Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

a) 2-(4-*tert*-butylbenzyl)propionaldehyde

and

b) 4-*tert*-butylbenzoic acid

and

c) 3-(4-*tert*-butylphenyl)propionaldehyde [1];
4-*tert*-butyltoluene [2];
4-*tert*-butylbenzaldehyde [3];
methyl 4-*tert*-butylbenzoate [4]

EC Numbers:

a. 201-289-8
b. 202-696-3
c. 242-016-2 [1]; 202-675-9 [2]; 213-367-9 [3]; 247-768-5 [4]

CAS Numbers:

a) 80-54-6
b) 98-73-7
c) 18127-01-0 [1]; 98-51-1 [2]; 939-97-9 [3]; 26537-19-9 [4]

Index Numbers:

a) 605-041-00-3
b) 607-698-00-1
c) TBD

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CONTENTS

1 PHYSICAL HAZARDS

Not evaluated in this dossier.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Study summaries are adapted from the publically available registration dossiers found at ECHA's dissemination site, or when scientific publications are used as references, from publically available abstracts.

2.1 Study 1: 3-(4-*tert*-butylphenyl)propionaldehyde

Toxicity Study by Oral Gavage Administration to Sexually Mature Male CD Rats for 5 Days.

Author: Givaudan

Year: 2009

At the end of a 5 Day study on 3-(4-tert-butylphenyl)propionaldehyde dosed at 25, 100 and 250 mg/kg bw, animals were placed overnight in an individual metabolism cage without food. Urine samples were collected after administration of the fifth dose for a maximum of 22 hours. Samples were subsequently analysed for 4-tert-butylbenzoic acid (TBBA), 4-iso-butylbenzoic acid and 4-iso-propylbenzoic acid. Bioanalysis was subject to the satisfactory validation of the bioanalytical method, which was performed as part of this study prior to analysis of the study samples. Treatment with 3-(4-tert-butylphenyl)propionaldehyde at these concentrations was associated with systemic toxicity and testicular/epididymal toxicity, and the urine contained TBBA, a known metabolite biomarker of testicular toxicity in rats.

The expected un-conjugated metabolite, tert-butyl benzoic acid (TBBA) was detected in the urine of rodents treated for 5 days with 3-(4-tert-butylphenyl)propionaldehyde in a dose dependent manner. Analysis of urine of male rats treated for 5 days at 25 and 100 mg/kg/day showed a mean of 35.75 and 274.8 ug/mL of TBBA detected.

2.2 Study 2: 4-*tert*-butyltoluene

Basic toxicokinetics in vivo

Title:The distribution of 14Cp-tert-butyltoluene (TBT) following inhalation by the rat: A whole body autoradiographic study.

Author: Ingebrigtsen K and Walde A

Year:1982

Bibliographic source:Acta pharmacol et toxicol 51: 203-208.

Reference 2

Title:Metabolism of p-tert.-Butyltoluene in the Rat and Guinea Pig.

Author:Walde A and Scheline RR

Year:1983

Bibliographic source:Acta Pharmacol Toxicol 53: 57-63.

Objective of study:excretion

GLP compliance:not specified

Test material

Reference substance name:4-tert-butyltoluene

EC Number:202-675-9

EC Name:4-tert-butyltoluene

Cas Number:98-51-1

Molecular formula:C11H16

IUPAC Name:1-tert-butyl-4-methylbenzene

Details on test material:

(methyl-14C)p-tert-butyltoluene; according to the authors, radiochemical purity was 98-99%; no further data

Radiolabelling:yes

Remarks:14C

Test animals

Species:rat, guinea pig

Strain:Wistar (rat), Dunin Hartley (guinea pig)

Sex:male

Administration / exposure

Route of administration: intragastric, inhalation

Vehicle:not specified

Doses / Concentrations:100 mg/kg bw

Results and discussion

The elimination of the test substance after intragastric and inhalational administration (doses of 100 mg/kg) was studied in male Wistar rats and in male Dunkin Hartley guinea pigs.

The test substance was well absorbed through the gastro-intestinal and respiratory tract, was quickly distributed, and was eliminated within a few days.

In rats, 73% of a single oral dose (100 mg/kg) was recovered in the urine and feces within 3 days. After 10 days, 83% of the administered dose was recovered; the ratio of urinary/fecal radioactivity was ca. 3.5 : 1. Elimination was biphasic with the slower elimination phase beginning on day 6 after dosing.

At day 3 after dosing, urinary excretion of radioactivity was 45% and 25% after oral and inhalational exposure, respectively, in rats and 42% and 41% after oral and inhalational administration, respectively, in guinea pigs**.**

2.3 Study 3: 4-*tert*-butyltoluene

Basic toxicokinetics in vivo

Title:Unnamed

Year:1982

Objective of study:metabolism

Principles of method if other than guideline:

Groups of rats are gavaged daily with graduated doses of the test article for 5 consecutive days. For 24 hours, urine is collected after the last application of the compound. Urinary metabolites are detected by appropriate method (GC)

GLP compliance:not specified

Test material

Reference substance name:4-tert-butyltoluene

EC Number:202-675-9

EC Name:4-tert-butyltoluene

Cas Number:98-51-1

Molecular formula:C11H16

IUPAC Name:1-tert-butyl-4-methylbenzene

Details on test material:p-tert-butyltoluene; no data on purity of the compound

Test animals

Species:rat

Strain:SPF albino

Sex:male/female

Administration / exposure

Route of administration:oral: gavage

Vehicle: rape oil

Doses / Concentrations:25, 100 mg/kg bw

No. of animals per sex per dose / concentration:

- Control: 4 male animals

- Dosed groups: 8 males/dose

Results and discussion

Metabolite characterisation studies

Metabolites identified:yes

Details on metabolites:

p-Tert-butylbenzoic acid (TBBA) was identified as metabolite in the 24 hours urine in rats treated with p-tert-butyltoluene (TBT), but not the secondary metabolite p-tert-butylhippuric acid.

Dose level TBT (mg/kg)	Weight of rats (g)	TBBA in last 24-h urine	
		(mg/l)	(mg/kg)
0	950	n.d.	n.d.
25	1826	56	2.7
100	1451	455	17.2

n.d. = not detectable

p-Tert-butylbenzoic acid (TBBA) was detected in a dose-related way in the urine samples of all treated rats. However, the corresponding p-tert-butyl hippuric acid (TBHA; i.e. the glycine conjugate of TBBA) could not be detected. GC/MS revealed additional peaks which were tentatively assigned to the trimethylsilyl derivatives of p-tert-hydroxybutylbenzoic acid and p-tert-carboxybutylbenzoic acid.

According to the authors, these results suggested that the test substance was metabolized by rats to a considerable degree to TBBA, which was eliminated in the urine (probably as glucuronide). Minor amounts of TBBA were further oxidized at the tertiary butyl group and then excreted in the urine. The oxidation reaction was likely to be a result of microsomal enzymes in the liver.The following metabolic pathway was proposed:tert-butyl toluene (1) is oxidized to p-tert-butyl benzoic acid (2) and, in parts, further oxidized to p-tert-hydroxybutyl benzoic acid (3).

Compounds (2) and (3) are excreted in the urine, probably as glucuronides.

Urinary glucuronides are known to be easily hydrolyzed by acidification. Since the analytical procedure, used in this study, included an acidification, glucuronides could not be detected. Under the experimental conditions chosen it was shown that the test material is metabolized by rats to a considerable degree to TBBA, which is eliminated in urine (probably as glucuronide).

2.4 Study 4: 4-*tert*-butyltoluene

Basic toxicokinetics in vivo

Title: Toxicology and Biochemistry of the Monocyclic Aromatic Hydrocarbons.

Author: Gerarde HW

Year: 1960

Bibliographic source: Med Bull NJ 20 (1): 74-90. Cited in: ACGIH (1991). p-tert-Butyltoluene. CAS No. 98-51-1. Documentation of the Threshold Limit Value, 186-189.

Objective of study:metabolism

GLP compliance:no

Test material

Reference substance name:4-tert-butyltoluene

EC Number:202-675-9

EC Name:4-tert-butyltoluene

Cas Number:98-51-1

Molecular formula:C11H16

IUPAC Name:1-tert-butyl-4-methylbenzene

Details on test material:p-tert-butyltoluene; no data on purity of the compound

Test animals

Species:rat

Administration / exposure

Route of administration:unspecified

Results and discussion

A metabolic pathway of the test substance was postulated. No change in the urinary sulfate ratio (inorganic/total) was observed in rats after dosing with the test substance. This result was taken as evidence that in situ oxidation of the aromatic ring system was not a pathway for the metabolism of the test substance. Thus, it was concluded that the p-methyl group or one of the methyl groups of the tertiary butyl moiety was oxidized in the liver to hydroxy- and carboxyl derivatives. These compounds were presumed to be eliminated as glucuronide or glycine conjugates.

Secondary literature; no further data.

2.5 Study 5: 4-*tert*-butyltoluene

Basic toxicokinetics in vivo

Title:Uptake, Distribution and Elimination of p-tert-Butyltoluene (TBT) in mice by inhalation.

Author:Rasmussen A et al.

Year:1980

Bibliographic source:Acta pharmacol. et toxicol. 47, 236.

Objective of study:distribution

GLP compliance:not specified

Test material

Details on test material:: p-tert-Butyltoluene

Test animals

Species:mouse

Administration / exposure

Route of administration:inhalation

Duration and frequency of treatment / exposure:8 hour(s)

Doses / Concentrations:

Males: 1000 ppm

Results and discussion

Data uptake in mesenterial fat and brain, as well as elimination data from these organs, do not suggest a marked tendency towards an accumulation of tert-butyltoluene in fat or nervous tissue. This war confirmed by the fact that no traces of tert-butyltoluene could be demonstrated in mesenterial fat tissue 24 h after one single exposure for 4 h to 1000 ppm tert-butyltoluene. Barely detectable amounts of tert-butyltoluene were found after the last exposure in a series of five subsequent daily exposures for 4 h, each to 1000 ppm.

2.6 Study 6: 4-*tert*-butyltoluene

Basic toxicokinetics in vivo

Title:Unnamed

Year:1985

Objective of study:metabolism

On the occasion of various 5 day oral toxicity studies of tert-butyltoluene with mice, guinea pigs and dogs, urine was collected and analyzed for the different metabolites by GC analysis.

GLP compliance:not specified

Test material

Reference substance name:4-tert-butyltoluene

EC Number:202-675-9

EC Name:4-tert-butyltoluene

Cas Number:98-51-1

Molecular formula:C11H16

IUPAC Name:1-tert-butyl-4-methylbenzene

Details on test material:

- Name of test material (as cited in study report): Ro 94-0522 (TBT)

- Analytical purity: no data given

Radiolabelling:no

Test animals

Species:mouse

Strain:other: SPF albino

Sex:male

Administration / exposure

Route of administration:oral: gavage

Vehicle:rape oil

Duration and frequency of treatment / exposure:daily for 5 consecutive days

Doses / Concentrations:

100 mg/kg bw/d

No. of animals per sex per dose / concentration:6

Control animals:no

Details on study design:

The urine samples were acidified with 37 % hydrochloric acid to pH 1-2 and extracted with ethylacetate using pre-packed extraction columns according to the procedure recommended by the manufacturer (Extrelut, Merck No. 11737/8).

After evaporation of the extraction solvent the residue was dissolved in 5 ml ethylacetate/methanol (4:1).

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness, taken up in 200 µl DMF and methylated by heating with 200 µl DMF-DMA (Fluka) for 15 min at 90 °C.

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness and heated with 1 ml acetic anhydride for 5 min at 95 °C in order to transform hippuric acid and TBHA into the corresponding molecules.

For GC analysis 1 µl of the reaction mixture was injected without further treatment. For GC reference pure TBBA was methylated with DMF-DMA.

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES

- Tissues and body fluids sampled: urine

- Time and frequency of sampling: 24 h urine was collected after the last application of the test material

- From how many animals: no data given

- Method type for identification: GC

Results and discussion

Metabolites identified:yes

Details on metabolites:

TBBA and TBHA were identified by GC retention time (co-injection on two GC systems).

TBHA was found to be the main metabolite in urine samples of mice, whereas TBBA levels were below the detection limit.

Table 1: Identification of TBBA and TBHA as metabolites of TBT in mice.

Dose TBT (mg/kg)	100
TBBA (mg/kg bw)	< 0.58
TBBA (mg/l)	< 28.6
TBBA (mg/24h)	< 0.2
TBBA (%)*	< 0.48
TBHA (mg/kg bw)	39.94
TBHA (mg/l)	1957.1
TBHA (mg/24h)	13.7
TBHA (%)**	25.2
Recovery (%)***	25.2

*%TBBA = TBBA in urine (µM/kg)/applied dose (µM/kg) * 100

**%TBHA = TBHA in urine (µM/kg)/applied dose (µM/kg) * 100

***Recovery = %TBBA + %TBHA

2.7 Study 7: 4-*tert*-butyltoluene

Basic toxicokinetics in vivo

Title:Unnamed

Year:1985

Objective of study:metabolism

On the occasion of various 5 day oral toxicity studies of tert-butyltoluene with mice, guinea pigs and dogs, urine was collected and analyzed for the different metabolites by GC analysis.

GLP compliance:not specified

Test material

Reference substance name:4-tert-butyltoluene

EC Number:202-675-9

EC Name:4-tert-butyltoluene

Cas Number:98-51-1

Molecular formula:C11H16

IUPAC Name:1-tert-butyl-4-methylbenzene

Details on test material:

- Name of test material (as cited in study report): Ro 94-0522 (TBT)

- Analytical purity: no data given

Radiolabelling:no

Test animals

Species:guinea pig

Strain:Himalayan

Sex:male

Administration / exposure

Route of administration:oral: gavage

Vehicle: rape oil

Duration and frequency of treatment / exposure:daily for 5 consecutive days

Doses / Concentrations:

100 mg/kg bw/d

No. of animals per sex per dose / concentration:5

Control animals:no

The urine samples were acidified with 37 % hydrochloric acid to pH 1-2 and extracted with ethylacetate using pre-packed extraction columns according to the procedure recommended by the manufacturer (Extrelut, Merck No. 11737/8).

After evaporation of the extraction solvent the residue was dissolved in 5 ml ethylacetate/methanol (4:1).

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness, taken up in 200 µl DMF and methylated by heating with 200 µl DMF-DMA (Fluka) for 15 min at 90 °C.

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness and heated with 1 ml acetic anhydride for 5 min at 95 °C in order to transform hippuric acid and TBHA into the corresponding molecules.

For GC analysis 1 µl of the reaction mixture was injected without further treatment. For GC reference pure TBBA was methylated with DMF-DMA.

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES

- Tissues and body fluids sampled: urine

- Time and frequency of sampling: 24 h urine was collected after the last application of the test material

- From how many animals: no data given

- Method type for identification: GC

Results and discussion

Metabolite characterisation studies

Metabolites identified:yes

Details on metabolites:

TBBA and TBHA were identified by GC retention time (co-injection on two GC systems).

TBHA was found to be the main metabolite in urine samples of guinea pig, whereas TBBA was found in the urine in very low concentrations only.

Table 1: Identification of TBBA and TBHA as metabolites of TBT in guinea-pig.

Dose TBT (mg/kg)	100
TBBA (mg/kg bw)	1.66
TBBA (mg/l)	18.95
TBBA (mg/24h)	4.6
TBBA (%)*	1.38
TBHA (mg/kg bw)	85.66
TBHA (mg/l)	565.9
TBHA (mg/24h)	237.7
TBHA (%)**	53.9
Recovery (%)***	55.28

*%TBBA = TBBA in urine (µM/kg)/applied dose (µM/kg) * 100

**%TBHA = TBHA in urine (µM/kg)/applied dose (µM/kg) * 100

***Recovery = %TBBA + %TBHA

2.8 Study 8: 4-*tert*-butyltoluene

Basic toxicokinetics in vivo

Title:Unnamed

Year:1985

Objective of study:metabolism

On the occasion of various 5 day oral toxicity studies of tert-butyltoluene with mice, guinea pigs and dogs, urine was collected and analyzed for the different expected metabolites by GC analysis.

GLP compliance:not specified

Test material

Reference substance name:4-tert-butyltoluene

EC Number:202-675-9

EC Name:4-tert-butyltoluene

Cas Number:98-51-1

Molecular formula:C11H16

IUPAC Name:1-tert-butyl-4-methylbenzene

Details on test material:

- Name of test material (as cited in study report): Ro 94-0522 (TBT)

- Analytical purity: no data given

Radiolabelling:no

Test animals

Species:dog

Strain:Beagle

Sex:male

Administration / exposure

Route of administration:oral: capsule

Vehicle:not specified

Duration and frequency of treatment / exposure:daily for 5 consecutive days

Doses / Concentrations:

100 mg/kg bw/d

No. of animals per sex per dose / concentration:2

Control animals:not specified

Details on study design:

The urine samples were acidified with 37 % hydrochloric acid to pH 1-2 and extracted with ethylacetate using pre-packed extraction columns according to the procedure recommended by the manufacturer (Extrelut, Merck No. 11737/8).

After evaporation of the extraction solvent the residue was dissolved in 5 ml ethylacetate/methanol (4:1).

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness, taken up in 200 µl DMF and methylated by heating with 200 µl DMF-DMA (Fluka) for 15 min at 90 °C.

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness and heated with 1 ml acetic anhydride for 5 min at 95 °C in order to transform hippuric acid and TBHA into the corresponding molecules.

For GC analysis 1 µl of the reaction mixture was injected without further treatment. For GC reference pure TBBA was methylated with DMF-DMA.

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES

- Tissues and body fluids sampled: urine

- Time and frequency of sampling: 24 h urine was collected after the last application of the test material

- From how many animals: no data given

- Method type for identification: GC

Results and discussion

Metabolite characterisation studies

Metabolites identified:yes

TBBA and TBHA were identified by GC retention time (co-injection on two GC systems).

TBBA was found to be the main metabolite in urine samples dogs, whereas TBHA was found in the urine in low concentrations only.

Table 1: Identification of TBBA and TBHA as metabolites of TBT in Beagle dogs.

Dose TBT (mg/kg)	100
TBBA (mg/kg bw)	4.68
TBBA (mg/l)	315.8

TBBA (mg/24h)	43.1
TBBA (%)*	3.89
TBHA (mg/kg bw)	1.54
TBHA (mg/l)	115.9
TBHA (mg/24h)	14.2
TBHA (%)**	0.97
Recovery (%)***	4.86

*%TBBA = TBBA in urine (µM/kg)/applied dose (µM/kg) * 100

**%TBHA = TBHA in urine (µM/kg)/applied dose (µM/kg) * 100

***Recovery = %TBBA + %TBHA

2.9 Study 9: 4-*tert*-butylbenzaldehyde

Basic toxicokinetics in vivo

Title: Unnamed

Year:1982

Objective of study:metabolism

Principles of method if other than guideline:

Groups of rats are gavaged daily with graduated doses of the test article for 5 consecutive days. For 24 hours, urine is collected after the last application of the compound. Urinary metabolites are detected by appropriate method (GC)

GLP compliance:not specified

Test material

Radiolabelling:no

Test animals

Species:rat

Strain:SPF albino

Sex:male

Administration / exposure

Route of administration:oral: gavage

Vehicle: rape oil

Duration and frequency of treatment / exposure:5 days

Doses / Concentrations:

0, 12.5 and 50 mg/kg bw

No. of animals per sex per dose / concentration:

- Control: 4 male animals

- Dosed groups: 8 males/dose

Control animals:yes

Details on study design:

The urine samples were acidified with 37 % hydrochloric acid to pH 1-2 and extracted with ethylacetate using pre-packed extraction columns according to the procedure recommended by the manufacturer (Extrelut, Merck No. 11737/8).

After evaporation of the extraction solvent the residue was dissolved in 5 ml ethylacetate/methanol (4:1).

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness, taken up in 200 µl DMF and methylated by heating with 200 µl DMF-DMA (Fluka) for 15 min at 90 °C.

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness and heated with 1 ml acetic anhydride for 5 min at 95 °C in order to transform hippuric acid and TBHA into the corresponding molecules.

For GC analysis 1 µl of the reaction mixture was injected without further treatment. For GC reference pure TBBA was methylated with DMF-DMA.

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES

- Tissues and body fluids sampled: urine

- Time and frequency of sampling: 24 h urine was collected after the last application of the test material

- From how many animals: no data given

- Method type for identification: GC-MS

Results and discussion

Metabolites identified:yes

Details on metabolites:

p-Tert-butylbenzoic acid (TBBA) was identified as metabolite in the 24 hours urine in rats treated with p-tert-butylbenzaldehyde (TBB), but not the secondary metabolite p-tert-butylhippuric acid.

