissue samples. They were placed in a lysing solution and kept in the dark for > 60 min, then chilled in

alkaline solution for 10 min in the dark at 0°C. Electrophoresis was conducted in the dark at 0°C for 15 min at 25V and at about 250 mA. The slides were then neutralised and stained with 50 µl of ethidiumbromide (20 µg/ml). Fifty nuclei were examined per organ per animal and photographed at 200x magnification. Migration was calculated as the difference between length and diameter of the comet. The mean migration from 50 nuclei was calculated for each organ of the individual animals. The Dunnett test after one-way ANOVA was used for statistical analysis. The level of significance was set to $p < 0.05$.

Results

No deaths or distinctive clinical or microscopic signs of toxicity were observed after treatment of the animals with *2,5-diaminotoluene Sulfate*. The mean migration values calculated for the organs isolated from mice at the three sampling times were not statistically different from the untreated control values in any tissue. Negative results were obtained for all rat organs at the three sampling times with the exception of the stomach. For rat stomach there was a statistically significant increase compared to the control values ($0.001 < p < 0.01$) in the mean migration calculated from 4 animals at the 8 h sampling time ($32.9 \pm 3.62 \mu\text{m}$ versus a control value of $14.1 \pm 2.84 \mu\text{m}$; net value $18.8 \mu\text{m}$).

Conclusion

Under the conditions of this *in vivo* comet assay *2,5-diaminotoluene Sulfate* did not induce DNA damage in any tissue in mice or in rat colon, liver, kidney, urinary bladder, lung, brain and bone marrow. *2,5-diaminotoluene Sulfate* did induce DNA-damage in rat stomach cells after the application of a single oral dose of 60 mg/kg bw. The effect was detectable 8 h after the administration of the test substance. 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't 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 (References: 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; Brendler-Schwaab, S.; Hartmann, A.; Pfuhrer, S.; Speit, G.; The *in vivo* comet assay: use and status in genotoxicity testing; MUTAGENESIS; 20, 245-254; 2005). 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 (Reference: Brendler-Schwaab, S.; Hartmann, A.; Pfuhrer, S.; Speit, G.; The *in vivo* comet assay: use and status in genotoxicity testing; MUTAGENESIS; 20, 245-254; 2005).

(Reference: 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; 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)

Mouse Spot Tests and Dominant Lethal Tests

Two mouse spot tests and two dominant lethal tests have been conducted with 2,5-*diaminotoluene* (free base or dihydrochloride). 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 2,5-*diaminotoluene* *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 2,5-*diaminotoluene* is not mutagenic *in vivo*.

Mouse Spot Test

Study 1. Female C57B1/6J mice were mated with C57B1/10J or T stock males. On day 10-14 of gestation, the female mice were administered 2,5-*diaminotoluene* (in HBSS) i.p. at 30 mg/kg bw. It was not reported if the applied dose induced toxic effects in the treated females. There was no significant increase in recessive spots in the 2,5-*diaminotoluene* exposed pups observed after birth. The positive control, triethylenemelanine, induced a significant increase in recessive spots demonstrating the validity of the test system. Under the conditions of this test, 2,5-*diaminotoluene* did not induce somatic mutations in fetal cells following transplacental absorption. The data supports the conclusion that 2,5-*diaminotoluene* is not mutagenic *in vivo*.

(Reference: 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)

Study 2. Female C57B1/6J mice were mated with T stock males. On days 9, 10, and 11 post fertilisation 2,5-*diaminotoluene* Dihydrochloride in cornoil was applied topically to the shaved dorsal surface at doses of 15, 150 and 1500 mg/kg bw. No toxic effects were observed in the treated animals and no affect on fertility or litter size. There was no significant increase in recessive spots. The positive control, Benz(a)pyrene 150 mg/kg bw i.p. induced a significant increase in recessive spots. Under the conditions of this study 2,5-*diaminotoluene* Dihydrochloride did not induce somatic mutations *in vivo*.

(Reference: Matheson, D. W.; Mutagenicity evaluation of Orex 111 in the somatic mutation assay; LITTON BIONETICS; 1978)

Dominant Lethal

Study 1. 2,5-*diaminotoluene* 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 fetuses, implantation and resorption sites. In total there were 460 live fetuses (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. 2,5-*diaminotoluene* under the conditions of this test did not induce dominant lethal mutations in male rats. Under the conditions of this test 2,5-*diaminotoluene* did not induce embryonic or fetal deaths by inducing chromosomal aberrations in germinal tissue. The test supports the conclusion that 2,5-*diaminotoluene Sulfate* is not mutagenic *in vivo* in germ cells.

(Reference: Burnett, C.; Loehr, R.; Corbett, J.; Dominant Lethal mutagenicity study on hair dyes; J. TOXICOL. ENVIRON. HEALTH; 2, 657-662; 1977)

Study 2. 2,5-*diaminotoluene* 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.

(Reference: Matheson, D. W.; *Mutagenicity evaluation of 2,5-Diaminotoluene, dihydrochloride in the mouse dominant lethal assay*; LITTON BIONETICS; 1978)

Summary in vivo mutagenicity

A number of in vivo genotoxicity tests, covering the relevant genetic endpoints were performed with 2,5-diaminotoluene (*Sulfate or free base or dihydrochloride*). The following test systems were considered in the evaluation of the in vivo genotoxicity:

- Mammalian Erythrocyte Micronucleus Test
- Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells In Vivo
- In Vivo Comet Assay
- In Vivo Mouse Spot Test
- In Vivo Mouse Dominant Lethal Test

In two mouse bone marrow micronucleus tests up to a maximum tolerated dose, there was no indication of micronuclei by 2,5-diaminotoluene *Sulfate* administered i.p. where evidence of systemic exposure was observed, or by oral gavage. In addition, there was a negative result in an in vivo/ in vitro UDS test in rats administered 2,5-diaminotoluene *Sulfate* up to a maximum tolerated dose by oral gavage. 2,5-diaminotoluene *Sulfate* administered by oral gavage was negative in an in vivo Comet assay in mice, and in all organs in rats except the stomach. Issues with this study with regard to interpretation and validity have already been discussed and these results give no convincing evidence that 2,5-diaminotoluene *Sulfate* is genotoxic *in vivo*. 2,5-diaminotoluene (dihydrochloride or free base) was negative in two in vivo mouse spot tests following dermal and intraperitoneal administration indicating lack of mutagenic activity *in vivo*. In addition, 2,5-diaminotoluene (dihydrochloride or free base) was negative in two dominant lethal assays indicating lack of clastogenic activity in germ cells in vivo. Taken together, the weight of the evidence supports the conclusion that 2,5-diaminotoluene *Sulfate* does not pose a significant genotoxic hazard in vivo.

General conclusion: Genotoxicity in vitro and in vivo

In vitro genotoxicity testing of 2,5-diaminotoluene *Sulfate* covered all of the relevant genetic endpoints. 2,5-diaminotoluene *Sulfate* induced gene mutations, chromosomal aberrations, and unscheduled DNA-repair synthesis in primary hepatocytes *in vitro*. On the basis of the described results from in vitro genotoxicity testing 2,5-diaminotoluene *Sulfate* is considered to be genotoxic *in vitro*.

The weight of the evidence indicates that 2,5-diaminotoluene *Sulfate* does not pose a genotoxic hazard *in vivo*. 2,5-diaminotoluene *Sulfate* was negative in two mouse bone marrow micronucleus tests, following oral and i.p. administration, an *in vivo/in vitro* UDS test following oral administration, and an *in vivo* Comet assay (oral gavage) in mice and rats in all organs evaluated except for the rat stomach. Issues with regard to interpretation and validity of the *in vivo* Comet assay result have been discussed, i.e. that effects observed only in the stomach may be due to localized irritation/toxicity, and that the validity of this study has been questioned in the scientific community for several reasons (see corresponding references on page 14:

Hartmann et al., 2003; Brendler-Schwaab et al., 2005). 2,5-diaminotoluene (dihydrochloride or free base) was negative in two *in vivo* mouse spot tests following dermal and intraperitoneal administration indicating lack of mutagenic activity *in vivo*. In addition, 2,5-diaminotoluene (dihydrochloride or free base) was negative in two dominant lethal assays indicating lack of clastogenic activity in germ cells *in vivo*. Overall, the weight of the evidence indicates 2,5-diaminotoluene Sulfate (or free base or dihydrochloride) does not pose a genotoxic hazard *in vivo* and therefore does not pose a risk to humans with regards to mutagenicity/genotoxicity.