Dose level TBB (mg/kg)	**Weight of rats (g)**	**TBBA in last 24-h urine**	
		(mg/l)	**(mg/kg)**
0	984	n.d.	n.d.
12.5	1742	29	1.2
50	1578	235	12.7

n.d. = not detectable

2.10 Study 10: 4-*tert*-butylbenzaldehyde

Basic toxicokinetics in vivo

Title:Unnamed

Year:1985

Objective of study:metabolism

Principles of method if other than guideline:

On the occasion of various 5 day oral toxicity studies of tert-butylbenzaldehyde with mice, guinea pigs and dogs, urine was collected and analyzed for the different metabolites by GC analysis.

GLP compliance:not specified

Test material

Radiolabelling:no

Test animals

Species:mouse

Strain: SPF albino

Sex:male

Administration / exposure

Route of administration:oral: gavage

Vehicle:rape oil

Duration and frequency of treatment / exposure:daily for 5 consecutive days

Doses / Concentrations:

100 mg/kg bw/d

No. of animals per sex per dose / concentration:6

Control animals:no

Details on study design:

The urine samples were acidified with 37 % hydrochloric acid to pH 1-2 and extracted with ethylacetate using pre-packed extraction columns according to the procedure recommended by the manufacturer (Extrelut, Merck No. 11737/8).

After evaporation of the extraction solvent the residue was dissolved in 5 ml ethylacetate/methanol (4:1).

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness, taken up in 200 µl DMF and methylated by heating with 200 µl DMF-DMA (Fluka) for 15 min at 90 °C.

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness and heated with 1 ml acetic anhydride for 5 min at 95 °C in order to transform hippuric acid and TBHA into the corresponding molecules.

For GC analysis 1 µl of the reaction mixture was injected without further treatment. For GC reference pure TBBA was methylated with DMF-DMA.

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES

- Tissues and body fluids sampled: urine

- Time and frequency of sampling: 24 h urine was collected after the last application of the test material

- From how many animals: no data given

- Method type for identification: GC

Results and discussion

Metabolites identified:yes

Details on metabolites:

TBBA and TBHA were identified by GC retention time (co-injection on two GC systems).

TBHA was found to be the main metabolite in urine samples of mice, whereas TBBA was found in the urine in very low concentrations only.

Table 1: Identification of TBBA and TBHA as metabolites of TBB in mice.

Dose TBT (mg/kg)	100

TBBA (mg/kg bw)	0.59
TBBA (mg/l)	< 15.4
TBBA (mg/24h)	< 0.2
TBBA (%)*	< 0.54
TBHA (mg/kg bw)	57.39
TBHA (mg/l)	1492.3
TBHA (mg/24h)	19.4
TBHA (%)**	39.6
Recovery (%)***	39.6

*%TBBA = TBBA in urine (µM/kg)/applied dose (µM/kg) * 100

**%TBHA = TBHA in urine (µM/kg)/applied dose (µM/kg) * 100

***Recovery = %TBBA + %TBHA

2.11 Study 11: 4-*tert*-butylbenzaldehyde

Basic toxicokinetics in vivo

Title:Unnamed

Year:1985

Objective of study:metabolism

Principles of method if other than guideline:

On the occasion of various 5 day oral toxicity studies of tert-butylbenzaldehyde with mice, guinea pigs and dogs, urine was collected and analyzed for the different metabolites by GC analysis.

GLP compliance:not specified

Test material

Radiolabelling:no

Test animals

Species:guinea pig

Strain:Himalayan

Sex:male

Administration / exposure

Route of administration:oral: gavage

Vehicle:other: rape oil

Duration and frequency of treatment / exposure:daily for 5 consecutive days

Doses / Concentrations:100 mg/kg bw/d

No. of animals per sex per dose / concentration:5

Control animals:no

Details on study design:

The urine samples were acidified with 37 % hydrochloric acid to pH 1-2 and extracted with ethylacetate using pre-packed extraction columns according to the procedure recommended by the manufacturer (Extrelut, Merck No. 11737/8).

After evaporation of the extraction solvent the residue was dissolved in 5 ml ethylacetate/methanol (4:1).

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness, taken up in 200 µl DMF and methylated by heating with 200 µl DMF-DMA (Fluka) for 15 min at 90 °C.

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness and heated with 1 ml acetic anhydride for 5 min at 95 °C in order to transform hippuric acid and TBHA into the corresponding molecules.

For GC analysis 1 µl of the reaction mixture was injected without further treatment. For GC reference pure TBBA was methylated with DMF-DMA.

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES

- Tissues and body fluids sampled: urine

- Time and frequency of sampling: 24 h urine was collected after the last application of the test material

- From how many animals: no data given

- Method type for identification: GC

Results and discussion

Metabolites identified:yes

Details on metabolites:

TBBA and TBHA were identified by GC retention time (co-injection on two GC systems).

TBHA was found to be the main metabolite in urine samples of guinea pig, whereas TBBA was found in the urine in very low concentrations only.

Table 1: Identification of TBBA and TBHA as metabolites of TBB in guinea pig.

Dose TBT (mg/kg)	100
TBBA (mg/kg bw)	1.42
TBBA (mg/l)	11.8
TBBA (mg/24h)	3.8
TBBA (%)*	1.29
TBHA (mg/kg bw)	108.21
TBHA (mg/l)	896.6
TBHA (mg/24h)	288.7
TBHA (%)**	74.6
Recovery (%)***	75.89

*%TBBA = TBBA in urine (µM/kg)/applied dose (µM/kg) * 100

**%TBHA = TBHA in urine (µM/kg)/applied dose (µM/kg) * 100

***Recovery = %TBBA + %TBHA

2.12 Study 12: 4-*tert*-butylbenzaldehyde

Basic toxicokinetics in vivo

Title:Unnamed

Year:1985

Objective of study:metabolism

Principles of method if other than guideline:

On the occasion of various 5 day oral toxicity studies of tert-butylbenzaldehyde with mice, guinea pigs and dogs, urine was collected and analyzed for the different expected metabolites by GC analysis.

GLP compliance:not specified

Test material

Radiolabelling:no

Test animals

Species:dog

Strain:Beagle

Sex:male

Administration / exposure

Route of administration:oral: capsule

Vehicle:not specified

Duration and frequency of treatment / exposure:daily for 5 consecutive days

Doses / Concentrations:100 mg/kg bw/d

No. of animals per sex per dose / concentration:2

Control animals:not specified

Details on study design:

The urine samples were acidified with 37 % hydrochloric acid to pH 1-2 and extracted with ethylacetate using pre-packed extraction columns according to the procedure recommended by the manufacturer (Extrelut, Merck No. 11737/8).

After evaporation of the extraction solvent the residue was dissolved in 5 ml ethylacetate/methanol (4:1).

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness, taken up in 200 µl DMF and methylated by heating with 200 µl DMF-DMA (Fluka) for 15 min at 90 °C.

0.5 ml of the ethylacetate/methanol extract was evaporated to dryness and heated with 1 ml acetic anhydride for 5 min at 95 °C in order to transform hippuric acid and TBHA into the corresponding molecules.

For GC analysis 1 µl of the reaction mixture was injected without further treatment. For GC reference pure TBBA was methylated with DMF-DMA.

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES

- Tissues and body fluids sampled: urine

- Time and frequency of sampling: 24 h urine was collected after the last application of the test material

- From how many animals: no data given

- Method type for identification: GC

Results and discussion

Metabolites identified:yes

Details on metabolites:

TBBA and TBHA were identified by GC retention time (co-injection on two GC systems).

TBBA was found to be the main metabolite in urine samples of dogs, whereas TBHA was found in the urine in low concentrations only.

Table 1: Identification of TBBA and TBHA as metabolites of TBB in Beagle dogs.

Dose TBT (mg/kg)	100	100
TBBA (mg/kg bw)	1.48	2.57
TBBA (mg/l)	119.4	125.4
TBBA (mg/24h)	17.2	25.7
TBBA (%)*	1.35	2.34
TBHA (mg/kg bw)	1.51	0.54
TBHA (mg/l)	121.5	26.5
TBHA (mg/24h)	17.5	5.44
TBHA (%)**	1.04	0.37
Recovery (%)***	2.39	7.78

*%TBBA = TBBA in urine (µM/kg)/applied dose (µM/kg) * 100

**%TBHA = TBHA in urine (µM/kg)/applied dose (µM/kg) * 100

***Recovery = %TBBA + %TBHA

2.13 Study 13: 3-(4-*tert*-butylphenyl)propionaldehyde and *p*-*tert*-butyltoluene

In vitro metabolism study with 3-(4-tert-butylphenyl)propionaldehyde, 4-tert-butyltoluene and 2-(4-tert-butylbenzyl)propionaldehyde (among other substances).

Publication: Laue et al. 2017. p-Alkyl-Benzoyl-CoA conjugates as relevant metabolites of aromatic aldehydes with rat testicular toxicity- studies leading to the design of a safe new fragrance chemical. Toxicological Sciences 160(2), 2017, 244-255.

Method: Rat hepatocytes in suspension were incubated in the presence of 100 µM of the test chemicals for 4 h. Benzoic acid derivatives were determined by GC-MS at 0.5, 4 and 22 h.

Formation of CoA conjugates following 0.5, 4 and 22 hours of exposure to the chemicals at 5 and 50 µM was also assessed by LC-HRMS

The abstract from the publication is given below:

Several aromatic aldehydes such as 3-(4-tert-butylphenyl)-2-methylpropanal were shown to adversely affect the reproductive system in male rats following oral gavage dose of ≥ 25 mg/kg bw/d. It was hypothesized that these aldehydes are metabolized to benzoic acids such as p-tert-butylbenzoic acid (TBBA) as key toxic principle and that Coenzyme A (CoA) conjugates may be formed from such acids. Here we performed a detailed structure activity relationship study on the formation of benzoic acids from p-alkyl-phenylpropanals and related chemicals in rat hepatocytes in suspension. Formation of CoA conjugates from either p-alkyl-phenylpropanals directly or from their benzoic acid metabolites was further assessed in plated rat hepatocytes using high resolution LC-MS. All of the test chemicals causing reproductive adverse effects in

male rats formed p-alkyl-benzoic acids in rat hepatocytes in suspension. Compounds metabolized to p-alkyl-benzoic acids led to accumulation of p-alkyl-benzoyl-CoA conjugates at high and steady levels in plated rat hepatocytes, whereas CoA conjugates of most other xenobiotic acids were only transiently detected in this in vitro system. The correlation between this metabolic fate and the toxic outcome may indicate that accumulation of the alkyl-benzoyl-CoA conjugates in testicular cells could impair male reproduction by adversely affecting CoA-dependent processes required for spermatogenesis. This hypothesis prompted a search for new p-alkyl-phenylpropanal derivatives which do not form benzoic acid metabolites and the corresponding CoA conjugates. It was found that such metabolism did not occur with a derivative containing an o-methyl substituent, ie, 3-(4-isobutyl-2-methylphenyl)propanal. This congener preserved the fragrance quality but lacked the male reproductive toxicity in a 28-day rat study, as predicted from its in vitro metabolism.

2.14 Study 14: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics

Title: Unnamed

Year:1995

GLP compliance: yes

Objective of study: toxicokinetics

Test guideline

Qualifier: equivalent or similar to guideline

Guideline: OECD Guideline 417 (Toxicokinetics)

Test material

Reference substance name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral, BMHCA)

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde

Cas Number: 80-54-6

Analytical purity of non-radioactive test substance: not determined

Radiolabelling: yes

Remarks: 14C

Test animals

Species: rat

Strain:Wistar derived RORO (Ibm:RORO (SPF)) albino rats

Sex: male

TEST ANIMALS

Source: Institut fuer biologisch-medizinische Forschung, Fuellindsorf, Switzerland

Age at study initiation: 5-7 weeks

Weight at study initiation: 207 ± 6 g

Individual metabolism cages: yes

Diet: ad libitum (LAD1, Special Diet Services, Manea, UK, Batches 6562 and 6882)

Water: ad libitum (tap water)

Acclimation period: 7-13 days

ENVIRONMENTAL CONDITIONS

Temperature (°C): 21 ± 2

- Humidity (%): 50 ± 10

- Photoperiod (hrs dark / hrs light): 12/12

Administration / exposure

Route of administration:

oral: gavage

Vehicle: rapeseed oil

Administration and exposure

PREPARATION OF DOSING SOLUTIONS:

The dosing solutions were prepared the day prior to dose administration. To prepare the doses 25 and 100 mg/kg, repurified 14C BMHCA (22.2 and 25.3 mg, respectively) was mixed with non radioactive BMHCA (17.1 and 135.6 mg, respectively) and rapeseed oil (14.1 and 14.3 g, repectively) to give solutions containing 2.8 and 11.2 mg BMHCA/g formulation

Administration volume: 2 ml

Duration and frequency of treatment / exposure: single administration

Doses / concentrations: 25 and 100 mg/kg bw

No. of animals per sex per dose / concentration: 4

Control animals: no

Details on study design:

TREATMENT OF ANIMALS:

- Fasting: Yes, overnight before application and for 2 hours after dose administration

Details on dosing and sampling:

PHARMACOKINETIC STUDY

- Tissues and body fluids sampled: blood (ca. 0.5 ml in to heparized tubes), plasma (generated after centrifugation of blood 14,000g)

- Time and frequency of sampling: prior to oral adminstration and at 0.5, 1, 2, 4, 6, 8, 24 and 48 h afterwards.

- Storage of samples: at -20°C until analysis was performed

Results and discussion

Metabolite characterisation studies

Metabolites identified: not measured

Table 1: Pharmacokinetic variables of radioactivity in plasma after single dose administration of 14C BMHCA to rats

Dose administered	C max (µg equivalents/ml)	T max (h)	AUC_{0-48} (µg.h/ml)	$T_{1/2}$ (h)
25 mg/kg bw	14.3 ± 6.4	3.5 ± 1.9	122	8*
100 mg/kg bw	52.0 ± 15.3	1.8 ± 0.5	937	9.8**

Tmax und Cmax represent the mean of Tmax and Cmax observed for single animals. AUC was estimated up to the last time at which concentrations were above the quantification limit by the linear trapezoidal rule.

* Calculated from the log linear plots of 2 animals only because a linear terminal elimination phase could not be identified for the other two animals

** Calculated from the log linear plots of 1 animal only because a linear terminal elimination phase could not be identified for the other 3 animals.

Table 2: Mean Plasma concentrations of radioactivity in rats following single doses of 14C BMHCA 25 and 100 mg/kg bw in rapeseed oil

Sample collection time (h)	25 mg/kg bw	100 mg/ kg bw
	µg equivalents/ml	
0.5	6.1 ± 2.2	33.9 ± 12.4
1	8.7 ± 4.3	46.9 ± 16.7
2	13.7 ± 7.3	49.5 ± 12.1
4	9.0 ± 5.1	41.6 ± 22.5
6	6.2 ± 4.1	43.5 ± 15.9
8	4.3 ± 3.4	27.4 ± 13.9
24	1.5 ± 0.5	22.6 ± 7.2
48	0.3 ± 0.03	2.8 ± 0.9

2.15 Study 15: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics in vivo

Title: Unnamed

Year: 2006

Objective of study: metabolism

Principles of method if other than guideline:

Plasmakinetics in rats were examined after single oral exposure to Lysmeral and Lysmerylic acid.

GLP compliance: no

Remarks: The study has no GLP status but was however conducted in accordance with the OECD principles for GLP

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Cas Number: 80-54-6

Molecular formula: C14H20O

Analytical purity: 99.1%
Radiolabelling: no

Test animals

Species: rat

Strain: Wistar

Sex: male

Administration / exposure

Route of administration: oral: gavage

Vehicle: Olive oil Ph EUR./DAB

Details on exposure:

Application Volume: 5 ml

Duration and frequency of treatment / exposure: single oral exposure

Doses / Concentrations:

Lysmeral: 50 mg/kg bw

Lysmerylic acid: 50 mg/kg bw

No. of animals per sex per dose / concentration:

Lysmeral: 5 male animals

Lysmerylic acid: 5 male animals

Control animals: no

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES
- Tissues and body fluids sampled: blood, from retroorbital venous plexus

- Anesthesia: yes, Isoflo (Essex GmbH Munich)

- Time and frequency of sampling: 3 days before gavage, directly after first application (10 min), after 2h, 4h, 8h, and 24h
- From how many animals: samples pooled for 5 animals

- Method type(s) for identification: LC/MS

Results and discussion

Details on absorption: not determined

Details on distribution in tissues: not determined

Transfer into organs

Observation: not determined

Details on excretion: not determined

Metabolite characterisation studies

Metabolites identified: yes

Details on metabolites:

Results are given for pooled samples of 5 animals/group. Lysmeral was not detected in any plasma sample, neither in plasma samples of animals that received 50 mg/kg Lysmeral, nor in the samples of animals that were dosed with 50 mg/kg bw Lysmerylic acid. Lysmerylic acid was detected in all plasma samples with the highest plasma concentration of 29.45 and 8.79 µg/g directly after application and 4h after application in animals that received Lysmerylic acid and Lysmeral, respectively. The AUC (0 -24h) for Lysmerylic acid was calculated to be 89.27 and 81.42 µg x h/g in the case of Lysmerylic acid and Lysmeral, respectively.

Kinetic Parameters of 2-(methyl)-3-(4-tert-butylphenyl) propionic acid after application of Lysmeral and Lysmerylic acid (2-(methyl)-3-(4-tert-butylphenyl) propionic acid) to male Wistar rats.

Compound gavaged to rat	Compound identified in plasma	T_{Max}	C_{Max} (µg/g)	AUC_{0-24h} (µg x h/g)	$T_{1/2}$ (h)
Lysmeral	Lysmerylic acid	4h	8.79	81.42	5.75
Lysmerylic acid	Lysmerylic acid	Directly after application	29.45	89.27	3.64

Parameters have been derived using TopFit 2.0 (Heinzel. G. et al.; TopFit 2.0, Pharmacokinetic and Pharmacodynamic data analysis system for the PC; Gustav Fischer Verlag, Stuttgart, Jena, New York; 1993). The AUC was determined according to the linear trapezoidal rule.

2.16 Study 16: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics in vivo

Title: Unnamed

Year: 1982

Objective of study: metabolism

Principles of method if other than guideline:

Groups of rats are gavaged daily with graduated doses of the test article for 5 consecutive days. For 24 hours, urine is collected after the last application of the compound. Urinary metabolites are detected by appropriate method (GC)

GLP compliance: not specified

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Cas Number: 80-54-6

Molecular formula: C14H20O

Analytical purity: not reported

Radiolabelling: no

Test animals

Species: rat

Strain: other: Albino (SPF)

Sex: male

Details on test animals or test system and environmental conditions:

Weight at study initiation: 880g (control), 1653 g (100 mg/kg bw), 1233 g (400 mg/kg bw)

Administration / exposure

Route of administration: oral: gavage

Vehicle: Rapeseed oil

Details on exposure:

Dose volume: 1ml/100 g bw /d

Duration and frequency of treatment / exposure: 5 days

Doses / Concentrations: 0; 100; 400 mg/kg bw/d (0; 490, 1958 µM/kg)

No. of animals per sex per dose / concentration:

Control: 4 male animals

Dosed groups: 8 males/dose

Control animals: yes, concurrent vehicle

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES
- Body fluids sampled: urine
- Time and frequency of sampling: 24 h urine after administration of last dose
- From how many animals: (samples pooled or not): not reported
- Method type(s) for identification: GC-MS, GC-retention time (Co-injection with reference material)

PREPARATION OF SAMPLES FOR GC (MS) DETECTION
TBBA Analysis
- Derivatization: Methylation, silylation
- Derivatization agent: DMF-DMA (dimethyl formamide-dimethylacetal) and N,O-Bis-(Trimethylsilyl)trifluoroacetamide/pyridine
- GC MS analysis conditions: Temperature 120°C; retention time 6.1 min
- Compound detected in GC MS after derivatization: TBBA-methylester

TBHA (tert-butylhippuric acid) analysis
- Derivatization: Cyclisation
- Derivatization agent: Acetic anhydride
- GC MS analysis conditions: Temperature 190°C; retention time 4.6 min
- Compound detected in GC MS after derivatization: TBHA azlactone
- Reference compound: hippuric acid

Results and discussion

Details on absorption: not determined

Details on distribution in tissues: not determined

Transfer into organs

Observation: not determined

Toxicokinetic parameters: not determined

Metabolite characterisation studies

Metabolites identified: yes

Details on metabolites:

TBBA was detected in a dose related manner except in control (table 1). The authors surmised that TBBA is eliminated in urine as a glucoronide. However, because sample preparation before GC detection requires acidification of the urine samples, glucoronides are hydrolysed, which make GC an unsuitable method to capture glucoronides. Tert-Butylhippuric acid (TBHA) was not detected in any sample (which means glycine conjugation to TBBA does not occur). 2 additional peaks were tentatively assigned to p-tert-hydroxybutylbenzoic and p-tert-carboxybutylbenzoic acids. However, no further work for a structure identification of these products was undertaken.

Table 1: Elimination of TBBA in last 24-h urine sample after oral 5-day application in rats

Dose level Lysmeral	para-tert-butylbenzoic acid in 24h urine

mg/kg	µM/kg	mg/l	mg/kg	µM/kg
0	0	Not detectable	Not detectable	Not detectable
100	490	162	12.7	71.3
400	1958	461	31.8	178.6

Urine values have been derived from rats dosed with p-tert-butylbenzaledhyde (TBB) or p-tert-butyltoluene (TBT), under the same testing conditions as described above for Lysmeral.

The following values have been derived:

	Dose level		TBBA in last 24h-urine		
Compound	mg/kg	µM/kg	mg/l	mg/kg	µM/kg
TBT	0	0	n.d.	n.d.	n.d.
TBT	25	169	56	2.7	15.4
TBT	100	675	455	17.2	96.8
TBB	0	0	n.d.	n.d.	n.d.
TBB	12.5	77	29	1.2	6.5
TBB	50	308	235	12.7	71.2

2.17 Study 17: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics in vivo

Title: Unnamed

Year: 2006

Materials and methods

Objective of study: metabolism

Principles of method if other than guideline:

Plasmakinetics in mice were examined after single oral exposure to Lysmeral and Lysmerylic acid.

GLP compliance: no

Remarks: The study was given no GLP status but was however conducted in accordance with the OECD principles for GLP

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Cas Number: 80-54-6

Molecular formula: C14H20O

Analytical purity: 99.1%

Radiolabelling: no

Test animals

Species: mouse

Strain: C57BL/6NCrl

Sex: male

Administration / exposure

Route of administration: oral: gavage

Vehicle: Olive oil Ph EUR./DAB

Details on exposure:

Application Volume: 5 ml

Duration and frequency of treatment / exposure: single oral application

Doses / Concentrations: Lysmeral: 50 mg/kg bw; Lysmerylic acid: 50 mg/kg bw

No. of animals per sex per dose / concentration: Lysmeral: 10 male animals; Lysmerylic acid: 10 male animals

Control animals: no

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES
- Tissues and body fluids sampled: blood, from retroorbital venous plexus
- Anesthesia: yes, Isoflo (Essex GmbH Munich)
- Time and frequency of sampling: 3 days before gavage, directly after first application (20 min), after 2h, 4h, 8h, and 24h
- From how many animals: samples pooled for 10 animals
- Method type(s) for identification: LC/MS

Results and discussion

Details on absorption: not determined

Details on distribution in tissues: not determined

Transfer into organs: not determined

Details on excretion: not determined

Metabolites identified: yes

Details on metabolites: Results are given for pooled samples of 10 animals. Lysmeral was not detected in any plasma sample, neither in plasma samples of animals that received 50 mg/kg Lysmeral, nor in the samples of the animals that were dosed with 50 mg/kg bw Lysmerylic acid. Lysmerylic acid was detected in all plasma samples with the highest plasma concentration of 22.09 and 18.38 µg/g for Lysmerylic acid and Lysmeral exposure, respectively. Time when peak Lysmerylic acid concentrations was detected was directly after application in animals that received Lysmerylic acid and Lysmeral, respectively. The AUC (0 -24h) for Lysmerylic acid was calculated to be 106.72 and 85.12 µg x h/g in the case of Lysmerylic acid and Lysmeral application, respectively.

Table: Kinetic Parameters of 2-(methyl)-3-(4-tert-butylphenyl) propionic acid after application of Lysmeral and Lysmerylic acid (2-(methyl)-3-(4-tert-butylphenyl) propionic acid) to male c57BL/6NCrl mice.

Compound gavaged to mouse	Compound identified in plasma	T_{Max}	C_{Max} (µg/g)	AUC_{0-24h} (µg x h/g)	$T_{1/2}$ (h)
Lysmeral	Lysmerylic acid	Directly after application	18.38	85.12	3.31
Lysmerylic acid	Lysmerylic acid	Directly after application	22.09	106.72	3.99

Parameters have been derived usingTopFit 2.0 (Heinzel. G. et al.; TopFit 2.0, Pharmacokinetic and Pharmacodynamic data analysis system for the PC; Gustav Fischer Verlag, Stuttgart, Jena, New York; 1993). The AUC was determined according to thelinear trapezoidal rule.

2.18 Study 18: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics in vivo

Title: Unnamed

Year: 1985

Materials and methods

Objective of study: metabolism

Principles of method if other than guideline:

Urine was collected from animals (mice, rats, guinea pigs, dogs and monkeys) in 5 day toxicity studies of LILIAL, and analyzed for the different expected acid metabolites. The method of analysis was GC

GLP compliance: not specified

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Cas Number: 80-54-6

Molecular formula: C14H20O

Analytical purity: not reported

Radiolabelling: no

Test animals

Species: other: see below; table 1

Sex: male/female

Administration / exposure

Route of administration: oral: unspecified

Vehicle: not specified

Duration and frequency of treatment / exposure: 5 days/daily exposure

Doses / Concentrations: see below; table 1

No. of animals per sex per dose / concentration: not reported

Control animals: yes, concurrent vehicle

Details on dosing and sampling:

METABOLITE CHARACTERISATION STUDIES
- Body fluids sampled: urine
- Time and frequency of sampling: 24 h urine after administration of last dose
- From how many animals: see below; table 1
- Samples pooled or not: not reported
- Method type(s) for identification: GC-MS (Varian Vista 44 GC-System), and GC-retention time (Co-injection on two GC different columns (packed and capillary))

PREPARATION OF SAMPLES FOR GC (MS) DETECTION
TBBA Analysis
- Derivatization: Methylation
- Derivatization agent: DMF-DMA (dimethyl formamide-dimethylacetal)

- GC MS analysis conditions: see below; table 2
- Target compound detected in GC MS after derivatization: TBBA-methylester

TBHA (tert-butylhippuric acid) analysis
- Derivatization: Cyclisation
- Derivatization agent: Acetic anhydride
- GC MS analysis conditions: see below; table 2
- Target compound detected in GC MS after derivatization: TBHA azlactone
- Reference compound: hippuric acid

Results and discussion

Details on absorption: not determined

Details on distribution in tissues: not determined

Transfer into organs: not determined

Details on excretion: not determined

Toxicokinetic parameters

Metabolites identified: yes

Details on metabolites: Tert-Butylbenzoic acid (TBBA) was found as main metabolite in urine samples of treated rats, dogs and monkeys, whereas urine samples of guinea pigs and mice contained very low concentrations of TBBA. Tert-Butylhippuric acid (TBHA) was found as main metabolite in urine samples of treated mice and guinea pigs and only in very low concentrations in urine samples of rats, dogs and monkeys.

Table 1: 5-day oral toxicity menagerie studies with mice, rats, guinea pigs, dogs and monkeys.

Study number	Species	Sex	No. of animals examined	Dose level Lysmeral (mg/kg bw)
723 A 83	Mouse	Male	5	100
727 A 84	Rat	Male	8	50, 100, 200, 400
65 A83*	Rat	Male/female	no data	50
723 A 83	Guinea pig	Male	5	100
749 A 83	Monkey	Male	2	100
91 A 83	Dog	Male and female	3/3	44.6

* Because of stability problems with the test substance admixed to food, no final report about the study was made

Table 2: GC condition for the detection of urinary metabolites

Conditions for Packed column	Column: Glass, 1= 2 m, 2 mm I.D., 5 % SE-30 On Chromosorb G, AW-DMCS. Flow: N2, 25 ml/min.	
Target compound	Retention time (min)	Temperature program
Hippuric acid azlactone	2.05	Start: 110°C/1' Rate: 20°C/min End: 190°C/10'
TBHA azlactone	6.48	

TBBA-methylester	8.7	Start: 120°C/5' Rate: 20°C/min End: 220°C/5'
Toluic-acid-methylester (internal standard)	3.45	
-		
Conditions for Capillary column	Column: Glass, 1 = 20 m, 0.29 mm I.D., SE-30.	
Target compound	Retention time (min)	Temperature program 1
Hippuric acid azlactone	3.2	Carrier gas: N2 Split: 1:40 Make-up gas: N2; 30 ml/min Start:110°C/4' Rate:20°C/min End:190°C/4'
TBHA azlactone	5.3	
TBBA-methylester	3.5	
-		
Conditions for Capillary column	Column: Glass, 1 = 20 m, 0.29 mm I.D., SE-30.	
Target compound	Retention time (min)	Temperature program 1
Toluic-acid-methylester (internal standard)	4.0	Carrier gas: H2 Split: 1:10 Inlet pressure 0.5 bar Make-up gas: N2; 30 ml/min Start: 80°C/1' Rate: 8°C/min End: 170°C/4'
TBHA azlactone	11.2	
TBBA-methylester	8.6	

Table 3: Identification of TBBA as urinary metabolite of Lysmeral "lilial"

Study	Species	Dosis (mg/kg bw)	Gender	Metabolite				Recovery %
				TBBA		TBHA		
				mg/kg	%*	mg/kg	%**	
723 A 83	Mouse	0	Male	<0.4	-	<1.2	-	-
		100	Male	<0.83	<0.96	14.52	12.6	12.6
	Guinea pig	0	Male	<0.03	-	<0.09	-	-
		100	Male	<0.03	<0.04	55.96	48.6	48.6
-								
727 A 84	Rat	0	Male	<0.27	-	<0.27	-	
		50	Male	4.77	10.9	<0.29	<0.5	10.9

		100	Male	5.85	6.7	0.84	0.70	7.4
		200	Male	23.27	3.7	1.32	0.60	4.3
		400	Male	36.91	3.3	1.4	0.30	3.6
-								
65 A 83	Rat	0	Male	<0.57	-	<0.85	-	
		50	Male	8.07	18.5	<0.89	<1.5	18.5
		0	Female	<0.64	-	<0.96	-	-
		50	Female	6.65	15.2	<1.14	<2	15.2
-								
749 A 83	Monkey	100	Male	9.87	11.3	0.16	0.14	11.4
		100	Male	2.44	2.8	0.04	0.03	2.8
-								
91 A 83	Dog	0	Male	<0.003	-	<0.004	-	-
		44.6	Male	1.1	2.8	0.63	1.2	4.0
		0	Female	<0.003	-	<0.004	-	-
		44.6	Female	1.41	3.6	0.54	1.1	19.17

*% TBBA = TBBA in urine (µM/kg) / applied dose (µM/kg)*100

**% TBHA = TBHA in urine (µM/kg) / applied dose (µM/kg)*100

Tert-Butylbenzoic acid (TBBA) was found as main metabolite in urine samples of treated rats, dogs and monkeys, whereas urine samples of guinea pigs and mice contained very low concentrations of TBBA. Tert-Butylhippuric acid (TBHA) was found as main metabolite in urine samples of treated mice and guinea pigs and only in very low concentrations in urine samples of rats, dogs and monkeys.

Urine values have also been derived from animals dosed with p-tert-butylbenzaledhyde (TBB) or p-tert-butyltoluene (TBT), under the same testing conditions as described above for Lysmeral.

The following values have been derived:

			Dosis	Gender	Metabolite				Recovery
					TBBA		TBHA		
	Study	Species	(mg/kg bw)		mg/kg	%*	mg/kg	%**	%
TBT	723A84	Dog	100	male	1.88	1.56	0.25	0.16	1.72
TBT	723A84	Dog	100	male	4.68	3.89	1.54	0.97	4.86
TBT	723A84	Dog	0	male	<0.09	-	0.33	-	-
TBT	720A84	Guinea pig	100	male	1.66	1.38	85.66	53.9	55.28
TBT	720A84	Guinea pig	0	male	<0.14	-	<0.14	-	-
TBT	722A84	mouse	100	male	<0.58	<0.48	39.94	25.2	25.2
TBT	722A84	mouse	0	male	<0.61	-	<0.31	-	-

			Dosis	Gender	Metabolite				Recovery
					TBBA		TBHA		
	Study	Species	(mg/kg bw)		mg/kg	%*	mg/kg	%**	%
TBB	736A84	Dog	100	male	1.48	1.35	1.51	1.04	2.39
TBB	723A84	Dog	100	male	2.57	2.34	0.54	0.37	7.78

TBB	720A84	Guinea pig	100	male	1.42	1.29	108.21	74.6	75.89
TBB	720A84	Guinea pig	0	male	<0.14	-	<0.14	-	-
TBB	722A84	mouse	100	male	0.59	<0.54	57.39	39.6	39.6
TBB	722A84	mouse	0	male	<0.61	-	<0.31	-	-

*% TBBA = TBBA in urine (µM/kg) / applied dose (µM/kg)*100

**% TBHA = TBHA in urine (µM/kg) / applied dose (µM/kg)*100

2.19 Study 19: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics in vitro / ex vivo

Title: Unnamed

Year: 1982

Materials and methods

Objective of study: metabolism

Principles of method if other than guideline:

- Incubation with 9000g supernatant of liver homogenates prepared from rats with the test article

- Incubation with hepatocytes isolated from rat liver with the test article

- Incubation with liver homogenates prepared from rats with the test article

GLP compliance: not specified

Test material

Radiolabelling: yes

Remarks: 14 C

Administration / exposure

Route of administration: other: not applicable

Results and discussion

The metabolism of the autoxydation product of Ro 82 -1763/002 (beta- 14C- lysmeral), Ro 83-2811 (lysmerylic acid) was studied in vitro in the 9000g supernatant of liver homogenate from male rats with and without phenobarbital induction, in rat liver homogenate and in rat liver hepatocytes.There was no metabolism of Ro 83-2811 under the experimental conditions neither in the 9000 g supernatant nor in the liver homogenate. In rat hepatocytes Ro 83-2811 was metabolized to Ro 02-3701 (p-tert-butylbenzoic acid (TBBA)) and to an unidentified metabolite up to 50% and 7%, respectively, during the period of 27 to 45.5 hours after plating

2.20 Study 20: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics in vitro / ex vivo

Title: Unnamed

Year: 2010

Materials and methods

Objective of study: metabolism

Principles of method if other than guideline:

Qualitative and quantitative investigation of the in vitro-metabolism of 14C-Lysmeral in microsomes and hepatocytes of rats, mice, rabbits and humans.

GLP compliance: yes (incl. QA statement)

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Cas Number: 80-54-6

Molecular formula: C14H20O

Analytical purity: 97.8 corr. area-% (12C-lysmeral)

Radiolabelling: yes

Test animals

Species: other: rat, mouse, rabbit, human

Strain: other: Han-Wistar rats, CD1-mice, white New Zealand rabbits

Sex: male

Details on test animals or test system and environmental conditions: not applicable

Administration / exposure

Route of administration: other: not applicable

Vehicle: DMSO

Details on exposure: not applicable

Duration and frequency of treatment / exposure: not applicable

Test concentrations: 10, 50, 100 µM

No. of animals per sex per dose / concentration: not applicable

Control animals: other: not applicable

Positive control reference chemical: The established positive control Testosterone was chosen to prove the validity of the systems, the applied incubation conditions as well as the methodology. (Additional comment: Testosterone is an appropriate positive control for in vitro-metabolism investigations, because in the Cytochrome P450 linked oxidation of Testosterone, specific oxidation sites can be attributed to specific isoenzyme activities. In addition, there is a long experience in the performing laboratory by applying Testosterone as a positive control which allows a comparison of received data with historical controls.)

Details on study design:

In vitro metabolism incubations in microsomes
Incubations with microsomes were carried out with 0.5 mg microsomal protein / ml incubate. The incubations were performed at 37°C for 2 hours in a cofactor containing buffer system. The cofactor was continuously reproduced by a NADPH generating system, consisting of Glucose-6-phosphate and Glucose-6-phosphate-dehydrogenase. In addition to the active incubations, heat deactivated control (HDC) incubations as well as controls that are directly stopped by the addition of one volume of acetone after the start of the enzymatic reaction ("t=0 controls") were performed. After incubation, the protein of incubates was precipitated by the addition of 1 vol. acetone and the supernatant was analysed for its metabolic profile. Recoveries were calculated by the ratio of the measured radioactivity in aliquots of the test substance preparation and the supernatants (after protein precipitation) of the incubates. When the recovery was below 80%, the activity was also analyzed in the protein pellet for mass balance calculation. All incubations were performed in triplicates. The isotope label was introduced to facilitate structure elucidation of formed metabolites. As positive control, Testosterone was incubated for 2 h at a final concentration of about 200

µM. Testosterone was added as a DMSO-stock solution. The final concentration of DMSO in the incubation mixture was 2.5 % (v/v). Controls were performed with heat deactivated in vitro-systems.

In vitro metabolism incubations in hepatocytes
The test substancewas incubated in hepatocytes in a serum free InVitroGRO HI medium. In addition to the active in vitro-system, directly stopped incubations (t=0) and medium controls (MC, without cells) were performed.
The incubations were performed at 37 °C in humidified atmosphere of 5% CO2under shaking. 500 000 cells/ml incubate were used for incubations. Incubations were performed with the test substance for about 4 hours in suspension in 24-well-plates.
DMSO, used as a carrier for the test substance was present with about 0.2 % (v:v).The incubations were stopped by adding 1 volume acetone and cells were solubilized by ultrasonication. After centrifugation the supernatant was analysed by Radio-HPLC. Recoveries were calculated by the measured radioactivity in aliquots of the medium at the beginning and at the end of each incubation. When the recovery in the medium was below 80%, the activity was also analyzed in the protein pellet for mass balance calculation. All incubates were performed in triplicates. Testosterone was incubated at a final test substance concentration of 200 µM as a positive control, to demonstrate the validity of the applied hepatocytes and the applied incubation conditions. Testosterone was added as a DMSO-stock solution. The final concentration of DMSO in the incubation was 0.2 % (v/v). After adding 1 volume of acetone, ultrasonication and centrifugation, aliquots of the resulted supernatants were analyzed directly without further purification.

Details on dosing and sampling:

Test substance preparation:
Microsomes:
For 100 µM lysmeral, a stock solution with a total concentration of 4 mM was prepared in DMSO (25 µl DMSO stock solution is added per ml microsomal incubate) containing a mixture of 12C-Lysmeral, 13C-Lysmeral and 14C-Lysmeral. The ratio of 12C-Lysmeral and 13C-Lysmeral was 1:1 (w:w). For 50 µM, a stock solution with a total concentration of 2 mM was prepared, containing a mixture of 12C-Lysmeral and 14C-Lysmeral. For 10 µM, a stock solution with a total concentration of 0.4 mM was prepared, containing 14C-Lysmeral.
For all concentrations used, specific activity of the DMSO solution was about 80 000 dpm / µl (correlating nominally to a specific activity of 2 000 000 dpm / ml microsomal incubate). This corresponded to a specific activity of lysmeral of about 100 000, 200 000 or 700 000 dpm/µg for the 100, 50, and 10 µM setup.

Hepatocytes:
For 10, 50 or 100 µM lysmeral in the cell culture medium, a stock solution with a total concentration of 5, 25 or 50 mM was prepared in DMSO (2 µl DMSO stock solution is added per ml medium). This stock solution contained a mixture of 12C-Lysmeral, 13C-Lysmeral and 14C-Lysmeral. The ratio of 12C-Lysmeral and 13C-Lysmeral was 1:1 (w:w) and the specific activity of the DMSO solution was about 500 000 dpm / µl (correlating nominally to a specific activity of 1 000 000 dpm / ml medium). This corresponded to a specific activity of Lysmeral of about 50 000, 100 000 or 400 000 dpm/µg for the 100, 50, and 10 µM setup.

Results and discussion

Details on absorption: not applicable

Details on distribution in tissues: not applicable

Details on excretion: not applicable

Metabolite characterisation studies

Metabolites identified: yes

Details on metabolites:

Microsomes:
In the microsomal incubates of all investigated species, 14C-Lysmeral was metabolized extensively and up to 9 metabolites could be separated by Radio-HPLC. All metabolites showed shorter retention times than

14C-Lysmeral and are therefore more polar than the parent compound from which they were formed. The metabolic pathway of 14C-Lysmeral in liver microsomes consists of two principles:
1) The oxidation of 14C-Lysmeral to its corresponding carboxylic acid (lysmerylic acid, M7).
2) The reduction of 14C-Lysmeral to its corresponding alcohol (lysmerol, M9) that was subsequently oxidized in a second step at the tert-butyl group of the molecule to form a hydroxy-metabolite of lysmerol (M3). These metabolites occurred in the microsomal incubates of all investigated species (rats, mice, rabbits and humans), but at different levels: Quantitatively, M3 was more pronounced in rats and rabbits than in mice and humans. Lysmerylic acid was quantitatively more pronounced in the metabolic profiles of rats and mice than in the metabolic profiles of rabbits and humans. Lysmerol had higher relative amounts in the metabolic profiles of humans than in those of rabbits, mice or rats.