Mutagenicity/Genotoxicity of mono- and di-acetylated metabolites of 2,5-diaminotoluene <i>in vitro</i>

In order to further support the absence of a genotoxicity potential under *in vivo* conditions we have also investigated the genotoxicity of the mono- and diacetylated metabolites of 2,5-diaminotoluene Sulfate which are the key metabolism products after systemic exposure (see chapters toxicokinetics *in vitro* and toxicokinetics in rats). The below described results have been taken from Zeller and Pfuhler, 2014 (Zeller A, Pfuhler S. N-acetylation of three aromatic amine hair dye precursor molecules eliminates their genotoxic potential. *Mutagenesis*. 2014; 29:37-48)

Ames Test

Study Design

Guideline:	According to ninth addendum to OECD guideline No. 471 (1997)
Species/strain:	Salmonella typhimurium / strains TA 1537, TA 98, TA 1535, TA 102, TA 100
Replicates:	Triplicates per test concentration; In accordance with current guidelines only one experiment was performed because a clearly positive result was obtained in experiment 1.
Assay conditions :	Plate incorporation assay with and without addition of S9 from phenobarbital/β-naphthoflavone induced rat liver.
Test substance:	N-acetylated metabolites of 2,5-diaminotoluene Sulfate: 4-amino-2-methylacetanilide, 2,5-diacetamino-toluene CAS 19039-27-1). Vehicle: DMSO
Purity:	HPLC: > 99.5 area % for both metabolites
Concentrations:	3; 10; 33; 100; 333; 1000; 2500; 5000 µg/plate
GLP:	In compliance

4-amino-2-methylacetanilide and 2,5-diacetamino-toluene were tested for mutagenicity in the reverse mutation assay in bacteria using the plate incorporation method both with and without addition of S9 (phenobarbital/β-naphthoflavone induced rat liver). Salmonella typhimurium / strains TA 1537, TA 98, TA 1535, TA 102, TA 100 were exposed to the test substances at concentrations of 3; 10; 33; 100; 333; 1000; 2500; 5000 µg/plate. For control purposes, solvent (DMSO) and positive controls (without S9 mix: 4-nitro-o-phenylene-diamine for strain TA98 and TA1537, sodium azide for strain TA100 and TA1535; methyl methane sulfonate for strain TA102; with S9 mix: 2-aminoanthracence for all tester strains) were evaluated in parallel.

Results

No mutagenic effects were observed or without metabolic activation with 4-amino-2-methylacetanilide or 2,5-diacetamino-toluene

The mutagenic response to appropriate positive controls demonstrated the sensitivity of the test system.

Conclusion

4-amino-2-methylacetanilide and 2,5-diacetamino-toluene in the presence and absence of S9 did not induce gene mutations under the experimental conditions reported.

(Reference: Zeller A, Pfuhler S. N-acetylation of three aromatic amine hair dye precursor molecules eliminates their genotoxic potential. Mutagenesis. 2014; 29:37-48)

Micronucleus assay *in vitro* (cultured human peripheral blood lymphocytes)

Study Design

Guideline:	According to OECD guideline no. 487 (2004; Draft Guideline)
Species/strain:	Human peripheral blood lymphocytes
Replicates:	Two cultures per concentration and controls Two independent experiments using pooled blood from male donors
Test substance:	N-acetylated metabolites of 2,5-diaminotoluene Sulfate: 4-amino-2-methylacetanilide and 2,5-diacetamino-toluene Vehicle: DMSO
Purity:	HPLC: > 99.5 area % for both metabolites
GLP:	In compliance

4-amino-2-methylacetanilide and 2,5-diacetamino-toluene were tested examined for its clastogenic and aneuploidogenic activity in the *in vitro* micronucleus assay using human lymphocyte cultures prepared from pooled blood of healthy male donors. Chromosomal defects (structural and/or numeric aberrations) are measured in this test system by means of micronuclei formed in binucleated cells (due to cytochalasin B treatment). Two independent assays were performed with and without metabolic activation (S9 mix from the liver of Aroclor induced male Sprague Dawley rats). In experiment I, cells were treated with the test item 24 hours after mitogen stimulation with phytohaemagglutinin (PHA). In the second experiment, the treatment started 48 hours after PHA stimulation. The exposure times for the test item in the presence and the absence of S9 mix were 3 and 20 hours, respectively. The recovery periods were 28 and 45 hours for the test without and with S9 mix. For this period, cytochalasin B was added to the cultures at 6 µg/ml to block the cell division but not the nuclear division. At the harvest time (72 h in experiment I, 96 h in experiment II), the culture medium was removed by centrifugation and fixation of the swollen cells was done by repeated treatment with ice-cold methanol/glacial acetic acid. After transfer of the fixed cell suspension onto slides, cells were stained with Giemsa and microscopically evaluated. To calculate the replication index (RI), 500 cells per replicate (1000 per dose) were examined for proportions of mononucleate, binucleate and multinucleate cells. Potential chemically-induced cell cycle delay or cytotoxicity was also evaluated. 1000 binucleate cells from each culture (2000 per dose) were analysed for the occurrence of micronuclei (coded slides). The number of cells containing micronuclei and the number of micronuclei per cell was noted for each slide.

A broad concentration range (from 7.449 to 1232 µg/ml), separated by narrow intervals, was evaluated for the test item to define as closely as possible the test concentration at which the

replication index (RI) was reduced by approximately 60 %. This test concentration was used as the highest concentration to be evaluated. The two/three other test concentrations to be evaluated were selected in order to cover the range of low (none) to medium cytotoxicity to allow a concentration-response analysis.

DMSO was used as solvent control, while 4-nitroquinoline 1-oxide and vinblastine in the absence of S9 mix and cyclophosphamide in the presence of S9 mix were evaluated as positive controls.

Results

Neither *4-amino-2-methylacetanilide* nor *2,5-diacetamino-toluene* caused any biological relevant or dose-dependent increase in the frequency of micronucleated binucleate (MNBN) cells. The findings with the solvent (negative) and the positive controls demonstrated the sensitivity and the validity of the test.

Conclusion

Based on these results, *4-amino-2-methylacetanilide* and *2,5-diacetamino-toluene* were considered non-mutagenic in this *in vitro* assay.

(Reference: Zeller A, Pfuhrer S. N-acetylation of three aromatic amine hair dye precursor molecules eliminates their genotoxic potential. Mutagenesis. 2014; 29:37-48)

Comet assay in vitro:

Since the *in vitro* Comet assay follows no OECD guideline and the experiments performed were exploratory in nature they are not described in detail here but more detail can be found in Zeller and Pfuhrer, 2013 (*Reference: Zeller A, Pfuhrer S. N-acetylation of three aromatic amine hair dye precursor molecules eliminates their genotoxic potential. Mutagenesis. 2014; 29:37-48*). In these experiments the authors did compare the genotoxic potential of *2,5-diaminotoluene Sulfate* in cell lines that are N-acetyltransferase proficient with cell lines that are not, thus demonstrating that NAT-proficient cells can detoxify *2,5-diaminotoluene Sulfate* and DNA damage does occur only at higher doses where NAT capacity is overloaded. This is shown by the observed shift of dose-response curves towards higher concentrations in the experiments with NAT-proficient cell lines and confirms the hypothesis of N-acetylation being a detoxification pathway. Relevant increases in Comet percent tail DNA occur at substantially higher concentrations, demonstrating the capacity of these cells to N-acetylate and thereby detoxify the parent.

General conclusion

The lack of genotoxicity observed for N-acetylated metabolites of *2,5-diaminotoluene Sulfate* confirms its overall favorable *in vivo* genotoxicity profile and provides an explanation as to why the *in vitro* findings from standard testing is not relevant for the *in vivo* situation.