Hepatocytes:
In hepatocytes of rats, mice and humans, 14C-Lysmeral was metabolized completely and in hepatocytes of rabbits, the metabolic turn over ranged from 99.2 – 99.7 %. In the experiments, up to 8 metabolites could be separated by Radio-HPLC. All metabolites showed shorter retention times than 14C-Lysmeral and are therefore more polar than the parent compound from which they were formed.
The metabolic pathway of 14C-Lysmeral in hepatocytes consists of the oxidation of 14C-Lysmeral to its corresponding carboxylic acid (lysmerylic acid, M7) and its dehydrogenation (most probably by hydroxylation and dehydration) to (E)-3-(4-tert-Butyl-phenyl)-2-methyl-acrylic acid (M16). A postulated decarboxylation of lysmerylic acid, followed by oxidation to the propanoic acid derivative and beta-oxidation leads to the identified metabolite p-tert-butyl-benzoic-acid (TBBA M15). This metabolite was conjugated with glycine to form p-tert-butyl-hippuric-acid (TBHA, M12) in rodents but not in rabbits or humans. In addition to these metabolites, glucuronic acid conjugates of metabolites M3, M7, M9, and M16 were identified.
Lysmerylic acid (M7) was quantitatively the main metabolite in hepatocytes of all species.
The metabolite M16 ((E)-3-(4-tert-Butyl-phenyl)-2-methyl-acrylic acid) was more pronounced in hepatocytes of rats (with amounts of 10.7 – 24.9 % ROI) than in hepatocytes of mice (5.8 – 9.7 % ROI) or humans (1.4 – 3.4 % ROI). M16 was not detected in hepatocytes of rabbits.
Species differences in metabolic profiles were seen for TBHA (M12) which was more pronounced in mice (with amounts of 4.9 – 27.1 % ROI) than in rats (3.5 – 3.6 % ROI). The conjugated metabolite M12 was not detected in incubates of hepatocytes of rabbits and humans.
Compared to other species, rats had the highest amounts of p-tert-butyl-benzoic-acid (TBBA, M15) in the metabolic profile and higher M15-levels were found with lower lysmeral concentrations in incubates (Table 1). Whereas this metabolite contributed to 8.3 – 29.3 % ROI in hepatocytes of rats, its amount was ≤ 0.5 % ROI in mice and ≤ 2.0 % ROI in rabbits. Furthermore, the levels observed in humans were found to be approx. 4 fold lower than in rat hepatocytes for all tested lysmeral concentrations, ranging from 1.9 – 7.5 % ROI. These highest amounts of TBBA in hepatocytes of rats versus human hepatocytes were statistically significant for all substrate concentrations.
Therewith species differences in the metabolism of lysmeral could be demonstrated in a quantitative (e.g. for M15 and M16) and qualitative (e.g. M12) manner.

Positive control incubations were carried out with the model substrate Testosterone that was incubated in the liver in vitro systems with a concentration of 200 µM and under identical incubation conditions as the test substance. In microsomes, Testosterone was metabolized to over 90 % in rats, 22.0 – 40.9 % in mouse, 40.7 – 56.9 % in human and 51.6 – 73.4 % in rabbit. In hepatocytes, Testosterone was metabolized under standard incubation conditions to over 95 % in all incubation series of all species.

Overall, it could be demonstrated that Testosterone was metabolized extensively in the applied in vitro systems, proving the activity of the systems and the appropriateness of the applied incubation conditions for all animal species tested.

The detected viability in hepatocytes incubated with lysmeral was for all test substance concentrations (0.1 – 500 µM) in all tested species (rats, mice, rabbits and humans) > 85 %. Triton X 100 treated hepatocytes (positive control) showed a viability of generally < 5%.

The recovery data demonstrate the capability of lysmeral to evaporate from in vitro incubates. This effect is observed in heat deactivated controls and medium control samples in which lysmeral was kept for 2 – 4

hours at 37°C. For the t=0 controls as well as for the active incubates acceptable recoveries (66 -92%) were detected within the performed experiments. No remarkable differences in the recovery values have been observed between species.

Table 1: Percentage of M15 (TBBA) in the metabolic profile of hepatocytes of tested species

Species	**Substrate conc.**	**ROI**	**SD**
	[µM]	[%]	[%]
Rat (*)	10	29.3	0.7
Mouse	10	0.0	0.0
Rabbit	10	1.3	0.2
Human (*)	10	7.5	0.4
Rat (*)	50	12.7	0.7
Mouse	50	0.5	0.3
Rabbit	50	2.0	0.4
Human (*)	50	3.1	1.3
Rat (*)	100	8.3	0.8
Mouse	100	0.0	0.0
Rabbit	100	1.3	0.7
Human (*)	100	1.9	0.3

2.21 Study 21: 2-(4-*tert*-butylbenzyl)propionaldehyde

Basic toxicokinetics in vivo

Title: Human metabolism and excretion kinetics of the fragrance lysmeral after a single oral dosage

Author: Scherer M, Koch HM, Schuetze A, Pluym N, Krnac D, Gilch G, Leibold E, Scherer G

Year: 2017

Bibliographic source: Int J Hyg Environ Health. 2017 Mar;220(2 Pt A):123-129. doi: 10.1016/j.ijheh.2016.09.005. Epub 2016 Sep 12.

Title: A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the human biomonitoring of non-occupational exposure to the fragrance 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral)

Author: Pluym N., Krnac D., Gilch G., Scherer M., Leibold E., Scherer G.

Year: 2016

Bibliographic source: Anal Bioanal Chem. 2016 Aug;408(21):5873-5882. doi: 10.1007/s00216-016-9702-x. Epub 2016 Jul 1.

Materials and methods

Principles of method if other than guideline:

Explorative study in human volunteers to develop a human biomonitoring method including identification of suitable biomarkers of exposure in human urine.

GLP compliance: no

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde

Cas Number: 80-54-6

Molecular formula: C14H20O

Test animals

Species: other: Human volunteers

Administration / exposure

Route of administration: other: oral

Vehicle: other: dissolved in ethanol and applied as a chocolate coated eatable waffle cup containing and approximately 20 mL coffee, milk or water

Duration and frequency of treatment / exposure: single exposure

Dose / conc.: 5.26 other: mg/subject

Results and discussion

Preliminary studies: A preliminary version of the analytical method was applied to a urine sample collected prior to and all fractions voided up to 48 h after using a lysmeral-containing sunscreen by a volunteer (male nonsmoker, 65 years old). Five grams of the sunscreen fortified to a lysmeral content of 6.5 mg/g was applied to the skin.
Lysmerol, lysmerylic acid, TBBA, and TBHA were present in their unconjugated form in percentages of 0.8, 7.0, 2.4, and 49.5 % of the total amount (conjugated and unconjugated) of the respective metabolite in urine, indicating that enzymatic hydrolysis of the conjugates is required as a first step in the sample work-up procedure.
Peak amounts of lysmerol and lysmerylic acid were excreted into the urine about 3–6 h after, whereas TBBA and TBHA appeared about 12 h after dermal application.
TBBA represented 0.67% of the applied dermal dose, followed by TBHA (0.04 %), lysmerol (0.02 %), and lysmerylic acid (0.012 %).
The lysmeral-related analytes measured in represented in total 0.75% of the dermally applied dose.

Toxicokinetic / pharmacokinetic studies

Details on excretion: The peak amounts of the 4 metabolites were excreted between 3 and 6 h after application with lysmerol and lysmerylic acid appearing slightly earlier in the urine than the secondary metabolites hydroxyl-lysmerylic acid and TBBA. A rapid urinary excretion was observed, since more than 90% of all measured lysmeral metabolites were excreted after 12 h, and the excretion was found to be complete by 48 h after the oral intake.

Toxicokinetic parameters:

half-life 1st: 1.19 h

Remarks: lysmerol

half-life 1st: 1.25 h

Remarks: lysmerylic acid

half-life 1st: 1.39 h

Remarks: hydroxyl-lysmerylic acid

half-life 1st: 1.40 h

Remarks: tert-butyl-benzoic acid (TBBA)

Tmax: 2.2 h

Remarks: lysmerol

Tmax: 4.64 h

Remarks: tert-butyl-benzoic acid (TBBA)

Tmax: 3.1 h

Remarks: lysmerylic acid

Tmax: 3.1 h

Remarks: hydroxyl-lysmerylic acid

Metabolite characterisation studies

Metabolites identified: yes

Details on metabolites: The sum of the 4 metabolites assessed in urine reflected about 16.5% of the applied dose. TBBA represented about 14.3% of the administered dose, followed by lysmerol, yielding 1.82% of the dose. The urinary fraction of hydroxy-lysmerylic acid and lysmerylic acid was 0.20% and 0.16% of the applied dose, respectively. Lysmeral itself was detectable after enzymatic deconjugation, but in very low amounts, i.e. <0.003% of the dose applied. Average times for peak excretion (tmax) were 2.2 h and 4.64 h for lysmerol and TBBA and 3.1 h for both lysmerylic acid and hydroxyl-lysmerylic acid. The elimination half-lives (t½) were found to be lower for the primary metabolites lysmerol and lysmerylic acid (1.19 h and 1.25 h, respectively) than for the secondary metabolites hydroxyl-lysmerylic acid and TBBA (1.39 h and 1.40 h, respectively), showing that the primary metabolites are excreted more rapidly.

Back-calculation based on the derived conversion factors resulted in median daily exposure doses of 224 µg/d lysmeral (range: 67-2218 µg/d) using all metabolites or 140 µg/d (range: 12-2249 µg/d) using all lysmeral specific metabolites (excluding TBBA as metabolite of potential lysmeral independent exposure sources).

2.22 Study 22: 2-(4-*tert*-butylbenzyl)propionaldehyde

Dermal absorption in vitro / ex vivo

Title: Unnamed

Year: 2016

Materials and methods

Guideline: OECD Guideline 428 (Skin Absorption: In Vitro Method)

GLP compliance: yes (incl. QA statement)

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral, BMHCA)

Cas Number: 80-54-6

Molecular formula: C14H20O

Radiolabelling: yes

Remarks: 3-14C

Administration / exposure

Details on in vitro test system (if applicable):

SKIN PREPARATION
- Source of skin: dermatomed human skin preparations; surgically removed skin from abdomen and/or breast
- Donors: age <65 years, without scars and strechmarks.

- Preparative technique: Dermatomized human skin preparations were supplied in suitable pieces frozen. Each skin preparation was hydrated in physiological saline for about 10 minutes before mounting to the diffusion cells which are filled up with physiological saline with protease inhibitor. The prepared diffusion cells were stored overnight in a refrigerator.
- Thickness of skin (in mm): 200 – 400 µm (217 - 389 µm, 261 – 400 µm, 200 – 389 µm, 238 – 386 µm for exp. 1&2, exp. 3&4, exp. 5&6, exp.7&8, respectively),
- Membrane integrity check: only visually intact skin with a TEER (impedance value) above 1 kΩ was used. Electrical resistance (TEER) was tested with a LCR bridge.
- Storage conditions: maximum storage time of 3 days in a refrigerator or 12 months at -20°C.

PRINCIPLES OF ASSAY
- Diffusion cells: Modified Franz cells, exposed skin area: 1 cm²
- Number of cells: 8 cells/group, minimum 4 donors/group
- Exposure time: 24 h
- Receptor fluid: tap water (exp. 1, 3, 5, 7) and tap water with 0.01 % (w/w), NaN3 (exp. 2, 4, 6, 8)
- Solubility of test substance in receptor fluid: The test substance is soluble in tap water with 0.033 g/L. Taking into account the amounts of a.i. administered for the high dose (target dose: 95 µg/cm²) and for the low dose (target dose: 5 µg/cm²), the amounts of test substance penetrated through the skin during the experiments as well as the volume of receptor fluid used (flow: 2.3 mL/h; corresponds to about 55 mL over the exposure period of 24 h), no rate limiting effects on the diffusion process by saturation of the aqueous receptor fluid were present.

- Flow-through system: continuous flow of 2.3 mL/h
- Test temperature: The temperature of the water was adjusted to 36 ± 1°C to realize a surface temperature of the skin preparation of 32 ± 1°C.
- Occlusion: charcoal filter and Fixomull® Stretch adhesive fleece to fix the filters after application (semi-occlusive conditions)
- Formuations: Four test-substance preparations (dose groups) of Lysmeral that reflect commercial applications were tested:
Formulation 1 - ethanol in water 70%
Formulation 2 - Oil in water
Formulation 3 - Water in oil
Formulation 4 - Silicone in oil

- Doses: The test-substance preparations were realized with pure 14C-Lysmeral and had the following target concentrations:
Dose group 1: 1.9 % 14C-Lysmeral in formulation 1
Dose group 2: 0.1 % 14C-Lysmeral in formulation 2
Dose group 3: 0.1 % 14C-Lysmeral in formulation 3
Dose group 4: 0.1 % 14C-Lysmeral in formulation 4
An application volume corresponding to about 5 mg/cm² was administered and lead to the following target doses of 14C-Lysmeral
Dose group 1: 95.0 µg Lysmeral / cm²
Dose group 2: 5.0 µg Lysmeral / cm²
Dose group 3: 5.0 µg Lysmeral / cm²
Dose group 4: 5.0 µg Lysmeral / cm²
Taking the specific activity of 14C-Lysmeral into account, the target doses correspond to the following radioactive target doses:
Dose group 1: 1035.5 kBq / cm²
Dose group 2: 54.5 kBq / cm²
Dose group 3: 54.5 kBq / cm²
Dose group 4: 54.5 kBq / cm²

- Experiments:
Experiment 1: dose group 1 (Formulation 1) 24h sampling, 24h skin wash, terminal procedure at 24h.

Experiment 2: dose group 1 (Formulation 1) 72h sampling, 24h+72h skin wash, terminal procedure at 72h. Methanol extraction of skin preparations.
Experiment 3: dose group 2 (Formulation 2) 24h sampling, 24h skin wash, terminal procedure at 24h.
Experiment 4: dose group 2 (Formulation 2) 72h sampling, 24h+72h skin wash, terminal procedure at 72h. Methanol extraction of skin preparations.
Experiment 5: dose group 3 (Formulation 3) 24h sampling, 24h skin wash, terminal procedure at 24h.
Experiment 6: dose group 3 (Formulation 3) 72h sampling, 24h+72h skin wash, terminal procedure at 72h. Methanol extraction of skin preparations.
Experiment 7: dose group 4 (Formulation 4) 24h sampling, 24h skin wash, terminal procedure at 24h.
Experiment 8: dose group 4 (Formulation 4) 72h sampling, 24h+72h skin wash, terminal procedure at 72h. Methanol extraction of skin preparations.
Experiment 9: dose group 1 (Formulation 1); Control group
Experiment 10: dose group 2 (Formulation 2); Control group

- Controls: Controls were performed to demonstrate the extractability of the test-substance on the skin preparations without exposure time. Therefore, the test-substance preparations were applied on untreated skin preparations and were extracted immediately after application.

- Sampling:
Receptor Fluid:
Experiment 1,3,5,7: pre-dose (collection about 15 minutes), 0-1; 1-2; 2-3; 3-4; 4-5; 5-6; 6-7; 7-8, 8-10; 10-12; 12-14; 14-16; 16-18; 18-21, 21-24 hours after application.
Experiment 2,4,6,8: pre-dose (collection about 15 minutes), 0-1; 1-2; 2-3; 3-4; 4-5; 5-6; 6-7; 7-8, 8-10; 10-12; 12-14; 14-16; 16-18; 18-21, 21-24, 24-48, 48-72 hours after application.
Samples for the mass balance:
Extract of charcoal filter
Post exposure wash: skin surface was thoroughly washed twice with washing fluid (Sodium-laurylethersulfate diluted 1:140 w/w in tap water). The washing fluid, tap water, pipette tips and cotton swabs used were stored for analysis. All parts of the diffusion cell (with the exception of the stainless steel clamp) were extracted in a suitable solvent.
Tape stripping: Twenty tape strips were taken. The tapes were pooled into three samples (the first 2 tapes as sample 1, the subsequent 9 tapes as sample 2 and the last 9 samples as sample 3) for analysis.
Remaining skin:
Experiment 1,3,5,7: The remaining skin was separated into dermis and epidermis by heat separation and was analyzed separately.
Experiment 2,4,6,8,9,10: The remaining skin were extracted immediately after the stripping procedure or application (for controls). Each skin piece was placed into Eppendorf reaction vials and two beads as well 0.8 mL methanol were added and extraction and homogenization was performed in the tissue lyzer following a defined protocol. The homogenates of each skin were centrifuged for 10 minutes at 4500 rpm. The supernatants were measured by LSC. Aliquots of the non extractable residues in the pellet were measured by LSC after addition of about 5 mL Soluene®-350 and incubation at room temperature for at least 3 days at room temperature.

- Test sample analyses: Liquid Scintillation Counting (LSC) of all study samples; prior digestion by Soluene for skin samples. High Performance liquid chromatography for radiochemical purity of stock preparation of 14C BMHCA and purity/ concentration in formulation vehicles.

Results and discussion

Time point: 24 h

Dose: 1.9% in Ethanol (70%)

Parameter: percentage

Absorption: 7.08 %

Remarks on result: other:

Remarks: (Absorbed dose) + (Epidermis + Dermis * 80%) = 5.31+((1.5+0.71)*(100-20)%) = 7.08

Time point: 24 h

Dose: 0.1 % in Oil-in-Water

Parameter: percentage

Absorption: 5.67 %

Remarks on result: other:

Remarks: (Absorbed dose) + (Epidermis + Dermis * 61%) = 4.77+((0.69+0.78)*(100-39)%) = 5.67%

Time point: 24 h

Dose: 0.1 % in Silicone-in-Water

Parameter: percentage

Absorption: 4.68 %

Remarks on result: other:

Remarks: (Absorbed dose) + (Epidermis + Dermis * 74%) = 3.5+((0.96+0.64)*(100-26)%) = 4.68%

Time point: 24 h

Dose: 0.1 % in Water-in-Oil

Parameter: percentage

Absorption: 5.83 %

Remarks on result: other:

Remarks: (Absorbed dose) + (Epidermis + Dermis * 68%) = 4.83+((0.74+0.73)*(100-32)%) = 5.83%

Table: Summary of [14C]-BMHCA penetration through and into human skin after single topical application of target doses of 95.0 µg/cm² and 5.0 µg/cm² of test substance formulated in different test-substance preparations expressed as % of applied doses.

%	Dose group		Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Exp. 6		Exp. 7		Exp. 8	
	Vehicle		EtOH 70%				Silicone in water				Water in oil				Oil in water			
	exposure/sampling		24h/24h	SD	24h/72h	SD	24h/24h	SD	24h/72h	SD	24h/24h	SD	24h/72h	SD	24h/24h	SD	24h/72h	SD
	target concentration	[mg/g]	19,0		19,0		1,0		1,0		1,0		1,0		1,0		1,0	
	target dose level of test substance	[µg/cm²]	95,0		95,0		5,0		5,0		5,0		5,0		5,0		5,0	
	mean actual nominal dose level test substance	[µg/cm²]	91,3		95,8		4,7		4,2		4,7		5,0		4,7		4,6	
Dislodgeable dose	charcoal filter	mean % of applied dose	55,35	2,37	56,49	12,93	55,13	4,15	62,88	4,48	32,87	6,61	28,24	9,99	35,11	4,41	23,92	3,10
	membrane washing after 24 hours	mean % of applied dose	11,60	3,55	13,20	4,00	14,45	6,41	12,28	1,76	53,85	8,85	50,37	13,07	47,75	5,01	56,23	5,43
	membrane washing after 72 hours	mean % of applied dose	-	-	1,80	1,19	-	-	2,78	0,88	-	-	1,21	0,97	-	-	0,82	0,33
	donor chamber washing	mean % of applied dose	1,65	1,46	1,99	1,06	4,03	2,47	2,62	1,85	1,06	0,68	0,96	0,61	1,71	0,90	0,92	0,47
	sum		68,60	3,77	73,48	12,35	73,61	5,57	80,55	4,28	87,77	4,85	84,86	14,82	84,58	4,40	81,89	5,14
Dose associated to tape strips	tape strip sample 1 (first two strips)	mean % of applied dose	0,90	0,33	1,52	1,17	1,19	0,42	0,88	0,46	1,16	0,75	0,62	0,51	1,88	0,77	0,22	0,17
	tape strip sample 2 (3-11)	mean % of applied dose	2,40	1,00	2,42	0,86	2,60	1,38	1,16	0,71	1,84	1,07	0,94	0,79	3,11	1,65	0,32	0,25
	tape strip sample 3 (12-20)	mean % of applied dose	1,02	0,85	0,31	0,24	0,58	0,27	0,12	0,16	0,26	0,13	0,11	0,17	0,41	0,17	0,02	0,03
Dose associated to remaining skin	epidermal membrane	mean % of applied dose	1,50	0,49	-	-	0,96	0,18	-	-	0,74	0,31	-	-	0,69	0,31	-	-
	dermal membrane	mean % of applied dose	0,71	0,28	-	-	0,64	0,23	-	-	0,73	0,35	-	-	0,78	0,17	-	-
	skin residue	mean % of applied dose	-	-	0,32	0,11	-	-	0,25	0,10	-	-	0,24	0,15	-	-	0,18	0,06

	skin extract	mean % of applied dose	-	-	1,31	0,33	-	-	0,71	0,24	-	-	0,50	0,48	-	-	0,28	0,12
Absorbed dose	sum receptor samples 0 - 24 h	mean % of applied dose	1,78	0,65	2,20	0,82	1,62	0,72	2,49	1,56	3,81	2,85	5,49	3,76	3,43	1,68	3,17	1,49
	receptor fluid	mean % of applied dose	0,04	0,02	0,00	0,00	0,03	0,01	0,00	0,00	0,13	0,08	0,00	0,00	0,12	0,03	0,00	0,00
	receptor chamber washing	mean % of applied dose	3,49	1,57	3,09	1,78	1,85	0,72	2,55	1,18	0,89	0,65	2,33	1,70	1,22	0,46	1,80	0,83
	sum		5,31	2,22	5,29	2,52	3,50	1,31	5,04	2,60	4,83	3,54	7,82	5,42	4,77	2,16	4,97	2,26
Total recovery		mean % of applied dose	80,44	1,83	84,67	13,80	83,08	3,28	88,72	2,97	97,32	3,91	91,01	13,82	96,21	2,98	87,88	3,44

2.23 Study 23: 2-(4-*tert*-butylbenzyl)propionaldehyde

Dermal absorption in vivo

Title: Unnamed

Year: 1995

Materials and methods

Qualifier: equivalent or similar to guideline

Guideline: OECD Guideline 427 (Skin Absorption: In Vivo Method)

GLP compliance: yes

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (Lysmeral, BMHCA)

Cas Number: 80-54-6

Molecular formula: C14H20O

Analytical purity of non-radioactive test substance: not determined

Radiolabelling: yes

Remarks: 14C

Test animals

Species: rat

Strain: other: Lister-Hooded (pigmented)

Sex: male

Details on test animals or test system and environmental conditions:

TEST ANIMALS
- Source: Charles River (U.K.)
- Age at study initiation: 5-7 weeks
- Weight at study initiation: 207 ± 6 g
- Individual metabolism cages: yes
- Diet: ad libitum (LAD1, Special Diet Services, Manea, UK, Batches 6562 and 6882)
- Water: ad libitum (tap water)
- Acclimation period: 7-13 days

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 21 ± 2
- Humidity (%): 50 ± 10
- Photoperiod (hrs dark / hrs light): 12/12

Administration / exposure

Type of coverage: occlusive

Vehicle: ethanol

Duration of exposure: 0.5; 1; 3; 6 h/ once a day (for more information: see "details on exposure; removal of test substance")

Doses: 6.75 mg/kg bw

No. of animals per group: 18

Control animals: no

Details on study design:

DOSE PREPARATION
- Preparation of test material: 18.54 mg (4 mCi, 215 .7 µCi/mg) 14C-BMHCA was diluted with ca. 520 mg non-radioactive BMHCA, dissolved in 2 ml hexane : ether (5 : 1 v/v) and the mixture repurified by silica gel column chromatography using hexane : ether (5 : 1 v/v).
- Identification of test material: The identity of 14C and non-radioactive BMHCA on silica gel thin-layer radiochromatograms was checked by EI-mass spectrometry prior to administration of topical dermal dose
- Dose Preparation: 14C-BMHCA, was accurately weighed (containing 3.8µCi/mg) and diluted in 70 % ethanol to provide two solutions containing 9.85 and 9.45 mg/ml, respectively (specific activity ca. 40 µCi/ml) for topical dermal administration on two consecutive days respectively. Each solution was prepared the day prior to dose administration. The concentration of the dose exceeded the target concentration by ca. 45 % and is thus a deviation from the protocol.

APPLICATION OF DOSE:
Amount per dose applied to skin: 0.2 ml (containing ca 8 µCi); concentration ca. 9.5 mg/ml (C14-lysmeral); -> ca. 0.2 mg/cm2

TEST SITE
- Area of exposure: back, (9 cm2)
- Type of cover / wrap if used: aluminium foil (occlusive) with gauze swab

REMOVAL OF TEST SUBSTANCE
- For animals sacrificed at times up to 6 h after dosing (0.5, 1, 3, 6h), the occlusions were removed at sacrifice and residual dose material at the site of application was removed using cotton wool swabs moistened with 70% ethanol. For animals sacrificed at later times (12, 24, 48, 72 and 120 h) the occlusions were removed at 6 h and residual dose material removed using ethanol-moistened cotton wool swabs. The treated area was then re-occluded with a clean piece of foil which was left in place until sacrifice .

SAMPLE COLLECTION
- Collection of blood: yes
- Collection of urine and faeces: yes
- Collection of expired air: yes
- Terminal procedure:
No of animals: 2 animals/sacrifice time point
Anesthesia: yes (halothane : oxygen mixture)
Method: cervical dislocation
- Analysis of organs: : Adrenals, lymph nodes, bone marrow , muscle, brain, pancreas, eyes, skin (treated skin separately), fat, small intestine (contents separately), heart, kidneys, spleen, large intestine (contents separately), stomach (contents separately), testes, liver, thymus, lungs, thyroid, carcass

Results and discussion

Signs and symptoms of toxicity: not specified

Dermal irritation: not specified

Percutaneous absorption

Dose: 6.75 mg/kg bw

Parameter: percentage

Absorption: 19 %

Remarks on result: other: 0-120 h

URINE

After topical application of 14C- BMHCA for 6 hours a mean cumulative total of 14.64% of dose was excreted in urine during 0- 120 h. A mean of 1.72% was excreted during 0- 6 h, 3.64% during 6 - 12 h, 5.62% during 12 - 24 h, 3.08 % during 24 - 48 h, 1.04 % during 48 - 72 h, 0.51 % during 72 - 96 h and 0.5% during 96-120h. The maximum urinary excretion rate (11.95 µg equivalents/hour) occurred during 6-12 hours. The excretion rate was about 11 µg/hour during 12-24 h and thereafter declined quite rapidly to 0.4 µg/hour during 72 - 120 h.

FAECES

Fecal excretion was low with a maximum excretion of about 2.04 % dose excreted during 0- 120 h.

RESPIRATED AIR

Radioactivity was not detectable in respirated air

Mean total recoveries of radioactivity in excreta (urine, feces, expired air) were 2.58 %, 6.92 %, 17.84 %, 15.96 %, 16.25 % and 17.57 % at 6, 12, 24, 48, 72 and 120 h respectively

Table 1: Total proportion of dose recovered from the site of application (on and in the skin), excreta and tissues

	Mean of 2 animals/time point (%)								
(h)	0.5	1	3	6	12	24	48	72	120
Dose removed from skin by washing at 6h or sacrifice before 6h	64.6	65.1	71.4	65.4	59.6	54.2	65.1	65.4	63.7
Dose on skin at sacrifice 12-120h	-	-	-	-	2.7	3.6	2.4	1.7	2.2
In skin at dose site at time of sacrifice	21.1	19.9	8.1	5.7	6.6	7.7	1.9	2.2	1.9
Excreta (urine, feces, expired air)	N	N	N	2.6	6.9	17.8	16.0	16.3	17.6
Tissues	3.5	4.1	9.5	12.6	11.1	6.2	2.0	1.5	1.2
Total % accounted for	89.3	89.2	88.9	86.3	86.8	89.6	87.4	86.1	86.5

N= not collected

TISSUE PHARMACOKINETICS

A comparison of Cmax values showed that in the liver Cmax was ca. 5-fold higher than in the large intestine, small intestine and stomach. Cmax values in the liver were ca. 15 - 32-fold higher than in untreated skin, pancreas and plasma. The times to reach these maximum levels (Tmax), were apparently directly related to the exposed blood perfusion rate of each tissue. Cmax was reached relatively rapidly in those tissues with a high perfusion rate (e.g. lungs, heart) and more slowly in poorly perfused tissues (e.g. skin, fat).

With the exception of the non-treated skin (i.e. not that of the dose site), the extent of exposure of each tissue to radioactivity (characterised by areas under the tissue radioactivity concentration-time curves), reflected the Cmax values. Overall, exposure of the liver to radioactivity was ca. 5 to 40 - fold higher than that of other tissues. In non-treated skin (i.e. not that of the dose site), radioactivity concentrations were more persistent than those in other tissues and the overall extent of exposure to radioactivity was relatively high. The tissue disposition of radioactivity was characterised by the respective terminal half-lives. In most tissues (large and small intestine, liver, lungs, stomach) radioactivity concentrations declined from peak levels with terminal half-lives of 34 - 93 h. Half-lives in heart, kidneys, plasma and blood (ca. 10 h) were presumably underestimated due to radioactivity concentrations falling below the quantification limit of the assay during the terminal phase. The relatively prolonged half-life in non-treated skin (152 h) is consistent with the

possibility of continued uptake of radioactivity throughout the tissue sampling period. Tissue concentrations of radioactivity relative to those in whole-blood (tissue: blood concentration ratio) tended to increase up to 12 hours post-dose at which time the ratio was > 1 in most tissues. Those tissues with relatively low tissue: blood concentration ratios (1) at 12 hours post-dose were brain, eyes, muscle, spleen, testes and thymus. In skin, the tissue : blood concentration ratio continued to increase up to at least 24 hours post-dose.

Mean relative concentrations found in tissues:

Table 1: Pharmacokinetic variables of radioactivity in plasma, whole-blood and selected tissues

	C Max (ng equiv./ml)	T Max (h)	AUC_1 (ng equiv.h/g)	AUC∞ (ng equiv.h/g)	Terminal rate constant (h)	Terminal half-life (h)
Fat	412	24	34882	-	-	-
Heart	1328	3	11732	12045	0.0644	10.8
Kidneys	568	6	15613	15793	0.0655	10.6
Large Intestines	2968	12	76247	86867	0.0110*	62.8
Liver	15601	6	337509	345304	0.0205	33.7
Lungs	261	1	7789	8597	0.0150*	46.1
Skin	930	24	74821	183203	0.0046*	151.6
Small intestine	2846	12	75341	77880	0.0177*	39.2
Pancreas	513	12	19382	-	-	-
Stomach	2518	0.5	16469	22610	0.0075*	92.8
Plasma	483	1	6190	8946	0.0492*	14
Whole blood	443	1	4580	6400	0.0521	13.3
Testes	150	1	-	-	-	-

* Period over which rate constant was calculated was 2-fold greater than the half-life itself; rate constants, half-lives and AUC∞ should only be regarded as approximations

2.24 Study 24: 2-(4-*tert*-butylbenzyl)propionaldehyde

Dermal absorption in vivo

Title: Unnamed

Year: 1994

Materials and methods

Guideline:

other: EEC Note for Guidance: Good Clinical Practice for Trials on Medicinal Products in the European Community (CPMP, 1990)

GLP compliance: yes

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral, BMHCA)

Cas Number: 80-54-6

Molecular formula: C14H20O

Radiolabelling: yes

Remarks: 14C BMHCA

Test animals

Species: human

Sex: male

Details on test animals or test system and environmental conditions:

TEST SUBJECTS
- Number of test subjects: 3
- Sex: male
- Age: mean/range 52 years/ 46-55 years
- Race/ethnicity: not reported
- Weight: Mean/range; 75.6 kg/70.8-82.0 kg
- Height: Mean/range; 175 cm/168-181 cm
- Diet: No consumption of xanthines containing food or drinks 10 h prior to dosing
- Fasting: from 10 pm (evening before dosing) till 4 hour post dosing
- Alcohol consumption: No consumption 24 h before dosing and throughout study period

Administration / exposure

Type of coverage: semiocclusive

Vehicle: ethanol

Duration of exposure: 6 hours

Doses: Dose: See table 1

No. of animals per group: 3

Control animals: no

Details on study design:

APPLICATION OF DOSE:
- Amount: 1 ml, given in 4 portions of 0.25 ml/portion
- Specific activity: 1.30 μCi/mg
- The treated area was recovered with gauze until 120 hours post dosing
-Sample preparation: An estimated 0.92 mg of the purified 14C-BMHCA (198 μCi) in solution was transferred to a preweighed volumetric flask and evaporated to dryness under a stream of argon gas . Non-radioactive BMHCA (151 .72 mg) was then added and the mixture was dissolved in 70% ethanol in water (10ml).

TEST SITE
- Area of exposure: back/100 cm2
- Type of cover / wrap if used: Gauze strips

REMOVAL OF TEST SUBSTANCE
Yes, removal by washing with ethanol soaked swabs

SAMPLE COLLECTION
- Collection of blood: yes, Blood samples (ca . 5 ml) were taken into heparinised tubes immediately before

application and at 0 .5,
1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, 48 and 72 hours afterwards . Blood samples were centrifuged soon after collection, and the plasma transferred to separate tubes .
- Collection of urine and faeces: yes, Urine samples were collected during the intervals 0- 2, 2 - 4, 4 - 6, 6 - 12, 12 - 24, 24 - 48,
48 - 72, 72 - 96, and 96 - 120 hours after application . Faeces samples were collected at 24-hour intervals for 5 days after application.
- Collection of expired air: no
- Terminal procedure: no
- Analysis of organs: no

SAMPLE PREPARATION
- Storage procedure: stored frozen at ca . -20°C
- Preparation details:
Urine samples were mixed thoroughly and after measurement of total volumes, duplicate 1 ml aliquots were mixed with MI31 scintillation fluid (10 ml) .
Faeces samples were homogenised in an approximately equal quantity of a water : methanol (1 : 1) mixture using a Colworth stomacher .
Weighed samples of plasma (0 .1 ml) were mixed with MI31 scintillation fluid (10 ml);
The swabs used to contain the dose application, those used to wash the site of application, and both sets of dressings used to cover the site of application, were subjected to Soxhlet extraction with methanol (250 ml) until radioactivity was no longer being extracted.
The "stripping tapes" applied to the skin at 6 hours and 120 hours were soaked in methanol for several days, and the residues were burned in oxygen in the automatic sample oxidiser . Weighed samples of the methanol extracts were mixed with M131 scintillation fluid .

ANALYSIS
Radioactivity was measured using an LKB 1410 liquid scintillation counter (LKB Wallac Oy, Turku, Finland) with automatic calibration using spectral quench parameters of the external standard . The validity of the calibration curve was checked at intervals of about 2 weeks . Samples were counted for at least 4 minutes or 900000 dpm . Radioactivity present at less than twice background in the sample under investigation was considered to be below the limit of accurate measurement and is recorded as not detected in the tabulated data . For the calculation of means ND was considered equal to zero.

Results and discussion

Dose: 10.3-13.1 mg

Parameter: percentage

Absorption: >= 0.8 - <= 2.4 %

Remarks on result: other: 0-120 h

A mean of 1.4% (range 0.8% to 2.4%) (table 2) of the applied radioactivity was excreted in urine by the three subjects within 24 hours. Radioactivity was below the limit of detection in all urine samples collected from 24 hours to 120 hours after application.

Radioactivity was below the limit of detection in faeces collected from all subjects during 0 to 120 hours after application.

Radioactivity in plasma samples was below the limit of detection at any time after application, corresponding to concentrations of less than 0.025 μg/ml.

A mean 63.12% ± 4.95 SD of the applied radioactivity was recovered from the gauze dressings used to occlude the site of application during 0 - 6 hours; a further mean 3.76% of dose ± 1 .95 SD was removed by

washing the skin with an ethanol-moistened swab at 6 hours and 3.06% ± 2.77 SD (range 1.4 - 6.3 %) was recovered from gauze dressings used to occlude the treated areas of skin during 6 - 120 hours.

The overall mean total recovery of radioactivity (excreta, gauze dressings, adhesive tape strippings and skin washings) following topical application of 14C-BMHCA was 71.43% ± 10.46 SD.

Table 1: Doses of 14C-BMHCA topically applied

Subject number	Dose dispended (µCi)	Amount of radioactivity absorbed by retaining gauze (µCi)	Dose administered	
			(µCi)	(mg)
1	20.76	3.83	16.93	13.1
2	20.76	7.41	13.35	10.3
3	21.16	7.33	13.83	10.7
Mean	20.89 ± 23	6.19 ± 2.04	14.70 ± 1.94	11.37 ± 1.51

Table 2: Excretion of radioactivity in urine collected for up to 120 h after topical application of 14 C-BMHCA to 3 healthy volunteers. Results are expressed as % dose

Sample collection time (h)	Subject number			Mean (SD)
	1	2	3	
0-2	0.01	0.02	ND	0.01
2-4	0.16	0.24	0.10	0.17 ± 0.07
4-6	0.24	0.18	0.16	0.19 ± 0.04
6-12	0.13	1.20	0.37	0.56 ± 0.56
12-24	0.30	0.76	0.26	0.44 ± 0.27
24-48	ND	ND	ND	-
48-72	ND	ND	ND	-
72-96	ND	ND	ND	-
96-120	ND	ND	ND	-
total	0.84	2.40	0.89	1.38 ± 0.89

Table 3: Proportions of radioactive dose recovered in swabs used for skin washing and in gauze dressings used to cover the site of application. Results are expressed as % dose.

Sample collection time (h)	Subject number			Mean (SD)
	1	2	3	
Ethanol moistened wash swab	3.52	5.82	1.95	3.76 ± 1.95
Gauze (0-6h)	60.09	68.82	60.45	63.12 ± 4.94

Gauze dressing (6-120 h)	1.56	6.26	1.36	3.06 ± 2.77
Total	65.23	81.09	63.85	70.06 ± 9.58

2.25 Study 25: 2-(4-*tert*-butylbenzyl)propionaldehyde

Toxicity to reproduction: other studies

Title: Inhibition of Hepatic Gluconeogenesis and Lipogenesis by Benzoic Acid, p-tert.-Butylbenzoic Acid, and a Structurally Related Hypolipidemic Agent SC-33459

Author: McCune S.; Durant P.; Flanders L.E.; Harris R.

Year: 1982

Bibliographic source: Archives of Biochemistry and Bioiphysics; Vol.214, No.1, March, pp. 124-133

Materials and methods

Principles of method if other than guideline: In vitro method using rat hepatocytes

GLP compliance: no

Test material

Purity: 99%

Test animals

Species: rat

Strain: Wistar

Sex: male/female

Details on test animals or test system and environmental conditions:

Hepatocytes were isolated from the livers of meal-fed female Wistar rats (220-280 g) or 48-h fasted male Wistar rats (180-220 g).

Administration / exposure

Details on study design:

The hepatocytes were incubated in 2 ml of Krebs-Henseleit buffer supplemented with 2.5% (w/v) bovine serum albumin under an atmosphere of 95% O2, 5% CO2 in stoppered 25-ml Erlenmeyer flasks. p-tert.-Butylbenzoic acid was dissolved in acetone and added to the incubation flasks. lncubations were conducted in a shaking water bath al 37°C aod for most experiments, terminated by adding 0.25 ml of 50% HCIO4 (w/v).
Metabolite assays were conducted on KOH-neutralized HClO4 extracts. For the determination of CoA and CoA esters, incubations were terminated by centrifuging an aliquot of the hepatocytc suspension at 1500g for 30s. Supernatants were discarded and the hepatocytes extracted once with 5% HClO4 (w/v) and twice with 3.5% HClO4 (w/v). CoA and acetyl-CoA were determined in the combined HCIO4 extracts.
The rate of fatty acid synthesis, expressed as micromoles of acetate equivalents per minute per gram wet weight of hepatocytes, was determined by incorporation of 3H2O into total lipid fatty acids. Oxidation of fatty acids was assayed by ketone body formation and the accumulation of acid-soluble radioactive products (mainly ketone bodies) and 14CO2 from [1-14C]oleate.

Results and discussion

- p-TBBA inhibited fatty acid synthesis (50% inhibition required 5-10 µM p-TBBA). Coadministration of glycine did not affect p-TBBA related inhibition. Coadministration of octanoate resulted in a less effective inhibition of the fatty acid synthesis by p-TBBA. In comparison, benzoic acid inhibited fatty acid synthesis with lower potency (50% inhibition required 70-80 µM Benzoic acid). Coadministration of glycine restored

benzoic acid inhibited fatty acid synthesis.
- p-TBBA did not affect glucose release
- p-TBBA increased lactate, pyruvat, medium- and long-chain acyl CoA levels (potentially p-tert.-butylbenzoyl-CoA)
- p-TBBA decereased citrate, CoA and acetyl-CoA levels
- p-TBBA inhibited gluconeogenesis from lactate (50% inhibition required 20 µM p-TBBA). In comparison, benzoic acid inhibited gluconeogenesis with lower potency (50% inhibition required 4 mM Benzoic acid). Coadministration of octanoate restored p-TBBA inhibited gluconegenesis. p-TBBA did not affect gluconeogenesis from proline or glycerol.
- p-TBBA decreased fatty acid oxidation (ketone body production from oleate and octanoate). Oleate/Octanoate dependent CO2 production was increased, indicating that acetyl-CoA was directed away from ketogenesis into the citric acid cycle.