Carcinogenicity

Survey of the carcinogenicity studies performed with *2,5-diaminotoluene Sulfate* or hair dye formulations containing *2,5-diaminotoluene Sulfate*:

Study Type	Source	Result
Oral carcinogenicity study in rats	US National Cancer Institute (NCI) Study	Negative
Oral carcinogenicity study in mice	US National Cancer Institute (NCI) Study	Negative
Dermal carcinogenicity studies in rats (2 studies) and mice (3 studies)	Published studies	Negative

Carcinogenicity study in rats (oral administration)

Study design

Guideline:	Not indicated.
Species/Strain:	Rat/Fischer 344
Group size:	50 animals per sex per dose level except for low dose control groups which had 25 animals per sex
Test substance:	<i>2,5-diaminotoluene Sulfate</i>
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 of observation
GLP:	No

2,5-diaminotoluene 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, esophagus, 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 tumors of the testis was observed in male rats (low dose 43/48 vs. 33/45 in low dose control; high dose 47/48 vs. 19/24 in high dose control), but this was not considered treatment-related since the spontaneous incidence of these tumors 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 vs. 12/41 in low dose control). A similar trend (not statistically significant) was seen in high dose male rats (high dose 3/40 vs. 3/21 in high dose control). The incidence of lung tumors (alveolar/bronchiolar adenomas or carcinomas) was significantly lower in high dose male rats (high dose 0/49 vs. 3/25 in high dose control), 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).

Conclusion

The conclusion drawn in the NCI report for this study was that sufficient evidence was not obtained to demonstrate the carcinogenicity of *2,5-diaminotoluene Sulfate* in Fischer 344 rats. This study involved practices that depart from the current study design for carcinogenicity studies (e.g., start of dosing of high and low dose animals at different times which necessitated separate control groups, housing of treated and control animals in different rooms). However, under the conditions of the study, *2,5-diaminotoluene Sulfate* did not show evidence of carcinogenicity in Fischer 344 rats. Despite the limitations of the study, the U.S. National Toxicology Program (NTP) designates the findings of this study as negative in male and female rats. (Reference: *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*)

(Reference: *Bioassay of 2,5-Toluenediamine sulfate for possible carcinogenicity; NATIONAL INSTITUTE OF HEALTH; 1978*)

Carcinogenicity study in mice (oral administration)

Study design

Guideline:	Not indicated.
Species:	Mouse/B6C3F1
Group size:	50 animals per sex per dose level
Test substance:	<i>2,5-diaminotoluene Sulfate</i>
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
GLP:	No

2,5-diaminotoluene Sulfate was administered in the diet to groups of 50 mice 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 C57BL/6 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 (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, gall bladder, pancreas, esophagus, 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 compared to the corresponding control. There was no treatment-related effect on survival in either males or females.

A statistically significant increase in lung tumors (alveolar/bronchiolar adenomas or carcinomas) was observed in high dose female mice (high dose 8/45 vs. 1/45 in high dose control). The incidence was not significantly increased in low dose female mice (low dose 6/42 vs. 4/46 in low dose control). The high dose results were not considered convincing evidence of a treatment-related effect because high dose mice were received in separate shipments from their controls and housed in separate rooms from their controls. The incidence of pituitary adenomas or carcinomas was significantly lower in high dose female mice (high dose 0/38 vs. 6/37 in high dose control). 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).

Conclusion

The conclusion drawn in the NCI report for this study was that sufficient evidence was not obtained to demonstrate the carcinogenicity of *2,5-diaminotoluene Sulfate* in B6C3F1. This study involved practices that depart from the current study procedures for carcinogenicity studies (e.g., start of dosing of high and low dose animals at different times which necessitated separate control groups, the use of animals from different shipments, housing of treated and control animals in different rooms). In addition, a maximum tolerated dose did not appear to have been reached in males. However, under the conditions of the study, *2,5-diaminotoluene Sulfate* did not show evidence of carcinogenicity in B6C3F1 mice. Despite the limitations of the study, the U.S. NTP designates the findings of this study as negative in male and female mice (*Reference: 50*).

(Reference: *Bioassay of 2,5-Toluenediamine sulfate for possible carcinogenicity; NATIONAL INSTITUTE OF HEALTH; 1978*)

Carcinogenicity studies (dermal application)

A number of published studies have been conducted in rats and mice to evaluate carcinogenicity following long term dermal application of 2,5-diaminotoluene Sulfate in hair dye formulations also containing other primary intermediates/couplers with or without addition of hydrogen peroxide. These studies were either conducted using a test material for which identity and purity were not adequately documents and/or the publication provided less detailed information than would be expected in a complete study report.

Therefore, these studies will not be discussed in detail, despite the fact that all results were favourable.

References:

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*

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*

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*

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*

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*

General conclusion: Carcinogenicity

Carcinogenicity studies conducted by the US National Cancer Institute in male and female Fischer 344 rats and B6C3F1 mice administered 2,5-diaminotoluene Sulfate in the diet showed no evidence of carcinogenicity in either species. Although certain aspects of the study execution differed from current practice in study conduct, the study findings were designated as negative by the US National Toxicology Program.

Toxicokinetics *in vitro*

Metabolism *in vitro*

Metabolic stability, metabolite profile, and species comparison in primary hepatocytes of human, rat, and mouse

Study design

Guideline:	None
Cells:	Hepatocytes from humans (pooled from 3 donors) (In Vitro Technologies, Inc) Hepatocytes from male Sprague Dawley rats (In Vitro Technologies, Inc.) Hepatocytes from male ICR/CD-1 mice (In Vitro Technologies, Inc.)
Cell density:	1.0 x 10 ⁶ cells/ml
Test substance:	2,5-diaminotoluene Sulfate
Batch:	2346
Purity:	98.3 weight % (NMR) 99.2 % (HPLC 254 nm) 99.8 % (HPLC 305 nm)
Test concentration:	10 µM
Incubation time:	4 h incubation
GLP:	In compliance

The metabolic profile of 2,5-diaminotoluene Sulfate was investigated *in vitro* in cryopreserved primary hepatocytes from male human donors, male Sprague Dawley rats and male ICR/CD-1 mice.

The metabolic capacity of the hepatocytes was characterised by the use of marker substrates for phase I activity, i.e., general cytochrome P450 activity using coumarin (human) and 7-ethoxycoumarin (rat and mouse), CYP 1A1/2 activity using 7-ethoxyresorufin, and CYP2E1 using chlorzoxazone. The activity of the phase II enzymes NAT 1 and NAT2 was characterised by the use of sulfamethazine and *p*-aminobenzoic acid, respectively. The three lots of human hepatocytes pooled for use in this study were genotyped and found to be NAT2 *5/*7, wt/*5, and wt/*6 and, while referred to in the study report as yielding an average rapid metaboliser phenotype, would be most appropriately characterised as yielding an average intermediate metaboliser phenotype.

Hepatocytes (~1.0 x 10⁶ cells/ml) were incubated with 10 µM 2,5-diaminotoluene Sulfate for 4 hours in 24 well microtitre plates. Cell viability was assessed by microscopic evaluation and samples of the supernatant were taken for metabolite analysis at 0, 0.5, 1.5, and 4 hours. Test conditions were chosen based on previous range finding experiments. Metabolic stability was assessed by assay for loss of parent compound by means of LC-MS/MS. The profile of metabolites was also investigated by LC/MS (Scan) and metabolites were identified/characterized as far as possible.

Results

Cell viability was ~90 %, 95 %, and 80 % in human, rat, and mouse hepatocytes, respectively, at the beginning of the incubations and appeared to be essentially unaffected over the incubation

period with a slight decrease of approximately 10 % after 4 hours. The marker enzymes demonstrated the functional metabolic capacity of the hepatocytes with respect to key enzymes involved in the metabolism of arylamines, the chemical class to which *2,5-diaminotoluene Sulfate* belongs.

2,5-diaminotoluene Sulfate was extensively metabolised by primary human, rat, and mouse hepatocytes in the order rat~mouse>human based on disappearance of parent compound from the supernatant. After incubation for 1.5 h 98.8 %, 99.4 %, and 71.0 % of the parent had disappeared from the supernatant in cultures of rat, mouse, and human hepatocytes, respectively. The N-acetylated metabolite was the major metabolite identified in all three species based on LC-MS (Scan). Mouse hepatocytes, but not human or rat hepatocytes, also appeared to produce a second major metabolite by hydroxylation based on the detection of an M+16 peak. The identity of this metabolite was not determined. The mouse also appeared to produce an unknown metabolite as evidenced by an M+324 peak. There was some evidence of a minor metabolite consistent with taurine conjugation (human and rat) and a minor metabolite that appeared to be a mercapturic acid metabolite (human), based on the presence of M+107 and M+161 peaks.

Conclusion

Under the conditions of this test, *2,5-diaminotoluene Sulfate* was extensively metabolised by hepatocytes from the three species. N-Acetylation was the major reaction in human, rat and mouse hepatocytes. The metabolite profile differed between the species with evidence of hydroxylation observed only in mouse hepatocytes.

(Reference: Krebsfänger, N.; *2,4-Diaminotoluene sulfate (23005): Metabolic stability, metabolite profile, and species comparison in primary hepatocytes of human, rat, and mouse; GENPHARMTOX; 2003*)

Human hepatic metabolism: *in vitro* analysis

Study design

Guideline:	None
Test System:	Hepatocytes from humans (pooled from 4 female donors) (In Vitro Technologies, Inc) Pooled human liver microsomes (Gentest, Woburn, MA, USA) Bacterially expressed human CYP isozymes CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Cypex, Dundee, UK)
Test substance:	[ring-U- ¹⁴ C]- <i>2,5-diaminotoluene Sulfate</i>
Batch:	CFQ13783, batch 1 - [ring- ¹⁴ C(U)]- <i>2,5-diaminotoluene Sulfate</i> Lot 16825DR Sigma Aldrich – non-radiolabelled <i>2,5-diaminotoluene Sulfate</i>
Purity:	Radiochemical purity: 98.2 % by HPLC - [ring- ¹⁴ C(U)]- <i>2,5-diaminotoluene Sulfate</i> , Batch CFQ13783, batch 1 Non-radiolabelled <i>2,5-diaminotoluene Sulfate</i> (Lot 16825DR) – 97.96 % by titration
Test concentration:	10 µM and 100 µM
Positive Control:	2-aminofluorene
Incubation time:	Hepatocytes: 4 h Hepatic microsomes: 20 min Recombinant human cytochrome P450s (CYPs): 60 min
GLP:	No

¹⁴C-2,5-diaminotoluene Sulfate was incubated with pooled human liver microsomal preparations for 20 min in the presence or absence of an NADPH-regenerating system and incubated for 60 min with recombinant human CYPs derived from a bacterial expression system coexpressing cytochrome P450 reductase. The metabolic capacity of both the microsomal preparations and the recombinant enzymes was documented by the supplier. In addition 2-aminofluorene was used as a positive control for aromatic amine oxidative metabolism. Reactions were stopped by the addition of acetonitrile. The samples were centrifuged and aliquots of the supernatants were analyzed by HPLC-MS for the detection of parent compound and metabolites. Potential for binding to microsomal protein was determined by liquid scintillation counting of repeatedly washed microsomal pellets from reactions conducted in the presence or absence of an NADPH generating system. Cryopreserved human hepatocytes from four female donors were pooled and incubated as a cell suspension (1X10⁶ viable cells/ml) for 4 h with ¹⁴C-2,5-diaminotoluene Sulfate. Metabolic capability for Phase I metabolism was confirmed via incubations of these cells with 7-ethoxycoumarin. 2-Aminofluorene and p-phenylenediamine were used as reference controls. Incubations were stopped by the addition of acetonitrile, samples were centrifuged, and supernatants were analyzed by HPLC-MS. For MS detection, total ion scanning in positive and negative mode was performed. Subsequently single ion recording was carried out for specific metabolites of interest in order to detect parent compound and mono-oxygenated, di-oxygenated, mono-acetylated, diacetylated, sulfated, and glucuronidated metabolites.

Results

No production of oxidised/hydroxylated metabolites of 2,5-diaminotoluene Sulfate was observed after incubation with human liver microsomes or after incubation with recombinant CYP isozymes (CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6, and CYP3A4). An acetylated metabolite of 2,5-diaminotoluene Sulfate, but no sulfated or glucuronidated metabolites, was observed after incubation with cryopreserved human hepatocytes. A number of mono-oxygenated metabolites were observed in human liver microsomes after incubation with the positive control compound, 2-aminofluorene. Isozyme-specific patterns of oxidative metabolism of 2-aminofluorene were also observed with 2-aminofluorene in incubations with the recombinant P450 enzymes. CYP1A1 and CYP1A2 showed the highest capacity for oxidative metabolism of 2-aminofluorene. In human hepatocytes 2-aminofluorene underwent mono-acetylation but there was no evidence of hydroxylation. This may have been the result of competing metabolic pathways (e.g. Phase I oxidative metabolism vs. Phase II N-acetylation) in the intact hepatocyte, whereas there is no competing Phase II metabolism in the incubations with microsomes or with recombinant CYP isozymes.

Conclusion

The results of this study demonstrate that 2,5-diaminotoluene Sulfate is acetylated in human hepatocytes. There was no evidence to suggest that 2,5-diaminotoluene Sulfate undergoes oxidative metabolism in human microsomes, in human recombinant P450 isozymes, or in human hepatocytes.

(Reference: Powrie, R.; *Human hepatic metabolism of 2,5-diaminotoluene: in vitro analysis*; CXR BIOSCIENCES LTD, 2005;

Skare JA, Hewitt NJ, Doyle E, Powrie R, Elcombe C. *Metabolite screening of aromatic amine hair dyes using in vitro hepatic models. Xenobiotica. 2009;39:811-25)*

Summary toxicokinetics in vitro

In vitro metabolism studies in human, mouse, and rat cryopreserved hepatocytes indicate that 2,5-diaminotoluene Sulfate is extensively metabolised by all three species. *N*-acetylation was the major metabolic pathway in all three species. The metabolite profile differed between species with evidence of hydroxylation occurring only in mouse hepatocytes. There was no evidence of hydroxylation in human hepatocytes. Further studies with human hepatic microsomes and human recombinant cytochrome P450 isozymes also showed no evidence of oxidative metabolism of 2,5-diaminotoluene Sulfate.

Toxicokinetics in vivo

Human studies

Consumer exposure to an oxidative hair dye. A [¹⁴C]-labelled 2,5-diaminotoluene mass balance study.

Study Design

Guideline:	None
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)
Test Substance Batch No.:	CFQ41174
Test Formulations:	Isotopic dilution of 2,5-Diamino[ring-U- ¹⁴ C]toluene sulfate in a dark shade oxidative hair dye formulation with a final on-head concentration of either 1.5% or 4.0% 2,5-diaminotoluene (expressed as free base) plus couplers, following mixing with hydrogen peroxide-containing developer (1:1, w/w).
Test Formulation Batch Nos.:	DTF 0938002 BF0 2 (1.5% 2,5-diaminotoluene formulation); DTF 0938002 AF0 3 (4% 2,5-diaminotoluene formulation)
GCP Status:	In compliance; ICH Guideline for Good Clinical Practice (ICH topic E6, adopted 01-05 1996 and implemented 17-01 1997)

This human exposure study was conducted at TNO, Zeist, and the study protocol was approved by the Medical Ethics Committee of Tilburg. In this study, two groups of male and female human subjects (N=16 per group, age range 18-45 years) received a single application of an oxidative hair dye mixture by professional hairdressers. The final on-head concentration of [¹⁴C]-2,5-diaminotoluene 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 radiolabeled 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 [¹⁴C] radioactivity by liquid scintillation counting to calculate an overall mass balance. Plasma samples were analysed for [¹⁴C] radioactivity to evaluate plasma kinetics of [¹⁴C]-2,5-diaminotoluene 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 ± 1.72 mg and 17.99 ± 8.76 mg 2,5-diaminotoluene equivalents for the low and high concentration hair dye mixtures, respectively.

Plasma kinetic results are summarised in the following table.

Summary of Plasma Kinetic Parameters

Parameter	Units	Mean	SD
		1.5% 2,5-diaminotoluene	
T_{max}	hr	2.4	0.8
C_{max}	ng eq/mL	99.1	38.4
$AUC_{(0-48hr)}$	ng eq·hr/mL	1189	390
$AUC_{(0-\infty)}$	ng eq·hr/mL	1241	402
$T_{1/2}$	hr	10.1	1.3
		4.0% 2,5-diaminotoluene	
T_{max}	hr	2.6	1.0
C_{max}	ng eq/mL	266	145
$AUC_{(0-48hr)}$	ng eq·hr/mL	3341	1474
$AUC_{(0-\infty)}$	ng eq·hr/mL	3353	1519
$T_{1/2}$	hr	11.3	2.6

These results indicate a C_{max} of 99.1 ± 38.4 ng/mL and 266 ± 145 ng/mL for the low and high concentration mixtures, respectively. T_{max} was similar in both treatment groups (10.1 ± 1.3 hr and 11.3 ± 2.6 hr for the low and high concentration mixtures, respectively). The mean $AUC_{(0-\infty)}$ was 1189 ± 390 ng eq·hr/mL and 3553 ± 1519 ng eq·hr/mL for the low and high concentration mixtures, respectively.