2.26 Study 26: - 2-(4-tert-butylbenzyl)propionaldehyde

Toxicity to reproduction: other studies

Title: Unnamed

Year: 2017

Materials and methods

Principles of method if other than guideline:

Assessment of CoA conjugate formation using plated rat hepatocytes.

GLP compliance: no

Type of method: in vitro

Test material

EC Number: 201-289-8

EC Name: 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral, BMHCA)

Cas Number: 80-54-6

Molecular formula: C14H20O

Further materials tested:
3-(3-tert-butylphenyl)-2-methylpropanal (meta- Lysmeral);
3-(4-tert-butylphenyl)-2-methylpropanoic acid (Lysmerylic acid);
3-(p-tert-butylphenyl)-2-methylpropanol (Lysmerol);
3-(4-tert-butylphenyl)-propanal (BHCA)
3-(4-isopropylphenyl)-2-methylpropanal (PMHCA)
3-(4-isopropylphenyl)-propanal (PHCA)
3-(3-isopropylphenyl)-3-methylpropanal (miP2MHCA)
3-(4-isobutylphenyl)-2-methylpropanal (iBMHCA)
p-tert-butyltoluene (TBT)
p-tert-butylbenzoic acid (TBBA)
p-isopropylbenzoic acid
3-(4-ethylphenyl)-2,2-dimethylpropanal (Floralozone)
alpha-methyl-1,3-benzodioxole-5-propanal (Tropional)
3-(4-methoxyphenyl)-2-methylpropanal (Fennaldehyde)
3-(4-tolyl)propanal (Jasmorange)
3-(4-isobutyl-2-methylphenyl)propanal (Nympheal)
Benzoic acid
p-Hydroxy-benzoic acid
ethyl 4-hydroxybenzoate (Ethylparaben)

Results and discussion

p-Alkyl benzoyl-Coenzyme A formation from TBBA or Lysmeral in plated rat hepatocytes

TBBA is rapidly transformed top-tert-butyl-benzoyl-CoA and accumulates to stable levels within 0.5 – 4 h (see Figure 2 in the document attached)

Saturation observed at test concentrations (5,10,50 µM).

After Lysmeral addition, TBBA is formed rapidly and is conjugated to CoA.

Concentration of the TBBA-CoA conjugate remains stable over time indicating that CoA conjugated benzoic acid is not rapidly and/or quantitatively transferred to secondary acceptors such as glycine or taurine.

TBBA-CoA conjugate concentrations (1-2µM) are higher compared to endogenous oleoyl-CoA, palmitoyl-CoA, arachidonoyl-CoA (<0.1 µM) -> Accumulation of the TBBA-CoA conjugates in treated cells may competitively inhibit other CoA dependent cellular processes.

Formation of benzoyl-Coenzyme A conjugates for Lysmeral-like materials and correlation to rat male reproductive toxicity

Similar accumulation (sustained accumulation for 22 h incubation) of the corresponding alkyl-benzoyl-CoA conjugate was observed for a number of chemicals with a para-substituent at the benzyl ring (BMHCA-acid, BMHCA-alcohol, PMHCA, PHCA, iBMHCA, p-tert-butyltoluene, p-isopropyl benzoic acid). They alsoexhibit male reprotoxic effects in the rat.

No accumulation of the corresponding alkyl benzoyl-CoA conjugate (i.e. especially low levels at the 22 h time point) was observed for m-BMHCA, m-iP2MHCA, Floralozone, Tropional, Fennaldehyde, Jasmorange, Nympheal™, benzoic acid, p-hydroxy benzoic acid and ethylparaben. No reprotoxic effects were detected for these chemicals in the rat in vivo.

Table: Structures, adverse reprotoxic effects on male rats and accumulation of benzoyl-CoA conjugates in plated rat hepatocytes.All chemicals were tested at 50 µM and selected chemicals were also tested at 5 µM in plated rat hepatocytes. Benzoyl-CoA conjugates refer to the CoA-conjugate formed from the benzoic acid derived from the test chemical and conjugated to CoA. Benzoyl-CoA conjugates were quantified versus a synthetic sample of TBBA-CoA using LC-HRMS analysis. Data for individual repetitions are expressed as % versus the formation of TBBA-CoA from Lysmeral at the given time point and at the corresponding test chemical concentration. Lysmeral was tested in all experiments as reference. bd, below detection level; nd, not determined.*only dose tested.

Name	CAS Nr.	LOAEL for male reprotoxic effects in rat	Benzoyl-CoA-conjugate (50 µM)		Benzoyl-CoA-conjugate (5 µM)	
		(mg/kg bw/day)	4 h	22 h	4h	22h
3-(4-tert-butylphenyl)-2-methylpropanal (Lysmeral)	80-54-6	50 (Givaudan 1986A)	100	100	100	100
3-(3-tert-butylphenyl)-2-methylpropanal (meta-Lysmeral)	62518-65-4	> 450 (BASF SE 2011A)	18	24	8	13
3-(4-tert-butylphenyl)-2-methylpropanoic acid (Lysmerylic acid)	66735-04-4	50* (BASF SE 2006A)	90	94	90	79
3-(p-tert-butylphenyl)-2-methylpropanol (Lysmerol)	56107-04-1	50 (BASF SE 2011B)	62	123	119	67

3-(4-tert-butylphenyl)-propanal (BHCA)	18127-01-0	25 (100) (RIFM 2016, ECHA 2017F)	115	143	95	102
3-(4-isopropylphenyl)-2-methylpropanal (PMHCA)	103-95-7	75 (ECHA 2017A)	64	66	59	82
3-(4-isopropylphenyl)-propanal (PHCA)	7775-00-0	75 (RIFM 2016)	96	81	90	42
3-(3-isopropylphenyl)-3-methylpropanal (m-iP2MHCA)	125109-85-5	> 250 (ECHA 2017B)	2	2	nd	nd
3-(4-isobutylphenyl)-2-methylpropanal (iBMHCA)	6658-48-6	25 (RIFM 2016)	53	44	24	17
p-tert-butyltoluene (TBT)	98-51-1	15 (Furuhashi 2007B)	127	116	nd	nd
p-tert-butylbenzoic acid (TBBA)	98-73-7	6 – 8 (Hunter 1965)	110	98	108	84
p-isopropylbenzoic acid	536-66-3	15 (Givaudan 2011)	77	69	56	35
Floralozone (3-(4-ethylphenyl)-2,2-dimethylpropanal)	67634-15-5	> 250 (RIFM 2016)	1	bd	nd	nd
Tropional (a-methyl-1,3-benzodioxole-5-propanal)	1205-17-0	> 1000* (BASF SE 2010B)	9	10	nd	nd
Fennaldehyde (3-(4-methoxyphenyl)-2-methylpropanal)	5462-06-6	> 1000* (BASF SE 2011B)	12	16	nd	nd
Jasmorange (3-(4-tolyl)propanal)	41496-43-9	> 1000* (BASF SE 2011B)	6	3	nd	nd
Nympheal™ (3-(4-isobutyl-2-methylphenyl)propanal		> 500 (Laue 2017)	bd	bd	nd	nd
Benzoic acid	65-85-0	> 900 (ECHA 2017C)	1	bd	nd	nd
p-Hydroxy-benzoic acid	99-96-7	> 1000 (ECHA 2017D)	bd	bd	nd	nd
Ethylparaben (ethyl 4-hydroxybenzoate)	120-47-8	> 1043 (ECHA 2017E)	1	bd	nd	nd

Coenzyme A conjugate formation from other xenobiotic acids in vitro

Only para-alkyl benzoic acids with the alkyl substituent being at least an ethyl group, a sustained accumulation of the CoA conjugate was observed.

The carboxylic acid CoA conjugate of Lysmerylic acid is only transiently formed at low levels at early time points and not detectable at 22 h incubation in contrast to TBBA-CoA.

TBBA-glycine conjugate was found in plated hepatocytes when incubated with benzoic acid but not with Lysmeral or TBBA.

Comparison of p-alkyl benzoyl-Coenzyme A formation from Lysmeral in rat vs. human plateable hepatocytes indicates species specificity

Around 5-times lower TBBA-CoA levels were detected after 0.5 h incubation with Lysmeral in plated human versus rat hepatocytes (see Figure 3 in the document attached).

While stable levels of TBBA-CoA are formed in plated rat hepatocytes, a strong decrease over time is observed in human hepatocytes.

Comparable results were observed with TBBA as test chemical (see Figure 4 in the document attached).

In human hepatocytes, amounts and kinetics of TBBA-CoA and Lysmerylic acid-CoA during Lysmeral incubation were almost identical (i.e. rapid decrease and no accumulation), whereas different amounts of these two CoA conjugates were detected in rat hepatocytes (see Figure 5 in the document attached).

In human hepatocytes, kinetics of TBBA-CoA formation during Lysmeral or TBBA treatment are similar to those observed for a number of non-reprotoxic chemicals in rat hepatocytes such as e.g. m-BMHCA, Fennaldehyde (see Figure 6 in the document attached), Tropional and Jasmorange.

Intrinsic formation of an endogenous CoA conjugate as marker for cell metabolic capacity

Octanoyl-CoA is the most predominant CoA-conjugate formed in control cells and it is formed with a similar kinetic in plated rat and human hepatocytes (see Figure 7 in the document attached). -> The decrease of TBBA-CoA in human hepatocytes at 22h is not due to a human hepatocyte specific loss in the ability of CoA-conjugation over time.

2.27 Study 27: 2-(4-*tert*-butylbenzyl)propionaldehyde

Comparative in vitro metabolism study with 2-(4-tert-butylbenzyl)propionaldehyde

Publication: Laue et al. 2020. Benzoyl-CoA conjugate accumulation as an initiating event for male reprotoxic effects in the rat? Structure-activity analysis, species specificity, and in vivo relevance. Archives of Toxicology 94:4115–4129.

Method: Plated rat, rabbit and human hepatocytes were exposed to 5 or 50 µM 2-(4-tert-butylphenyl)-propionaldehyde for 0.5, 4, 8 (only rabbit hepatocytes) and 22 h in triplicate. Coenzyme A conjugates were analysed by LC-HRMS

Phase I and phase II metabolites were determined in rat and human hepatocytes by GC-MS and LC-HRMS at 0.5, 4 and 22 hours of exposure to 50 µM 2-(4-tert-butylphenyl)-propionaldehyde

The abstract from the publication is given below:

A number of para-substituted benzoic acids (p-BA) and chemicals metabolized to p-BA have been found to confer adverse effects in male rats on sperm viability, motility, and morphology. These effects are putatively associated with the metabolism of p-BA to toxic intermediates. We had shown that p-BA lead to accumulation of high levels of p-alkyl-benzoyl-CoA conjugates in plated primary rat hepatocytes. Here we further investigated the relevance of this metabolic pathway for the reprotoxic effects in rats and rabbits. We extended the structure-activity relationship to a set of 19 chemicals (nine reprotoxic and ten non-reprotoxic) and confirmed a very strong correlation between p-alkyl-benzoyl-CoA accumulation in rat hepatocytes and the toxic outcome. Species specificity was probed by comparing rat, rabbit and human hepatocytes, and p-

benzoyl-CoA accumulation was found to be specific to the rat hepatocytes, not occurring in human hepatocytes. There was also very limited accumulation in hepatocytes from rabbits that are a non-responder species in in vivo studies. Tissues of rats treated with 3-(4-isopropylphenyl)-2-methylpropanal were analysed and p-isopropyl-benzoyl-CoA conjugates were detected in the liver and in the testes in animals at toxic doses indicating that the metabolism observed in vitro is relevant to the in vivo situation and the critical metabolite does also occur in the reproductive tissue. These multiple lines of evidence further support benzoyl-CoA accumulation as a key initiating event for a specific group of male reproductive toxicants, and indicate a species-specific effect in the rat.

2.28 Study 28: 2-(4-*tert*-butylbenzyl)propionaldehyde

Biomonitoring study

Publication: Murawski et al. 2020. Metabolites of the fragrance 2-(4-tert-butylbenzyl)propionaldehyde (lysmeral) in urine of children and adolescents in Germany – Human biomonitoring results of the German Environmental Survey 2014-2017 (GerES V). International Journal of Hygiene and Environmental Health, 229:113594, 2020.

Method: Urine samples were collected in the population-representative German Environmental Survey for Children and Adolescents 2014-2017 from German residents aged 3-17 years (N=2133) with the aim to analyse urine metabolites of the fragrance lysmeral.

The abstract from the publication is given below:

The synthetic fragrance 2-(4-tert-butylbenzyl)propionaldehyde, also known as lysmeral, butylphenyl methylpropional, lilial, or lily aldehyde, is widely used in cosmetics, personal care products, laundry detergents, and air fresheners. It is classified as suspected to be harmful to fertility and possibly endocrine disrupting. Its maximum concentration in cosmetics is limited. First-morning void urine samples (N = 2133) were analysed for several metabolites of lysmeral (Chemical Abstract Service (CAS) No.: 80-54-6). Samples were collected in the population-representative German Environmental Survey for Children and Adolescents 2014-2017 (GerES V) from German residents aged 3-17 years. Four main metabolites tert-butylbenzoic acid, lysmerol, lysmerylic acid, and hydroxy-lysmerylic acid were found in quantifiable amounts in 100%, 99%, 40%, and 23% of the samples, respectively, with geometric mean concentrations of 10.21 μg/L (8.658 $\mu g/g_{crea}$) for tert-butylbenzoic acid, 1.528 μg/L (1.296 $\mu g/g_{crea}$) for lysmerol, and below the limit of quantification of 0.2 μg/L and 0.4 μg/L for lysmerylic acid and hydroxy-lysmerylic acid, respectively. Girls had higher urinary concentrations of lysmeral metabolites than boys. Usage of fragrances, fabric softener, and personal care products, especially perfume, was positively associated with urinary concentrations of lysmeral metabolites. Source identification builds a basis to derive proposals for reduction of exposure. These results can also provide the foundation for developing reference values for urinary metabolite concentrations of lysmeral in children and adolescents in Germany that will facilitate recognising future exposure trends.

2.29 Study 29: 2-(4-*tert*-butylbenzyl)propionaldehyde

Biomonitoring study

Publication: Scherer et al. 2021. Human biomonitoring in urine samples from the Environmental Specimen Bank reveals a decreasing trend over time in the exposure to the fragrance chemical lysmeral from 2000 to 2018. Chemosphere, 2021 Feb;265:128955.

Method: In total 329 urine samples from the Environmental Specimen Bank collected between 2000 and 2018 were analysed for metabolites of the fragrance lysmeral.

The abstract from the publication is given below:

2-(4-tert-butylbenzyl)propionaldehyde (trade names, e.g. lysmeral or lilial) is a fragrance chemical frequently used in cosmetic products where it is labelled as Butylphenyl methylpropional. A recently developed LC-MS/MS method for the analysis of four lysmeral metabolites (tert-butylbenzoic acid (TBBA), lysmerol, lysmerylic acid, and hydroxy-lysmerylic acid) was applied to 329 urine samples from the

Environmental Specimen Bank collected between 2000 and 2018. The two major metabolites TBBA and lysmerol were found in quantifiable concentrations in almost all samples in this study and correlated significantly. Hence, both analytes proved to be specific biomarkers indicating the broad exposure to lysmeral. A significant decline was found for TBBA and lysmerol for the monitored years with the most pronounced decrease from 2012 to 2015. The daily intake (DI) was used to evaluate potential health risks with respect to the derived no-effect level (DNEL) as a threshold for exposure of the general population. The median DI (1.63 µg/kg bw/d) and the 95th percentile (4.69 µg/kg bw/d) corresponded to 2.6% and 7.5% of the lowest DNEL (62.5 µg/kg bw/d for oral administration), respectively. Even though a decreasing trend in exposure was observed the data still calls for efforts to reduce the exposure towards lysmeral since metabolites of lysmeral were detected in nearly all samples and adverse effects cannot be excluded. Clearly, these results need to be substantiated by HBM campaigns in population representative samples like the German Environmental Survey in adults (GerES VI) to provide more robust data for the adult population.

3 HEALTH HAZARDS

3.1 Acute toxicity - oral route

Not evaluated

3.2 Acute toxicity - dermal route

Not evaluated.

3.3 Acute toxicity - inhalation route

Not evaluated.

3.4 Skin corrosion/irritation

Not evaluated.

3.5 Serious eye damage/eye irritation

Not evaluated.

3.6 Respiratory sensitisation

Not evaluated.

3.7 Skin sensitisation

Not evaluated.

3.8 Germ cell mutagenicity

Not evaluated.

3.9 Carcinogenicity

Not evaluated.

3.10 Reproductive toxicity

3.10.1 Animal data

3.10.1.1 Study 1: 3-(4-*tert*-butylphenyl)propionaldehyde

Reference*:* Unnamed 2019

Study period: 27 Dec 2018 to 16 Dec 2019

Test Guideline*:* OECD Guideline 422 (Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test)

GLP compliance: yes

Limit test: no

Justification for study design: ECHA requirement - Screening for reproductive/developmental toxicity (Annex VIII, Section 8.7.1.; OECD 421 or 422) in rats, oral route.

Justification of dose selection rationale: Doses were selected from a 14-day Dose-Ranger Finding study to determine the preliminary effects of 3-(4-tert-butylphenyl)propionaldehyde following daily exposure for 14 consecutive days to male and female rats, and to provide information for selection of dose levels to be used in subsequent OECD 422 study. A total of 40 Crl:CD(SD) Sprague Dawley P generation rats (20 rats/sex) were randomly assigned to dose groups, 5 rats/sex/group. Formulations of the test substance, 3-(4-tert-butylphenyl)propionaldehyde, or the control substance, Corn Oil, were administered orally by gavage three times (approximately 6 hours apart) daily for 14 consecutive days at 0 (Control), 5, 25, and 50 mg/kg/day.

Study parameters included: viability, clinical observations, body weights and body weight changes, food consumption, urinalysis (acid metabolites), macroscopic and microscopic observations, organ weights, and sperm evaluations (motility and concentration).

There were no 3-(4-tert-butylphenyl)propionaldehyde-related mortalities in females at any dose or males at 5 and 25 mg/kg/day. There were two mortalities at 50 mg/kg/day in males. One male was found dead on SD 14. Although there were no clinical signs prior to death or macroscopic findings during necropsy examination the likelihood of 3-(4-tert-butylphenyl)propionaldehyde-related toxicity cannot be eliminated. The other male was euthanized on SD 1 due to adverse clinical condition. This early death was considered by study authors to be unrelated to 3-(4-tert-butylphenyl)propionaldehyde because the death was attributed to the jugular blood collection procedure based on the macroscopic findings and timing of death. All other animals survived to scheduled euthanasia on SD 15.

3-(4-tert-butylphenyl)propionaldehyde-related clinical signs were limited to females in the 25 and 50 mg/kg/day dose groups, and included suspected dehydration (based on skin turgor) and a low incidence of hunched posture and thin appearance. There were no 3-(4-tert-butylphenyl)propionaldehyde-related clinical observations in males at any dose.

In females, mean body weight losses of -15.4 g and -33.0 g were observed at 25 and 50 mg/kg/day, respectively, compared to a mean body weight gain of +13.3 g in controls for the interval of SD 1 to 15. In addition, lower mean body weights were observed in females on SD 7 and SD 15 at 25 mg/kg/day (91% to 96% of control) and on SD 3 and SD 15 at 50 mg/kg/day (82% to 95% of control). There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on mean body weights or mean body weight gain in males at any dose.

Lower mean food consumption was observed in females at 25 mg/kg/day for the intervals of SD 1 to 5, SD 5 to 8, and SD 12 to 14 (84% to 94% of control) and at all tabulated intervals between SD 1 and SD 14 and

overall for the interval of SD 1 to 14 at 50 mg/kg/day (36% to 83% of control). There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on mean absolute food consumption in males at any dose.

At necropsy examination, 3-(4-tert-butylphenyl)propionaldehyde-related macroscopic findings were limited to decreased testicular size which was observed in one male at 50 mg/kg/day. In addition, 3-(4-tert-butylphenyl)propionaldehyde-related differences in mean absolute and relative organ weights were limited to the testes (50 mg/kg/day) and liver (≥5 mg/kg/day) in the males and the ovaries (≥25 mg/kg/day) and uterus (50 mg/kg/day) in females. Of note: due to prominently decreased food consumption and weight loss indicative of sustained negative energy balance in females, the relationship of uterine and ovarian findings cannot be definitively determined (direct test substance effect, vs. secondary to negative energy balance) due to the impact of negative energy balance on reproductive structures that are non-essential for survival. With the exception of the decreased ovarian weights, each of the changes in the testes, liver, and uterus had a histologic correlate of hypertrophy or atrophy/degeneration.