(Reference: Consumer exposure to an oxidative hair dye. A [^{14}C]-labelled 2,5-diaminotoluene mass balance study. Study Number V9317, TNO, Zeist, The Netherlands, January 2012)

2,5-diaminotoluene Sulfate [Me- ^{14}C] absorption, disposition and elimination in human subjects

Study Design

Guideline:	None
Species:	Human
Group size:	5 Healthy adult male subjects between the ages of 20 and 45 years and within 15 % of their ideal body weight
Test substance:	[Me- ^{14}C] - 2,5-diaminotoluene (7.63 $\mu\text{Ci}/\text{mg}$)
Vehicle:	A representative formulation containing 0.825 % resorcinol and 0.825 % 2,5-diaminotoluene Sulfate, mixed 1:1 (w/w) with either distilled deionized water (formulation I) or 6 % hydrogen peroxide (formulation II)
Batch No.:	Not indicated
Purity:	Not indicated
GLP/GCP:	No

The test formulation was prepared by addition of [Me- ^{14}C]-2,5-diaminotoluene plus unlabeled 2,5-diaminotoluene Sulfate to a hair dye cream formulation containing 0.825 % resorcinol to yield a final concentration of 1.65 % 2,5-diaminotoluene Sulfate. 100 g of this formulation was then mixed with either 100 g of water (formulation I) or 100 g of 6 % hydrogen peroxide (formulation II). 45 g of Formulation 1 or formulation 2, each containing a total of 371 mg 2,5-diaminotoluene was applied to an area of 250 cm^2 of the scalp of 5 male subjects, respectively.

After 30 minutes the formulation was thoroughly rinsed off, and the hair was shampooed. The amount of radioactivity was quantified in urine up to 48 hours and in faeces up to 72 hours after application. The amount of radioactivity in whole blood was measured in samples taken before the application and at 0.5, 1, 2, 4, and 6 hours after application. Recovery was estimated as a percent of applied dose by measure the amount of radioactivity found in shampoo washings and rinses and in disposable items used during the procedure (towels, tissues, gloves, spatulae) plus the amount excreted in urine and faeces.

Results

For formulation I (containing coupler plus water) the total elimination or radioactivity in urine and faeces ranged from 2.84 % to 6.06 % of the applied dose (mean: 4.81 ± 0.624 S.E.M.). For formulation II (containing coupler plus hydrogen peroxide) it ranged from 0.97 % to 1.79 % of the applied dose (mean: 1.31 ± 0.136). The difference between the formulations was statistically significant. Based on these data a mean absorption of 2,5-diaminotoluene of approximately 17.8 + 2.3 mg and 4.9 ± 0.5 mg was obtained for formulations 1 and II, respectively. The mean amount of 2,5-diaminotoluene absorbed per cm^2 of scalp was estimated to be approximately 71 $\mu\text{g}_{\text{eq}}/\text{cm}^2$ (formulation I, without peroxide) and 20 $\mu\text{g}_{\text{eq}}/\text{cm}^2$ (formulation II, with peroxide).

Levels of 2,5-diaminotoluene equivalents in whole blood were greatest at the earlier time points in subjects administered formulation I and declined with a half life ranging from 1.2 to 2.7 hours, whereas levels in subjects administered formulation II were much lower than for formulation I and were non-detectable in one subject. Whole blood $\text{AUC}_{(0-\infty)}$ calculated for subjects

administered formulations I and II was 41.61 ± 1.74 ng_{eq}hr/ml and 9.18 ± 3.10 ng_{eq}hr/ml, respectively.

The total recovery of radioactivity was 91.2 ± 1.73 % for formulation I and 47.64 ± 3.43 % for formulation II. It is reasonable to assume that the lower recovery with formulation II was due to a higher retention of radiolabel in the hair as a result of the oxidative hair colouring process. Samples of hair were not obtained and analysed to confirm this.

(Reference: 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)

Relevant published literature

In a publication by Schettgen et al. (2010), urinary excretion of 2,5-diaminotoluene was measured in two female subjects after use of either a brown-reddish colour or a brown-black colour commercial hair dye containing 2,5-diaminotoluene. The amount of 2,5-diaminotoluene excreted in the urine within 48 hours was 700 µg in the subject using the brown-reddish colour and 1500 µg in the subject using the brown-black colour. Adjusting for expression of the results as 2,5-diaminotoluene sulfate rather than as the free base, these urinary excretion values are 1260 and 2700 µg, respectively. Although the concentration of 2,5-diaminotoluene in the hair dyes used by these two subjects was not available, the brown-black colour likely contained a higher concentration of this ingredient than the brown-reddish colour.

(Reference: Schettgen T, Heinrich K, Kraus T, and Gube M. Determination of 2,5-touylenediamine (2,5-TDA) and aromatic amines in urine after personal application of hair dyes: kinetics and doses. Arch. Toxicol. 2011; 85:127-33)

Summary toxicokinetics in humans:

The most recent study indicates that the human systemic exposure to 2,5-diaminotoluene Sulfate following a typical hair dyeing process is in the range of $0.86 \pm 0.27\%$ and $0.87 \pm 0.45\%$ of the applied radioactivity for the concentrations of 1.5% and 4%, respectively with nearly all of this found in urine (0.83% of the applied radioactivity for both concentrations). Plasma kinetic results indicated a mean systemic exposure [plasma area under the curve (AUC_(0-∞))] of 1241 ± 402 ng eq·hr/mL and 3553 ± 1519 ng eq·hr/mL for the low and high concentration formulations, respectively. Mean T_{max} and T_{1/2} values were similar at both concentrations. This is in line with data from previous human studies.

Rat studies

Absorption, distribution, metabolism and excretion have been investigated in the female Wistar Kyoto rat, a strain with a slow acetylator phenotype. For comparison, toxicokinetics after oral and dermal administration have also been evaluated in Sprague-Dawley rats.

Absorption, distribution, metabolism and excretion of ¹⁴C-2,5-diaminotoluene Sulfate in the female Wistar Kyoto rat after a single oral or dermal dose

Study design

Guideline: OECD Guideline no. 417 (1984)

	OECD no. 427 (draft document, 2000)
Species/strain:	Rat / Wistar Kyoto, WKY/NR CrI BR (inbred)
Group size:	4 Females in the mass balance groups (groups 1, 2, 3) 6 Females in the toxicokinetics groups (groups 4, 5, 6)
Test substance:	<i>2,5-diaminotoluene Sulfate</i>
Vehicle:	Oral administration: milli-Q (adjusted to pH 7 with 2 M NaOH) Dermal administration: milli-Q:acetone 1:1 w/w (adjusted to pH 7 with 2M NaOH)
Batch No.:	3362-259 [ring- ¹⁴ C(U)]- <i>2,5-diaminotoluene Sulfate</i> CFQ13783, batch 1 [ring- ¹⁴ C(U)]- <i>2,5-diaminotoluene Sulfate</i> , substance B, applied to one animal in group 1) 2346 (non-radiolabelled <i>2,5-diaminotoluene Sulfate</i>)
Purity:	Radiochemical purity: 99.3 % by HPLC - [ring- ¹⁴ C(U)]- <i>2,5-diaminotoluene Sulfate</i> , Batch 3362-259 Radiochemical purity: 98.2 % by HPLC - [ring- ¹⁴ C(U)]- <i>2,5-diaminotoluene Sulfate</i> , Batch CFQ13783, batch 1 Non-labelled <i>2,5-diaminotoluene Sulfate</i> (Batch 2346): 99.2 area % (254 nm) and 99.8 area % (305 nm) by HPLC; 98.3 weight % by NMR
Dose levels:	Oral administration: 2.5, 25 mg/kg bw/day (containing approximately 1.5 Mbq of radioactivity per dose) Dermal administration: 50 mg/ml containing approximately 0.5 Mbq of radioactivity (equal to 33.3 mg/kg bw/day and 0.5 mg/cm ² skin)
Dose volume:	Oral administration: 5 ml/kg Dermal administration: 0.1 ml/animal
Route:	Oral (gavage), dermal
Dosing schedule:	Oral: Single administration Dermal: Single application for 30 min
GLP:	In compliance

A single dose of ¹⁴C- *2,5-diaminotoluene Sulfate* was administered orally by gavage at 2.5 mg/kg bw/day and 25 mg/kg bw/day to fasted rats or by dermal application at a dose of 0.5 mg/cm² (equal to 33.3 mg/kg bw/day) on the back of the animals. For animals dosed by dermal application, ingestion of test substance via grooming was prevented by collaring the animals or by wrapping the application site with an adhesive bandage.

Animals in the mass balance groups were housed in metabolism cages in order to obtain a total ¹⁴C-radioactivity material balance. Urine and faeces were collected over the following time intervals after dosing: 0-8 h, 8-24 h, 24-48 h, 48-72 h, 72-96 h. Animals in the oral administration groups were euthanized 96 h after dose administration, and several tissues and organs were collected. In the dermally dosed group, additional urine and faeces samples were collected at a 24 hour interval and animals were sacrificed at 120 hours. Total radioactivity in urine, faeces, tissues, and organs was determined. For metabolism evaluation, urine and faeces samples were pooled per group, and the metabolite profile of the pooled samples was obtained by radio-HPLC and LC-MS/MS.

In the toxicokinetics groups, blood was sampled alternatively from several animals per time point at 0.25, 0.5, 1, 2, 4, 8, 24, and 72 h after dosing. Total radioactivity and *2,5-diaminotoluene Sulfate* equivalent concentrations were determined.

Results

2,5-diaminotoluene Sulfate was readily absorbed and rapidly excreted after oral administration in Wistar Kyoto rats. Mean cumulative recovery of radioactivity in the urine after 96 h was 62.2 ± 3.6 % (low dose) and 72.9 ± 3.5 % (high dose) of applied dose. Mean cumulative recovery of radioactivity in faeces was 31.4 ± 5.8 % (low dose) and 22.0 ± 3.5 % (high dose) of applied dose. Mean residual radioactivity in the carcass, tissues, and blood was 0.89 % (low dose) and 1.35 % (high dose) of the applied dose. Less than 5 % of the total radioactivity was recovered in the cage wash. The mean mass balance was 97.8 ± 2.5 % (low dose) and 98.6 ± 4.2 % (high dose). The presence of radioactivity in the faeces most likely represents biliary excretion rather than unabsorbed radiolabeled test substance since a similar finding was observed after intravenous administration.

After dermal application, the mean cumulative recovery of radioactivity was 10.9 ± 7.9 % of the applied dose for the urine and 2.7 ± 1.9 % of the applied dose for the faeces. Mean residual radioactivity in the carcass, tissues, and blood was 5.5 ± 2.1 %, and the majority of this was recovered from treated skin. Less than 1 % of the total radioactivity was recovered in the cage wash. The mean mass balance was 99.8 ± 2.3 %.

After oral dosing, three major metabolites were observed in the urine. The largest peak was identified as N,N-diacetyl-2,5-diaminotoluene. The second and third most abundant metabolites have unknown structure but are likely N-acetylated. No parent compound was observed in the urine. Radioactivity in urine after dermal application was too low for separation and detection of metabolites. However, LC-MS/MS analysis suggested a quantitatively similar profile of metabolites as that seen after oral administration.

Plasma kinetics results indicate that absorption after oral administration is rapid, with T_{max} values of 0.5h (low dose) and 0.25 h (high dose). C_{max} values were 1.56 mg/L (low dose) and 19.99 mg/L (high dose). $AUC_{0 \rightarrow \infty}$ values were 17.59 and 174 $mg_{eq} hr/L$ for the low and high dose groups, respectively, demonstrating good dose proportionality. After dermal administration the time to peak plasma *2,5-diaminotoluene* equivalents was delayed ($T_{max} = 2$ h) and the total systemic exposure was lower ($AUC_{0 \rightarrow \infty} = 4.39$ $mg_{eq} h/L$, excluding one outlying animal from the calculation). Radio-HPLC of plasma from the oral dose group animals indicated the presence of several metabolites (not identified/characterized). Plasma concentrations of parent compound were very low or non-detectable.

The data from this study were used in combination with the results from the intravenous dosing study in Wistar Kyoto rats described below to calculate bioavailability (F_{abs}) after oral and dermal administration. The bioavailability of *2,5-diaminotoluene Sulfate* after oral administration calculated from the toxicokinetics groups was high (95 % for the low dose oral group and 91 % for the high dose oral group), whereas the bioavailability following dermal application was low (2 %) when calculated from dose-normalized $AUC_{0 \rightarrow \infty}$ values.

(Reference: Wenker, M. A. M.; *Absorption, distribution, metabolism and excretion of ^{14}C -2,5-diaminotoluene sulphate in the female Wistar Kyoto rat after a single oral or dermal dose; NOTOX; 2005*)

Absorption, distribution, metabolism and excretion of ^{14}C -2,5-diaminotoluene Sulfate in the female Wistar Kyoto rat after a single intravenous dose.

Study design

Guideline: OECD Guideline no.: 417 (1984)

Species/strain:	Rat/Wistar Kyoto, WKY/NR CrI BR (inbred)
Group size:	6 Females
Test substance:	A solution of <i>2,5-diaminotoluene Sulfate</i>
Vehicle:	Milli-Q (adjusted to pH 7 with 2 M NaOH)
Batch No.:	CFQ13783, batch 1 [ring- ¹⁴ C(U)]- <i>2,5-diaminotoluene Sulfate</i> 2346 (non-radiolabelled <i>2,5-diaminotoluene Sulfate</i>)
Purity:	Radiochemical purity 98.2 % by HPLC (radiolabelled <i>2,5-diaminotoluene Sulfate</i> , batch CFQ13783, batch 1) 99.2 area % (254 nm) and 99.8 area % (305 nm) by HPLC (non-radiolabelled <i>2,5-diaminotoluene Sulfate</i> , batch 2346); 98.3 weight % by NMR
Dose levels:	2.5 mg/kg bw (containing approximately 1.5 Mbq of radioactivity)
Dose volume:	2 ml/kg
Route:	Intravenous
Dosing schedule:	Single application
GLP:	In compliance

A single dose of ¹⁴C-*2,5-diaminotoluene Sulfate* (2.5 mg/kg bw) was administered by intravenous injection. Animals in the mass balance group were housed in metabolism cages in order to obtain a total ¹⁴C-radioactivity material balance. Urine and faeces were collected over the following time intervals after dosing: 0-8 h, 8-24 h, 24-48 h, 48-72 h, 72-96 h. Animals were euthanized 96 h after dose administration, and several tissues and organs were collected. Total radioactivity in urine, faeces, tissues, and organs was determined. For metabolism evaluation, urine and faeces samples were pooled per group, and the metabolite profile of the pooled samples was obtained by radio-HPLC and LC-MS/MS.

In the toxicokinetics groups, blood was sampled alternatively from several animals per time point at 5, 15, and 30 min and at 1, 2, 4, 8, 24, 48, and 72 h after dosing. Total radioactivity and *2,5-diaminotoluene Sulfate* equivalent concentrations were determined.

Results

The mean percent recovery of radioactivity in urine and faeces, when corrected assuming 100 % recovery of radiolabel in the i.v. study, were comparable to those observed after oral administration in the study described above. The recovery of radioactivity in the faeces suggests that the test material is probably excreted in the bile after intravenous administration. The same three major urinary metabolites observed after oral dosing were detected after i.v. dosing. The plasma kinetics data from this study were used in combination with the study described above to calculate the bioavailability (F_{abs}) after oral and dermal application. Radio-HPLC of plasma from the oral dose group animals indicated the presence of several metabolites (not identified/characterized). Plasma concentrations of parent compound were detectable but quite low.