In males, 3-(4-tert-butylphenyl)propionaldehyde-related microscopic findings were observed in the testes at ≥5 mg/kg/day (vacuolation and degeneration of seminiferous tubular epithelium, Sertoli cell vacuolation) with secondary effects in the epididymides at ≥25 mg/kg/day (cribriform change, cellular debris, and hypospermia). The vacuolation noted in the seminiferous tubule was characterized by fine microvesicular vacuolation within the cytoplasm of seminiferous tubule epithelium and uniformly affected all stages of spermatogenesis (spermatogonia, spermatocyte, and spermatid). In females, 3-(4-tert-butylphenyl)propionaldehyde-related microscopic findings were observed in the uterus at 50 mg/kg/day (uterine atrophy). 3-(4-tert-butylphenyl)propionaldehyde-related microscopic findings were also observed in the liver of both males and females, including periportal to midzonal hepatocellular vacuolation at ≥5 mg/kg/day and centrilobular hepatocellular hypertrophy in males at ≥25 mg/kg/day and in females at 50 mg/kg/day.

3-(4-tert-butylphenyl)propionaldehyde-related effects in sperm motility were observed at all doses, including reductions in sperm motility at 5 mg/kg/day (77% vs. 84% in controls) and little to no sperm at ≥25 mg/kg/day (18% and 3%, respectively, vs. 84% in controls). All sperm samples that were analyzed at ≥25 mg/kg/day contained headless and detached sperm, with the exception of one sperm sample at 25 mg/kg/day. The infrequent increased spermatid head retention by the Sertoli cells, degeneration of maturing spermatids, round spermatids, and/or elongating spermatids, exfoliation/degeneration of germ cells, increased cellular debris, and moderate to marked hypospermia that was observed microscopically in the seminiferous tubules or epididymides may have contributed to the overall decrease in sperm motility.

On SD 1, 3-(4-tert-butylphenyl)propionaldehyde acid and 4-tBBA were below the limit of detection in all male and female predose plasma samples and from males and females administered 5 mg/kg/day. Mean 3-(4-tert-butylphenyl)propionaldehyde acid concentrations were below the level of detection or generally lower than 4-tBBA concentrations from 25 and 50 mg/kg/day samples and tended to be higher in females. Mean 4-tBBA concentrations were slightly higher in females at 25 mg/kg/day but were much similar to males at 50 mg/kg/day.

On SD 14, 4-tBBA plasma concentrations quickly increased and maintained steady state from 0.5 to 24 hr in males and females at all dose levels. At doses ≥ 25 mg/kg/day, 4-tBBA concentrations in females were almost twice that of males and increased in a nearly dose proportional manner. Mean 3-(4-tert-butylphenyl)propionaldehyde acid concentrations were below the limit of detection or ≤ 1.6 μM in males and females at 5 mg/kg/day. Overall, mean 3-(4-tert-butylphenyl)propionaldehyde acid concentrations at SD 14 were 1.5 to 6x and 9 to 15x lower at 25 and 50 mg/kg/day, respectively, then concentrations observed on SD 1.

Based on these results, the no-observed-adverse-effect level (NOAEL) for 3-(4-tert-butylphenyl)propionaldehyde could not be estab-lished in the 14-day DRF study. Therefore, the maximum tolerated dose was determined to be 5 mg/kg/d, for both males and females based on the adverse effects observed at this dose and with consideration to the significantly longer exposure time for animals in the OECD 422 study (orally by gavage once daily with P generation male rats actual 42 to 45 doses and P generation female rats actual 38 to 56 doses).

Test material

Reference substance name :3-(4-tert-butylphenyl)propionaldehyde,

EC Number: 242-016-2

Cas Number:18127-01-0

Reference substance name:3-(4-tert-butylphenyl)propionaldehyde

Details on test material:

- Name of test material (as cited in study report): 3-(4-tert-butylphenyl)propionaldehyde

- Physical state: liquid

- Analytical purity: 99.0%

- Lot/batch No.: VE00121957

- Expiration date of the lot/batch:October 15, 2011

Test animals

Species:rat

Strain:Sprague-Dawley

The Crl:CD(SD) strain was used because 1) it is one mammalian species accepted for use in toxicity studies and it has been widely used throughout industry; 2) this species and strain has been demonstrated to be sensitive to reproductive and developmental toxicants; and 3) historical data and experience exist at the Testing Facility.

Sex:male/female

- Source:Charles River Laboratories, Inc., Raleigh, NC,

- Females (if applicable) nulliparous and non-pregnant: yes

- Age at study initiation:29 to 35 days

- Weight at study initiation:191-230g for males, 283-329g for females

- Housing:Upon arrival, P generation rats were co-housed in solid-bottomed cages (2/sex/cage), except during the cohabitation period and postpartum periods (see Appendix 2, Deviations). During cohabitation, each pair of P generation rats was housed in the male rat's nesting box (1:1). P generation females with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed in situ were considered to be at Gestation Day (GD) 0 and assigned to individual housing in nesting boxes. Each P generation dam and delivered litter were housed in a common nesting box during the postpartum period, except during motor activity and functional observational battery testing. Following cohabitation, P generation males were returned to the previous premating cage mate and were monitored for incompatibility issues post-cohabitation.

Controls were housed on a separate rack from test substance treated rats (see Appendix 2, Deviations).

- Diet (e.g. ad libitum):Rats were given Certified Rodent Diet® #5002 pelleted food (PMI® Nutrition International) available ad libitum from individual feeders throughout the study, except during designated procedures

- Water (e.g. ad libitum):ad libitum from individual bottles attached to the nesting boxes

DETAILS OF FOOD AND WATER QUALITY:

The food was analyzed for environmental contaminants and results of the analysis are on file at the Testing Facility. There were no known contaminants in the food that would interfere with the objectives of the study.

Periodic analysis of the water was performed, and results of these analyses are on file at the Testing Facility.

ENVIRONMENTAL CONDITIONS

- Temperature (°C):19-23°C

- Humidity (%):40-70%

- Photoperiod (hrs dark / hrs light):12h/12h

IN-LIFE DATES:

Study Initiation Date: 27 Dec 2018

Initiation of Dosing: 15 Jan 2019

Completion of In-life: 12 Mar 2019

Administration / exposure

Route of administration:oral: gavage

Vehicle:corn oil

The control substance, Corn Oil, NF, was aliquoted once weekly for administration to Group 1, stored at room temperature until use, and dispensed as necessary. The control substance, Corn oil, NF to be used as the vehicle to prepare the test substance dose formulations had an assigned 7 day use period. Therefore, the Corn Oil, NF parent container was used for preparation and or/aliquoted in single use aliquots for up to 7 days from the initial date of opening of the parent container. The single use aliquots were used for the duration of the study, up to the date of expiration.

The test substance, 3-(4-tert-butylphenyl)propionaldehyde, was formulated in the control substance and prepared at the appropriate concentrations. The prepared test substance dose formulation was prepared once daily, and maintained at ambient conditions, protected from light throughout the duration of use for dose administration and/or sampling. The test substance dose formulation was fully used within 4 hours of preparation. The dosing formulations were stirred continuously for at least 30 minutes prior to dosing, throughout dose administration and post-dose sampling.

P generation male rats were administered the test or control substance formulations once daily via oral gavage beginning 14 days before cohabitation with treated females, during cohabitation and continuing through the day prior to scheduled euthanasia. P generation males were exposed to the test or control substance for 42 to 45 days.

P generation female rats were administered the test or control substance formulations once daily via oral gavage beginning 14 days before cohabitation with treated males and continuing through Lactation Day (LD) 12 (rats that deliver a litter) or GD 25 (rats that did not deliver a litter).

Doses were adjusted based on the most recently recorded body weight. The gavage needle was wiped clean prior to dose administration for each rat.

F1 generation pups were not directly exposed to the test or control substance, but may have been possibly exposed during maternal gestation (in utero exposure), via maternal milk during the lactation period, or from exposure to maternal urine/feces. The doses were selected from a 14-day Dose-Range Finding (DRF) study (CRL 20153551). Based on this study the maximum concentration dose was determined to be 5 mg/kg/dose. Animals received the test material or vehicle control formulations orally at a volume-dosage of 15 ml/kg/dose.

Details on mating procedure: During cohabitation, each pair of rats was housed in the male rat’s nesting box (1:1). P generation females with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed in situ was considered to be at GD 0 and assigned to individual housing in nesting boxes. Each dam and delivered litter were housed in a common nesting box during the postpartum period, except during motor activity and functional observational battery testing. Following cohabitation, P

generation males were returned to the previous premating cage mate and were monitored for incompatibility issues post-cohabitation.

Analytical verification of doses or concentrations:yes

Details on analytical verification of doses or concentrations: Duplicate (1 mL, aliquot weights to be measured to at least 0.001 g) sets of top, middle, and bottom test substance samples for the sampling time points were sent to the analytical laboratory. Concentration results were considered acceptable if mean sample concentration results were within or equal to ± 15% of theoretical concentration. Each individual sample concentration result was considered acceptable if it was within or equal to ± 20% of theoretical concentration. For homogeneity, the criteria for acceptability were a relative standard deviation (RSD) of concentrations of ≤ 10% for each group.

There was no 3-(4-tert-butylphenyl)propionaldehyde detected in the control samples for all pre- and postdose first, mid study, and last preparation samples.

Duration of treatment / exposure: 3-(4-tert-butylphenyl)propionaldehyde, or the control substance, corn oil, were administered orally by gavage through mating and continuing for at least 28 days (P generation male rats) or through parturition until Day 12 of lactation (P generation female rats) at 0 (Control), 0.5, 1, and 5 mg/kg/dose.

Frequency of treatment:Once daily beginning before cohabitation.

Details on study schedule:

Study Initiation Date: 27 Dec 2018

Initiation of Dosing: 15 Jan 2019

Completion of In-life: 12 Mar 2019

Experimental Start Date (OECD): 27 Dec 2019

Experimental Start Date (EPA): 15 Jan 2019

Experimental Completion Date (OECD): 09 Dec 2019

Experimental Termination Date (EPA): 09 Dec 2019

Doses/concentrations:

1 Dose / conc.:0.5 mg/kg bw/day (nominal)

2 Dose / conc.:1 mg/kg bw/day (nominal)

3 Dose / conc.:5 mg/kg bw/day (nominal)

No. of animals per sex per dose:10

Control animals:yes

Details on study design: P generation male rats were administered the test or control substance formulations once daily via oral gavage beginning 14 days before cohabitation with treated females, during cohabitation and continuing through the day prior to scheduled euthanasia. P generation males were exposed to the test or control substance for 42 to 45 days. P generation female rats were administered the test or control substance formulations once daily via oral gavage beginning 14 days before cohabitation with treated males and continuing through Lactation Day (LD) 12 (rats that deliver a litter) or GD 25 (rats that did not deliver a litter).

F1 generation pups were not directly exposed to the test or control substance, but may have been possibly exposed during maternal gestation (in utero exposure), via maternal milk during the lactation period, or from exposure to maternal urine/feces.

Doses were adjusted based on the most recently recorded body weight. The gavage needle was wiped clean prior to dose administration for each rat.

The in-life procedures, observations, and measurements listed below were performed for all P generation rats:The rats were assessed for viability at least twice daily during the study.

General Appearance: The P generation rats were observed for general appearance at least once during the acclimation period, at least once weekly during the predose estrous evaluation (females), daily during the exposure period, and on the day of scheduled euthanasia.

Detailed Clinical observations: The P generation rats were observed for detailed clinical observations once prior to initiation of exposure (baseline), and once weekly thereafter. During the weeks of Functional Observational Battery (FOB) testing, detailed clinical observations were not

conducted on the 5 rats/sex/group.

Postdose Observations: Postdose observations were recorded between 1 and 2 hours following each daily dose administration.

Body Weights: P generation male rats had body weights recorded on the day after arrival at the Testing Facility, on the first day of exposure, at least once weekly thereafter, and on the day of scheduled euthanasia.

P generation female rats had body weights recorded on the day after arrival at the Testing Facility, on the first day of exposure, at least once weekly thereafter, and on GD 0, 4, 7, 11, 14, 17, 20, and 25 (for P generation females with no confirmed mating date), and on LD 0, 3, 6, 9, and 12. A terminal weight was also recorded.

Food Consumption: In P generation males, food consumption was recorded at least once weekly during the exposure period and once during the week of euthanasia (food left value). In P generation females, food consumption was recorded at least once weekly during the exposure period, on GD 0, 4, 7, 11, 14, 17, 20, and 25 (for P generation females with no confirmed mating date) and on LD 0, 3, 6, 9, and 12.

Estrous Cycle Evaluations: Estrous cycles were evaluated by examining the vaginal cytology of samples obtained by vaginal lavage. Samples were collected from P generation females beginning 13 days prior to treatment, the first 14 days of treatment and during cohabitation, and then until spermatozoa were observed in a smear of the vaginal contents and/or a copulatory plug was observed in situ during the cohabitation period. Also, on the day of scheduled euthanasia (LD 13), an examination of vaginal cytology was performed prior to necropsy examination to determine the stage of estrous cycle.

Cohabitation: P generation rats were assigned to cohabitation (i.e., pairing), one male per one female (within each dose group). The cohabitation period consisted of a maximum of 14 days. P generation females that did not mate with a P generation male within the first 7 days of cohabitation were assigned an alternate P generation male (same dose group) that successfully mated with a P generation female from the same dose group and remained in cohabitation for a maximum of 7 additional days. P generation females observed with spermatozoa in a smear of the vaginal contents and/or a copulatory plug observed in situ were considered to be GD 0 and assigned to individual housing. P generation female 1420 (Group 1, 0 mg/kg/dose) did not mate after completion of the 14-day cohabitation period and was considered to be GD 0 on the last day of cohabitation. This female was assigned to individual housing (solid bottom cage) and euthanized at the discretion of the Study Director.

Natural Delivery Observations: P generation females were evaluated for adverse clinical signs, the duration of gestation (GD 0 to the time the first pup was observed), litter size (defined by all pups delivered), and pup viability at birth.

Maternal Observations: Maternal observations were recorded daily during the postpartum period.

Functional Observational Battery (FOB): A functional observational battery (FOB) evaluation was conducted on one occasion for 5 rats/sex/group, during the exposure period, when possible. Due to early deaths, the evaluations were conducted on 4 female rats in Groups 1, 2, and 3 to ensure sufficient data for clinical pathology analysis. The FOB 3,4,5,6 was conducted by an observer who was unaware of the group assignment of the rat. The observer examined the rat in its home cage, while handling the animal, and/or in an open field to assess parameters including, but not limited to the following: lacrimation, salivation, palpebral closure, prominence of the eye, pupillary reaction to light, piloerection, respiration, and urination and defecation (autonomic functions); sensorimotor responses to visual, acoustic, tactile and painful stimuli (reactivity and sensitivity); reactions to handling and behavior in the open field (excitability); gait pattern in the open field, severity of gait abnormalities, air righting reaction, visual placing response, and landing foot splay (gait and sensorimotor coordination); forelimb and hindlimb grip strength; and abnormal clinical signs

including but not limited to convulsions, tremors and other unusual behavior, hypotonia or hypertonia, emaciation, dehydration, unkempt appearance and deposits around the eyes, nose, or mouth. Body temperature was measured at the completion of the FOB.

Motor Activity Evaluation:

Motor activity was conducted on one occasion during the exposure period using the same five rats/sex/group that were selected for FOB evaluation, when possible.

The rats were placed in an individual enclosure held within a Smartframe containing 7 x 15 photobeams utilizing infra-red pyroelectric detectors. Movement was detected in 2 dimensions anywhere in the enclosure and was differentiated into fine movement and ambulation. Each animal was monitored for one session of 60 minutes. For the purpose of data tabulation, activity data files were reduced to Excel® format into successive periods of 10 minutes each at the completion of testing. Fine movements and ambulation were analyzed in these six 10-minute periods and compared across the dose groups.

In-life Procedures, Observations, and Measurements - F1 Generation

Preweaning: The in-life procedures, observations, and measurements listed below were performed for all F1 generation litters, with the litter as the unit of measure.

Viability Checks: Litters were observed for dead pups at least twice daily and the pups in each litter were counted once daily during the preweaning period.

Clinical Observations: Litters were observed at least once daily.

Body Weights: F1 generation pups had body weights recorded on Day 0 (birth), 3, 6, 9, and 12 postpartum.

Anogenital Distance: On Day 0 (birth) anogenital distance was recorded for all F1 generation pups using a calibrated stereomicroscope, micrometer, and ruler. The anogenital distance was measured from the cranial edge of the anus, which comes to a point, to the base of the

genital tubercle.

Nipple Retention: On Day 12 postpartum (LD 12) nipple presence was evaluated and the number present was recorded for all F1 generation male pups.

Laboratory Evaluations:

Clinical Pathology: Blood samples were collected under isoflurane/oxygen anesthesia from the inferior vena cava from 5 rats/sex/group.

Hematology Parameters:Red blood cell count; Hemoglobin concentration; Hematocrit; Mean corpuscular volume; Red blood cell distribution width; Mean corpuscular hemoglobin concentration; Mean corpuscular hemoglobin; Reticulocyte count (absolute); Platelet count; White blood cell count; Neutrophil count (absolute); Lymphocyte count (absolute); Monocyte count (absolute); Eosinophil count (absolute); Basophil count (absolute); Large unstained cells.

Coagulation Parameters: Activated partial thromboplastin time; Fibrinogen; Prothrombin time

Clinical Chemistry Parameters: Alanine aminotransferase, Aspartate aminotransferase, Alkaline phosphatase, Gamma-glutamyltransferase, Creatine Kinase, Total bilirubina, Urea nitrogen, Creatinine, Calcium, Phosphorus, Total protein, Albumin, Globulin (calculated), Albumin/globulin ratio, Glucose, Cholesterol, Triglycerides, Sodium, Potassium, Chloride.

Thyroid Sample Collection: P Generation On the day of scheduled euthanasia, blood samples were collected from all P generation male and female rats. F1 Generation Pups: On Day 3 postpartum, blood samples were collected from 2 culled pups/litter/group. On Day 12 postpartum, blood samples were collected from 1 pup/sex/litter/group.

Thyroid Sample Analysis: Serum samples were analyzed for Thyroxine (T4) levels using a validated analytical procedure.

Ovarian and Uterine Examinations: The reproductive tract was dissected from the abdominal cavity. The number of implantation sites was recorded.

Necropsy: All P generation males and females were subjected to a complete necropsy examination.

Organ Weights: The organs were weighed at necropsy for all P generation rats at scheduled euthanasia. Paired organs were weighed together, unless otherwise indicated.

Tissue Collection and Preservation: Representative samples of the tissues were collected from all rats and preserved in 10% neutral buffered formalin.

Histology: Tissues were embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin.

Histopathology:Histopathological evaluation was performed by a board-certified veterinary pathologist. The following tissues were evaluated:

• Thyroid and parathyroid were evaluated from all P generation males and females in the control and high dose groups.

• Gross lesions were evaluated in all P generation males and females in all groups.

• Tissues identified were evaluated from all P generation males and females in the control and high dose groups.

• Special attention was paid to the stages of spermatogenesis in the testes, epididymides, and interstitial testicular cell structures.

• Target tissues identified by the study pathologist during microscopic evaluation were communicated to the Study Director; tissues were evaluated, processed, and reported.

Thyroid and parathyroid were evaluated from one F1 generation pup/sex/litter in the control and high dose groups

STATISTICAL ANALYSIS: Descriptive statistics including number, mean, percentages and/or standard deviation were reported as appropriate.

Positive control:none

Examinations

Parental animals: Observations and examinations:

CAGE SIDE OBSERVATIONS: Time schedule: twice daily during the study

DETAILED CLINICAL OBSERVATIONS: Time schedule

BODY WEIGHT: Time schedule for examinations:once weekly

FOOD CONSUMPTION AND COMPOUND INTAKE:Food consumption for each animal determined and mean daily diet consumption calculated as g food/kg body weight/day. Compound intake calculated as time-weighted averages from the consumption and body weight gain data.

FOOD EFFICIENCY: No

WATER CONSUMPTION AND COMPOUND INTAKE (if drinking water study): No

HAEMATOLOGY: Time schedule for collection of blood:

- Anaesthetic used for blood collection: Yes isoflurane/oxygen anesthesia

- Animals fasted: Yes

- How many animals:5/sex/group

CLINICAL CHEMISTRY:

-Time schedule for collection of blood:

- Animals fasted: Yes

- How many animals: :5/sex/group

- Parameters checked in table [No.?] were examined.

URINALYSIS:

- Time schedule for collection of urine:5/sex/group

- Metabolism cages used for collection of urine: Yes

- Animals fasted: Yes

NEUROBEHAVIOURAL EXAMINATION:

- Time schedule for examinations:

- Dose groups that were examined:all groups

- Battery of functions tested: sensory activity / grip strength / motor activity / other: Functional Observation Battery, Motor Activity.

Oestrous cyclicity (parental animals):

Daily vaginal lavage was performed beginning 14 days prior to treatment (pretest period), the first 14 days of treatment and during cohabitation, until evidence of spermatozoa were observed in a smear of the vaginal contents and/or a copulatory plug was observed in situ during the cohabitation period. On the day of scheduled euthanasia (LD 14), an examination of vaginal cytology was performed prior to necropsy examination to determine the stage of estrous cycle.