(Reference: Wenker, M. A. M.; *Absorption, distribution, metabolism and excretion of ¹⁴C-2,5-diaminotoluene sulphate in the female Wistar Kyoto rat after a single oral or dermal dose*; NOTOX; 2005;
Wenker, M. A. M.; *Absorption, distribution, metabolism and excretion of ¹⁴C-2,5-diaminotoluene sulphate in the female Wistar Kyoto rat after a single intravenous dose*; NOTOX; 2005)

Plasmakinetics of ^{14}C -2,5-diaminotoluene Sulfate in the female Sprague-Dawley rat after a single oral or dermal dose

Study design

Guideline:	OECD Guideline no. 417 (1984) OECD Guideline no. 427 (draft document, 2000)
Species/strain:	Rat / Sprague-Dawley, CrI: CD (outbred)
Group size:	6 Females
Test substance:	Oral administration (groups 1 and 2): 2,5-diaminotoluene Sulfate in milli-Q (adjusted to pH 7 with 2M NaOH) Dermal administration (group 3): 2,5-diaminotoluene Sulfate in milli-Q:acetone 1:1 w/w (adjusted to pH 7 with 2M NaOH) Dermal administration (group 4): 2,5-diaminotoluene Sulfate in vehicle 81905108B (composed of water, cetearyl alcohol, sodium laureth sulfate, ammonia, lanoline alcohol, sodium sulfite, ascorbic acid, disodium EDTA)
Batch No.:	CFQ13783, batch 1 [ring- ^{14}C (U)]-2,5-diaminotoluene Sulfate 2346 (non-radiolabelled 2,5-diaminotoluene Sulfate)
Purity:	Radiochemical purity: 98.2 % by HPLC [ring- ^{14}C (U)]-2,5-diaminotoluene Sulfate) Non-radiolabelled 2,5-diaminotoluene Sulfate): 99.2 area % (254 nm) and 99.8 area % (305 nm) by HPLC; 98.3 weight % by NMR
Dose levels:	Oral administration: 2.5, 25 mg/kg bw (containing approximately 3 Mbq of radioactivity per dose) Dermal administration: 50 mg/ml containing approximately 0.5 Mbq of radioactivity (equal to 33.3 mg/kg bw respectively 0.5 mg/cm ² skin)
Route:	Oral (gavage), dermal
Dosing schedule:	Oral: Single administration Dermal: Single administration for 30 min
GLP:	In compliance

A single dose of ^{14}C -2,5-diaminotoluene Sulfate was administered orally by gavage at 2.5 mg/kg bw and 25 mg/kg bw to fasted animals or by dermal application at a dose of 0.5 mg/cm² (equal to 33.3 mg/kg bw) on the back of the animals. For animals dosed by dermal application, ingestion of test substance via grooming was prevented by wrapping the application site with an adhesive bandage. Blood was sampled alternatively from several animals per time point at 0.25, 0.5, 1, 2, 4, 8, 24, and 72 h after dosing. Total radioactivity and 2,5-diaminotoluene Sulfate equivalent concentrations were determined.

Results

Oral absorption of 2,5-diaminotoluene Sulfate was rapid with T_{max} values of 1 h in both the low and high dose groups. C_{max} values were 1.28 mg/L and 18.03 mg/L and $\text{AUC}_{0 \rightarrow \infty}$ values were 8.53 mg_{eq}h/L and 112 mg_{eq}h/L at the low and high dose, respectively, demonstrating reasonable dose proportionality.

After dermal administration the time to peak plasma 2,5-diaminotoluene equivalents was delayed ($T_{\text{max}} = 2$ h) and the total systemic exposure was lower. The $\text{AUC}_{0 \rightarrow \infty}$ was 5.00 mg_{eq}h/L in the group dosed using the milli-Q:acetone vehicle and 2.27 mg_{eq}h/L in the group dosed using the formulation vehicle (excluding one outlying animal from the calculation). When normalised for applied dose to correct for somewhat higher applied dose with the milli-Q:acetone vehicle, the

corresponding values were 0.18 and 0.12 mg_{eq}h*kg bw/mg*L, indicating a modest reduction in systemic exposure with the milli-Q:acetone vehicle vs. the formulation vehicle, although the low plasma concentrations after dermal dosing make it difficult to assess quantitative differences between groups. Radio-HPLC of plasma indicated the presence of several metabolites (not identified/characterised). Concentrations of parent compound were very low or non-detectable.

The data from this study were used in combination with the results from the intravenous dosing study in Sprague-Dawley rats described below to calculate bioavailability (F_{abs}) after oral and dermal administration. The bioavailability of 2,5-diaminotoluene Sulfate after oral administration was reasonably high (53 % for the low dose oral group and 69 % for the high dose oral group), whereas the bioavailability following dermal application was low (2-3 % for both vehicles) when calculated from dose-normalised AUC_{0→∞} values.

(Reference: Wenker, M. A. M.; *Pharmacokinetics of 14C-2,5-diaminotoluene sulphate in the female Sprague-Dawley rat after a single oral or dermal dose; NOTOX; 2005*)

Plasmakinetics of ¹⁴C-2,5-diaminotoluene Sulfate in the female Sprague-Dawley rat after a single intravenous dose

Study design

Guideline:	OECD Guideline no.: 417 (1984)
Species/strain:	Rat/Sprague-Dawley, Crl: CD (outbred)
Group size:	6 Females
Test substance:	2,5-diaminotoluene Sulfate
Vehicle:	Milli-Q (adjusted to pH 7 with 2 M NaOH)
Batch No.:	CFQ13783, batch 1 - [ring- ¹⁴ C(U)]-2,5-diaminotoluene Sulfate/ 2346 - (non-radiolabelled 2,5-diaminotoluene Sulfate)
Purity:	Radiochemical purity: 98.2 % by HPLC [ring- ¹⁴ C(U)]-2,5-diaminotoluene Sulfate) Non-radiolabelled 2,5-diaminotoluene Sulfate): 99.2 area % (254 nm) and 99.8 area % (305 nm) by HPLC; 98.3 weight % by NMR
Dose levels:	2.5 mg/kg bw containing approximately 1.5 Mbq of radioactivity;
Route:	Intravenous
Dosing schedule:	Single application
GLP:	In compliance

A single dose of 14C-2,5-diaminotoluene Sulfate (2.5 mg/kg bw) was administered by intravenous injection. Blood was sampled alternatively from several animals per time point at 5, 15, and 30 min and at 1, 2, 4, 8, 24, 48, and 72 h after dosing. Total radioactivity and 2,5-diaminotoluene Sulfate equivalent concentrations were determined. Radio-HPLC was conducted with plasma samples.

Results

The plasma kinetics data from this study were used in combination with the study described above to calculate the bioavailability (F_{abs}) after oral and dermal application. Radio-HPLC of plasma indicated the presence of several metabolites (not identified/characterised) plus low concentrations of parent compound detectable through the 8 h time point.

(Reference: Wenker, M. A. M.; *Pharmacokinetics of 14C-2,5-diaminotoluene sulphate in the female Sprague-Dawley rat after a single intravenous dose; NOTOX; 2005*;

Wenker, M. A. M.; Pharmacokinetics of ¹⁴C-2,5-diaminotoluene sulphate in the female Sprague-Dawley rat after a single oral or dermal dose; NOTOX; 2005)

Summary toxicokinetics in rats

Absorption, distribution, metabolism and excretion were investigated in the female Wistar Kyoto rat, a strain with a slow acetylator phenotype and in Sprague-Dawley rats. The results indicate extensive metabolism in both strains after both oral and dermal administration. In both strains, bioavailability (F_{abs}) after oral administration was reasonably high to high (53-95 %) whereas bioavailability after dermal application was relatively low (2-3 %). Efforts to characterise the urinary metabolites in Wistar Kyoto rats indicate the presence of *N*-acetylated metabolites, including *N,N*-diacetyl-2,5-diaminotoluene plus two other metabolites that were not identified but are likely to be *N*-acetylated.

Appendix II

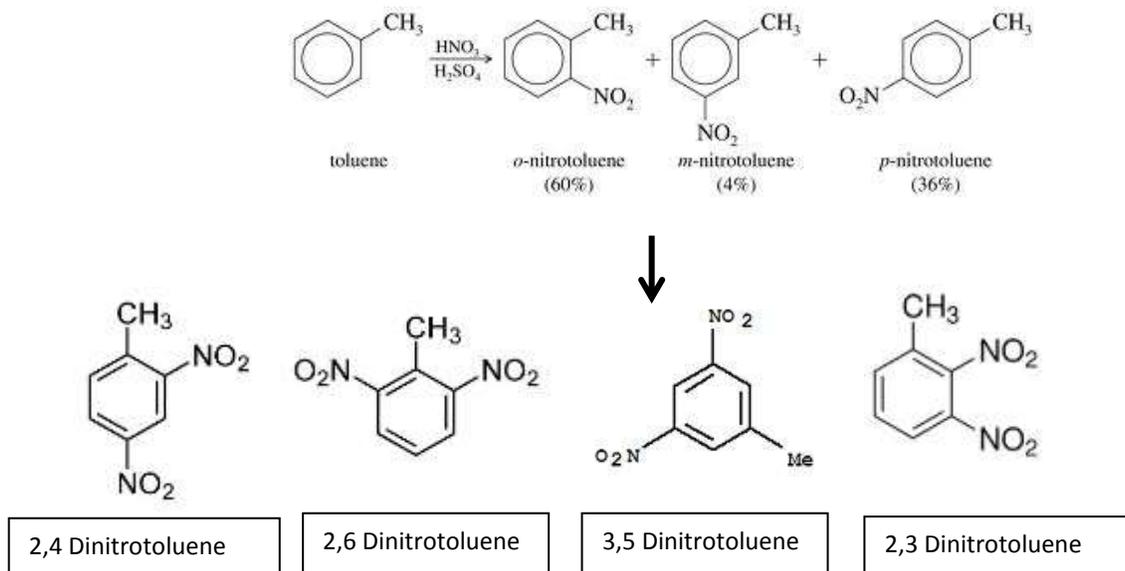
Relevant Information - Diaminotoluenes

Question: Why is 2,5 Diaminotoluene not included in the Diaminotoluene (mixed) definition?

Answer: The answer lies in the synthetic pathway. Industrially, amino substitutions on the aromatic ring are achieved by reacting toluene (methylbenzene) with nitric acid to form nitrotoluene. Then this nitro-substituted toluene is reduced to form the amino toluene.¹ This seems to be the favored industrial pathway to synthesize diaminotoluenes as well by adding two nitro groups to the ring before reduction.

Nitrosation of toluene is governed by molecular stabilization or resonance energies that control reaction mechanisms of electrophilic aromatic substitution. This gives certain substitution patterns over others. The methyl group on benzene is considered “activating” and *ortho* and *para* directing when undergoing further reaction (please see figure below).² In this type of reaction the product will **always** be a **mixture** of products because of resonance.³ Some small amount of *meta* substituted product is seen because the methyl group **activates** the ring to electrophilic reaction since it **donates** electrons to the π electron cloud of the aromatic ring even though it is not favored.⁴

Toluene will react with nitric acid in sulfuric acid (HNO₃ in H₂SO₄) to give substitution on the ring, the reaction proceeds as follows:



¹ http://books.google.com/books?id=AO5HyPI_X0wC&pg=PA378&lpg=PA378&dq=nitration+of+m-nitrotoluene&source=bl&ots=uKUchmCDGI&sig=46xeovfw1ds49nabyXenDL4k3fQ&hl=en&sa=X&ei=d6VbU4K4EM2nsAS61IHgBA&ved=0CIABEOgBMA4#v=onepage&q=nitration%20of%20m-nitrotoluene&f=false

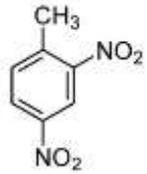
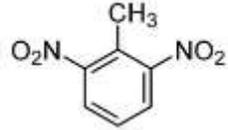
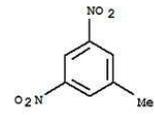
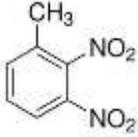
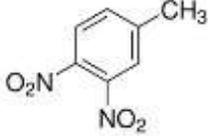
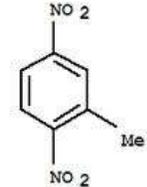
² Carey, F.S., Sundberg, R.J. Advanced Organic Chemistry Part A: Structure And Mechanism, Plenum Press, 1977 p 394-5; <http://science.uvu.edu/ochem/index.php/alphabetical/o-p/ortho-para-directing-group/>; alternatively any fundamental Organic Chemistry text on Electrophilic Aromatic Substitution will provide the same information.

³ Any fundamental Organic Chemistry text; <http://science.uvu.edu/ochem/index.php/alphabetical/q-r/resonance-theory/>

⁴ Again any fundamental Organic Chemistry text will provide the same information.; <http://www.mhhe.com/physsci/chemistry/carey/student/olc/graphics/carey04oc/ref/ch12substituenteffects.html>

Further nitration will then give another mixture of products since the nitro group is a strong *meta* directing *deactivating* group and counteracts to some extent the *ortho, para* direction from the methyl group. The most favored reaction pathways are those that satisfy both drivers. The reaction products are a mixture of 2,4-, 2,6- and 2,3 and 3,5-(minor from *meta* nitrotoluene). Please refer to the Table 1.

Table 1: Reaction Products of Nitration of Nitrotoluene

Structure	Name	Comment
	2,4 Dinitrotoluene	Result of: 1. 2 nd nitration that is <i>para</i> to methyl and <i>meta</i> to 2-nitrotoluene 2. 2 nd nitration that is <i>ortho</i> to methyl and <i>meta</i> to 4 nitrotoluene Most likely product (76%)
	2,6 Dinitrotoluene	Result of nitration that is <i>ortho</i> to methyl and <i>meta</i> to 2- nitrotoluene Second most likely (19%) but not favored due to steric hinderence (bulky groups adjacent to one another)
	3,5 Dinitrotoluene	Result of 2 nd nitration that is <i>meta</i> to methyl and <i>meta</i> to m-nitrotoluene (m-nitrotoluene is least likely product). This is not the most likely synthetic pathway of the 3,5 DNT (more likely to alkylate DNT)
	2,3 Dinitrotoluene	Result of: 1. 2 nd nitration <i>ortho</i> to methyl and <i>ortho</i> to nitro group of 3-nitro (the same <i>ortho</i>) 2. 2 nd nitration that is <i>ortho</i> to nitro group of 2-nitrotoluene (unlikely) and <i>meta</i> to methyl group Extremely unlikely
	3,4 Dinitrotoluene	Result of: 1. 2 nd nitration <i>ortho</i> to nitro and <i>para</i> to methyl group of 3-nitrotoluene 2. 2 nd nitration <i>ortho</i> to nitro and <i>meta</i> to methyl group of of 4-nitrotoluene Extremely unlikely
	2,5 Ditnitrotoluene	Result of 1. 2 nd nitration that is <i>para</i> to nitro and <i>meta</i> to methyl group of 2-nitrotoluene 2. 2 nd nitration that is <i>para</i> to nitro and <i>meta</i> to methyl group of 3-nitrotoluene Extremely unlikely

Because of the mechanism of electrophilic aromatic substitution or the phenomenon of resonance the possibility of producing 2,5 dinitrotoluene is negligible because the second nitro group will have to attack

the ring in a way that is energetically very unfavorable from a resonance perspective. Nitro groups deactivate the ring and direct subsequent substitutions to the *meta* position never *ortho*, *para*. Similarly, the methyl group activates the ring and directs further reaction on the ring to the *ortho* or *para* not *meta*.

Once the dinitro products are obtained the synthesis of the amino version is a relatively simple reduction using a palladium catalyst or Raney Nickel under a hydrogen atmosphere.

Because of the extreme unlikelihood of this occurrence of this product, synthesis of 2,5 diamino toluene is not so straightforward as substitution on a toluene. It was first accomplished in 1877 by R. Nietzki.⁵ Its preparation was accomplished by cleavage of o-amido-azotoluene by means of tin/hydrochloric acid to o-toluidine and 2,5-diaminotoluene. The product is then purified by extraction, distillation and then recrystallization. The current industrial process is probably along similar lines.⁶

OEHHA does not note a CAS number for its listing of diaminotoluene (mixed) but the Code of Federal Regulations, 40 CFR §372.65, does cite a CAS number 25376-45-8.⁷ This CAS number brings back a list of synonyms none of which are 2,5 Diaminotoluene⁸ and refers to the 2,4 and 2,6 isomers.⁹

Summary: The synthetic pathways are what separate the diaminotoluenes. Starting from toluene gives one discrete set of products that is a mixture primarily of 2,4 and 2,6 diaminotoluene. The synthesis of 2,5 diaminotoluene must commence from different starting materials because of the impossibility of isolating any amount of this product from a pathway that starts at toluene. The mixed diaminotoluenes are made by nitrosation of toluene as cited in reference 1.

⁵ R. Nietzki, Chemische Berichte 10, 662 (1877)

⁶ <http://www.google.com/patents/US20130123540>

⁷ <http://www.gpo.gov/fdsys/pkg/CFR-2011-title40-vol28/pdf/CFR-2011-title40-vol28-sec372-65.pdf>

⁸ <http://environmentalchemistry.com/yogi/chemicals/cn/Toluenediamine.html>

⁹ http://www.chemicalbook.com/ChemicalProductProperty_EN_CB1875166.htm ;

http://www2.basf.us/corporate/ehs/pss_pdfs/TolueneDiamineProductSafetySummary.pdf