Estrous cycles were evaluated by examining the vaginal cytology of samples obtained by serial vaginal lavage procedures.

Sperm parameters (parental animals):Stage dependent qualitative evaluation of spermatogenesis in the testis, epididymides, and interstitial testicular cell structures was performed.

Litter observations:

- Litter Size (defined as all pups delivered);

- Litters were observed for dead pups at least twice daily;

- F1 generation pups had body weights recorded on Day 0 (birth), 3, 6, 9, and 12 postpartum;

- Anogenital distance was recorded for all F1 generation pups using a calibrated stereomicroscope;

- Nipple presence was evaluated and the number present (if any) was recorded for all F1 generation male pups;

Postmortem examinations (parental animals):

- Clinical Pathology;

- Coagulation;

- Clinical Chemistry;

- Hematology;

- Thyroid Sample Analysis and Evaluation;

- Ovarian and Uterine Examinations;

- Necropsy;

Necropsy

Postmortem examinations (offspring):

- Thyroid hormone (T4 assessment);

- Thyroid and parathyroid gland (histology);

- Organ Weights;

- Tissue Collection and Preservation

Statistics:

Descriptive statistics including number, mean, percentages and/or standard deviation were reported as appropriate. Litter values were used where appropriate. Additional procedures and/or analyses may be performed, if appropriate. Clinical and necropsy observations data were summarized but no inferential statistical analysis were performed. Statistically significant pair-wise comparison probabilities were reported as either $p \leq 0.05$ or $p \leq 0.01$, unless otherwise noted below.

Reproductive indices:

- Duration of Gestation: The duration of gestation was calculated from GD 0 to the day the first pup is observed;

- Fertility Index: Percentage of matings that result in pregnancies. (Number of Animals Pregnant x 100)/ (Number of Animals Mated);

- Mating Index: Percentage of animals in cohabitation that mated. (Number of Animals Mated x 100)/ Number of Animals in Cohabitation

- Pregnancy Rate: Percentage of animals in cohabitation that result in pregnancies. (Number of Animals Pregnant x 100)/ (Number of Animals in Cohabitation)

- Gestation Index: Percentage of pregnancies that result in birth of live litters. (Number of Animals with Live Offspring x 100)/(Number of Animals Pregnant)

- Number of offspring per litter: Live and dead pups;

- Number of implantation sites;

- General condition of dam and litter during the postpartum period

Offspring viability indices:

- Viability Index: Percentage of pups born that survive 4 days postpartum. (Number of Live Pups on Day 4 Postpartum x 100)/(Number of Liveborn Pups on Day 1 Postpartum);

- Lactation Index: Percentage of pups that survive 134 days postpartum. (Number of Live Pups on Day 134 Postpartum x 100)/ (Number of Live Pups on Day 4 Postpartum)

Results and discussion

Results: P0 (first parental generation)

General toxicity (P0)

Clinical signs:All P generation males survived to scheduled euthanasia, and there were no 3-(4-tert-butylphenyl)propionaldehyde-related clinical signs in the P generation males at any dose. Mean body weights, mean body weight gains, and mean food consumption values were similar across all groups in the P generation males. There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on any neurobehavioral parameter (functional observation battery or motor activity) at any dose. There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on any mating and fertility parameter in the P generation males at any dose. There were no 3-(4-tert-butylphenyl)propionaldehyde-related macroscopic or microscopic observations or alterations in organ weights at any dose. In addition, there were no 3-(4-tert-butylphenyl)propionaldehyde related effects on hematology, clinical chemistry, or coagulation parameters in the P generation males at any dose. There were no 3-(4-tert-butylphenyl)propionaldehyde-related changes in serum T4 concentrations in the P generation males at any dose. In the P generation males, mean serum T4 concentrations were 104%, 87%, and 90% of controls in the 0.5, 1, and 5 mg/kg/dose groups, respectively, on DS 43/44. This effect was not dose-dependent and not associated with any macroscopic or microscopic observations or alteration in thyroid weight.

Mortality:There was no 3-(4-tert-butylphenyl)propionaldehyde-related mortality in the P generation females at any dose.

Body weight and weight changes:no effects observed

Food consumption and compound intake :no effects observed

Food efficiency:no effects observed

Water consumption and compound intake:no effects observed

Haematological findings:All differences in hematology parameters were considered unrelated to 3-(4-tert-butylphenyl)propionaldehyde because: 1) the observations were not dose dependent; 2) the differences were of small magnitude; and/or 3) the differences were inconsistent in direction.

Clinical biochemistry findings:There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on clinical chemistry parameters in the P generation males and females at any dose.

All differences in clinical chemistry parameters, including those that reached statistical significance, were considered unrelated to 3-(4-tert-butylphenyl)propionaldehyde because: 1) the observations were not dose dependent; 2) the differences were of small magnitude; and/or 3) the differences were inconsistent in direction.

There were no 3-(4-tert-butylphenyl)propionaldehyde-related changes in serum T4 concentrations in the P generation males at any dose. In the P generation males, mean serum T4 concentrations were 104%, 87%, and 90% of controls in the 0.5, 1, and 5 mg/kg/dose groups, respectively, on DS 43/44. This effect was not dose-dependent and not associated with any macroscopic or microscopic observations or alteration in thyroid weight.

Behaviour (functional findings):no effects observed

Description (incidence and severity):There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on any neurobehavioral parameter (functional observation battery or motor activity) at any dose.

There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on ambulation or fine movement during motor activity testing in the P generation males at any dose.

Organ weight findings including organ / body weight ratios:effects observed, non-treatment-related

Histopathological findings: non-neoplastic:The test substance, 3-(4-tert-butylphenyl)propionaldehyde, and vehicle control formulations were administered to male and female Crl:CD(SD) Sprague-Dawley rats at doses of 0, 0.5, 1, or 5 mg/kg/dose by once daily oral gavage 7 days a week for a minimum of 28 days. Parental (P generation) male rats were administered the test or control substance formulations once daily via oral gavage beginning 14 days before cohabitation with treated females, during cohabitation and continuing through the day prior to scheduled euthanasia on Days 43 through 46. P generation males were exposed to the test or control substance for a minimum of 28 days. P generation female rats were administered the test or control substance formulations once daily via oral gavage beginning 14 days before cohabitation with treated males and continuing through Lactation Day 12 (rats that delivered a litter) or Gestation Day 24 (rats that did not deliver a litter).

F1 generation pups were not directly exposed to the test or control substance, but may have been possibly exposed during maternal gestation (in utero exposure), via maternal milk during the lactation period, or from exposure to maternal urine/feces.

In the P generation, female No. 1407 in the 1 mg/kg/dose group was found dead on Day 39, and female No. 1434 in the 0.5 mg/kg/dose group was found dead on Day 45. The causes of death were not evident microscopically and considered undetermined and unrelated to the test substance.

There were no microscopic changes attributed to 3-(4-tert-butylphenyl)propionaldehyde in the 5 mg/kg/dose P generation males or females, or in the thyroid glands or testes/epididymides of any groups.

No microscopic changes in the P generation animals were attributed to the test substance.

Histopathological findings: neoplastic:no effects observed

Reproductive function / performance (P0)

Reproductive function: oestrous cycle:no effects observed

Reproductive function: sperm measures:not examined

Reproductive performance:There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on mating and fertility. The days in cohabitation (2.8 to 3.5 days), mating index (90% or 100%), and fertility index (90% or 100%) in the 0.5, 1, and 5 mg/kg/dose groups were similar to the control group values.

Pregnancy occurred in 9 (90%), 10 (100%), 9 (90%), and 10 (100%) of the 10 mated females in the 0, 0.5, 1, and 5 mg/kg/dose groups, respectively. Of these pregnant females, 8 to 10 females across the groups delivered their litters and one dam in the 1 mg/kg/dose group was found deadn on GD 22, as previously described. There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on any natural delivery or litter parameter at any dose. The mean number of implantation sites per delivered litter, dams with stillborn pups, dams with no liveborn pups, gestation index (number of rats with live offspring/number of pregnant rats), mean number of dams with all pups dying (Days 0 to 3 postpartum and Days 4 to 12 postpartum), mean number of pups delivered (liveborn and stillborn), pups found dead or presumed cannibalized, percent male pups per number of pups sexed per litter, surviving pups per litter, lactation index, and live litter size were similar among the four dose groups.

There was a statistically significant decrease ($p \leq 0.01$) in the number of pups found dead between Days 1 and 3 postpartum at 0.5 and 1 mg/kg/dose resulting from an increase in pup mortality in the control group during this same period. Consequently, the viability index in the 0.5 and 1 mg/kg/dose groups was higher ($p \leq 0.01$) than the control group.

There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on mating and fertility. The days in cohabitation (2.8 to 3.5 days), mating index (90% or 100%), and fertility index (90% or 100%) in the 0.5, 1, and 5 mg/kg/dose groups were similar to the control group values. There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on gross pathology, body weight gain, food consuption, organ weight.

Results: P1 (second parental generation)

General toxicity (P1)

Clinical signs:not examined

Mortality:not examined

Body weight and weight changes:not examined

Food consumption and compound intake (if feeding study):not examined

Food efficiency:not examined

Water consumption and compound intake (if drinking water study):not examined

Ophthalmological findings:not examined

Haematological findings:not examined

Clinical biochemistry findings:not examined

Urinalysis findings:not examined

Behaviour (functional findings):not examined

Immunological findings:not examined

Organ weight findings including organ / body weight ratios:not examined

Gross pathological findings:not examined

Neuropathological findings:not examined

Histopathological findings: non-neoplastic:not examined

Histopathological findings: neoplastic:not examined

Results: F1 generation

General toxicity (F1)

Clinical signs:There were no 3-(4-tert-butylphenyl)propionaldehyde-related clinical signs observed in the F1 generation pups at any dose.

Mortality / viability:no mortality observed

Body weight and weight changes:There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on mean body weights in the F1 generation pups at 0.5 and 1 mg/kg/dose. The mean pup body weight was statistically significantly reduced ($p \leq 0.05$ or $p \leq 0.01$) in the 5 mg/kg/dose group compared to control values on Days 9 and 12 postpartum (88% to 89% of controls). The reduced pup weights were considered unrelated to the 3-(4-tert-butylphenyl)propionaldehyde because they were within the range observed historically at the Testing Facility.

Food consumption and compound intake (if feeding study):no effects observed

Food efficiency:not examined

Ophthalmological findings:not examined

Haematological findings:not examined

Clinical biochemistry findings:There were no 3-(4-tert-butylphenyl)propionaldehyde-related changes in serum T4 concentrations in the F1 generation males or females at any dose. In the F1 generation male pups, mean serum T4 concentrations were 98%, 82%, and 78% of controls in the 0.5, 1, and 5 mg/kg/dose groups, respectively, on Day 12 postpartum. In the F1 generation female pups, mean serum T4 concentrations were 82%, 74%, and 74% of controls in the 0.5, 1, and 5 mg/kg/dose groups, respectively, on Day 12 postpartum. There were no 3-(4-tert-butylphenyl)propionaldehyde-related microscopic changes in the thyroid or parathyroid glands of the single F1 generation pup/sex/litter that was microscopically examined from 5 mg/kg/dose group. The differences observed in mean serum T4 concentrations in the F1 generation females did not reflect any other evidence at the tissue level, therefore, were considered unrelated to administration of 3-(4-tert-butylphenyl)propionaldehyde.

Urinalysis findings:not examined

Sexual maturation:not examined

Anogenital distance (AGD):There were no 3-(4-tert-butylphenyl)propionaldehyde-related differences in mean anogenital distance in the F1 generation males or females at any dose on Day 1 postpartum.

Nipple retention in male pups:There were no 3-(4-tert-butylphenyl)propionaldehyde-related differences on nipple retention in the F1 generation male pups in any dose group.No male pups had nipples present on PND 12.

Organ weight findings including organ / body weight ratios:not examined

Gross pathological findings:There were no 3-(4-tert-butylphenyl)propionaldehyde-related macroscopic findings noted in the F1 generation males or females at any dose. The macroscopic findings observed were considered incidental, of the nature commonly observed in this strain and age of Sprague-Dawley rats and/or were of similar incidence in control and treated animals and, therefore, were considered unrelated to administration of 3-(4-tert-butylphenyl)propionaldehyde.

Histopathological findings:There were no 3-(4-tert-butylphenyl)propionaldehyde-related microscopic findings in the thyroid gland or parathyroid gland in the single F1 generation pup/sex/litter that was microscopically examined from the 5 mg/kg/dose group.

Developmental neurotoxicity (F1)

Behaviour (functional findings):not examined

Developmental immunotoxicity (F1)

Developmental immunotoxicity:not examined

There were no 3-(4-tert-butylphenyl)propionaldehyde-related clinical signs observed in the F1 generation pups at any dose. There were no 3-(4-tert-butylphenyl)propionaldehyde-related effects on mean body weights in the F1 generation pups at 0.5 and 1 mg/kg/dose. There were no 3-(4-tert-butylphenyl)propionaldehyde-related changes in serum T4 concentrations in the F1 generation males or females at any dose. There were no 3-(4-tert-butylphenyl)propionaldehyde-related differences on nipple retention in the F1 generation male pups in any dose group. There were no 3-(4-tert-butylphenyl)propionaldehyde-related macroscopic findings noted in the F1 generation males or females at any dose. The macroscopic findings observed were considered incidental, of the nature commonly observed in this strain and age of Sprague-Dawley rats and/or were of similar incidence in control and treated animals and, therefore, were considered unrelated to administration of 3-(4-tert-butylphenyl)propionaldehyde. There were no 3-(4-tert-butylphenyl)propionaldehyde-related microscopic findings in the thyroid gland or parathyroid gland in the single F1 generation pup/sex/litter that was microscopically examined from the 5 mg/kg/dose group.

In conclusion, Bourgeonal was administered via oral gavage to male and female Crl:CD(SD) Sprague Dawley rats once daily beginning before cohabitation, through mating and continuing for at least 28 days (P generationmale rats;actual; 42 to 45 doses) or through parturition until Day 12 of lactation (P generation femalerats;actual:38 to 56 doses) at doses of 0.5, 1, or 5 mg/kg/dose. Administration of Bourgeonal did not produce any mortality or clinical signs in the P generation males or females at any dose. There were no Bourgeonal-related effects on mating and fertility in the P generation males or females or any effects on estrous cycling and natural delivery parameters in the P generation females.

The mean pup body weight was statistically significantly reduced in the 5 mg/kg/dose group compared to control values on Days 9 and 12 postpartum (88% to 89% of controls). The Study authors considered the reduced pup weights were unrelated to the 3-(4-tert-butylphenyl)propionaldehyde because they were within the range observed historically at the Testing Facility. There were no Bourgeonal-related macroscopic or microscopicfindings in the P generation adults or F1 generation pupsor alterations in the organ weights in the P generation adults.

3.10.1.2 Study 2: 3-(4-*tert*-butylphenyl)propionaldehyde

Reference: Unnamed 2009

Toxicity Study by Oral Gavage Administration to Sexually Mature Male CD Rats for 5 Days

Study period: Between 4 December 2008 and 7 July 2009

Test guideline: The systemic toxic potential and testicular and epididymal toxicity of 3-(4-tert-butylphenyl)propionaldehyde to Crl:CD® (SD)IGS BR rats by oral gavage administration was assessed over a period of 5 days. Each test material was administered to three groups each comprising six sexually mature male CD rats that received treatment at doses of 25, 100 or 250 mg/kg/day. A similarly constituted Control group received the vehicle, corn oil, at the same volume dose (5 mL/kg). A further group comprising 6 male rats was included in the study to act as a positive Control group and received the substance, Lilial, at a treatment dose of 250 mg/kg/day.

GLP compliance:yes

Test material:

Reference substance name:3-(4-tert-butylphenyl)propionaldehyde

EC Number:242-016-2

Cas Number:18127-01-0

Description : Clear colourless liquid

Storage conditions : At ambient temperature under nitrogen.

Batch number : VE000010051

Date of receipt : 20 November 2008

Expiry date : 4 August 2009

Purity : 98.4%

Test animals:

Species:rat

Strain: Crl:CD® (SD)IGS BR

Sex: male

- Source:Charles River (UK) Ltd.

- Age at study initiation:12 weeks

- Weight at study initiation:380 to 473 g.

- Fasting period before study:Not specified.

- Housing:Animals were housed inside a barriered rodent facility (Building 8, Room 08). The facility was designed and operated to minimise the entry of external biological and chemical agents and to minimise the transference of such agents between rooms. Before the study the room was cleaned and disinfected.

- Diet:The animals were allowed free access to a standard rodent diet (Rat and Mouse No. 1 Maintenance Diet), except when urine was being collected on Day 5. This diet contained no added antibiotic or other chemotherapeutic or prophylactic agent.

- Water:Potable water taken from the public supply was freely available via polycarbonate bottles fitted with sipper tubes.

- Acclimation period:14 days

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 19 to 23°C

- Humidity (%): 40 to 70%

- Air changes (per hr):Each animal room was kept at positive pressure with respect to the outside by its own supply of filtered fresh air, which was passed to atmosphere and not re-circulated.

- Photoperiod (hrs dark / hrs light):Artificial lighting was controlled to give a cycle of 12 hours continuous light and 12 hours continuous dark per 24 hours, except on Day 1 of treatment when the light cycle was overridden on 3 occasions to accommodate technical procedures required by the Study Protocol.

Administration / exposure

Route of administration:oral: gavage

Vehicle:corn oil

Analytical verification of doses or concentrations:yes

Details on analytical verification of doses or concentrations:Analytical procedure - 3-(4-tert-butylphenyl)propionaldehyde

High performance liquid chromatograph (HPLC): Comprising any suitable pump, autosampler and UV detector with sufficient precision and sensitivity

Chromatography data handling: Waters Empower 2

Balances fitted with printers: Capability of weighing to 5 or 6 decimal places General laboratory apparatus and glassware.

Control vehicle: Corn oil

Methanol: HPLC solvent

Tetrahydrofuran (THF): HPLC solvent

Water: Reverse osmosis

Mobile phase: Methanol / water (65/35 v/v).

Analytical column: Waters Symmetry C18, 5 μm, 150 × 3.9 mm

Column temperature: Ambient

Flow rate: 1.0 mL/minute

Detector wavelength: 210 nm

Injection volume: 10 μL

Approximate retention time: 11.6 – 14.2 minutes

The analytical procedurse were validated with respect to linearity of detector response, precision of injection, specificity of chromatographic analysis, limit of detection, accuracy and precision.

The mean concentrations of 3-(4-tert-butylphenyl)propionaldehyde in test formulations analysed for the study were within +10%/-15% of nominal concentrations, confirming accurate formulation.

Duration of treatment / exposure:5 consecutive days.

Frequency of treatment:Once each day at approximately the same time each day

Doses / concentrations: 0, 25, 100, 250 mg/kg bw/day actual ingested

No. of animals per sex per dose: 6

Control animals: yes, concurrent vehicle

Details on study design:

- Dose selection rationale:

The doses used in this study (0, 25, 100 and 250 mg/kg/day) were selected in conjunction with the Sponsor. The known LOAEL for testicular effects in male rats for the positive control, Lilial, is 50 mg/kg/day over 5 days and the NOAEL was established as 25 mg/kg/day. Effects were clearly seen for the positive control at higher doses (100 and 200 mg/kg/day). The dose levels were therefore chosen for the test substances as those expected to cover the range where effects would be noted, without causing significant systemic toxicity.

- Rationale for animal assignment:On arrival, the animals were removed from the transit boxes and allocated to study cages. Using the sequence of cages in the battery, one animal at a time was placed in each cage with the procedure being repeated until each cage held the appropriate number of animals.

Groups were dispersed in batteries so that possible environmental influences arising from their spatial distribution were equilibrated, as far as was practicable. Additionally, batteries of cages were rotated around the room at a weekly interval to further minimise possible spatial variations.

Each animal was assigned a number and identified uniquely within the study by a tail tattoo. Each cage label was colour-coded according to group and was numbered uniquely with cage and study number, as well as the identity of the occupants.

Before the start of treatment, four males with bodyweights at the extreme of the weight range were replaced with spare animals of suitable weight from the same batch.

Positive control:one positive control group of male rats

Examinations

Parental animals: Analysis of urine from males treated with 100 or 25 mg/kg/day revealed the presence of the metabolite, 4-tert-butylbenzoic acid (TBBA).

Mortality

Two males (Nos. 19 and 20) treated at 250 mg/kg/day were killed for welfare reasons approximately 5 hours after administration of the first dose due to poor clinical condition. Last in-life signs included underactivity, prostrate posture, reduced body temperature, partially closed eyelids, irregular breathing and piloerection. Macroscopic examination revealed that both animals had a dark liver with animal No. 20 also showing a distended stomach. Male No. 23 treated at 250 mg/kg/day was killed for welfare reasons approximately 10 hours after administration of the first dose due to poor clinical condition. Last in-life signs included underactivity, hunched posture, partially closed eyelids, reduced body temperature, irregular breathing and piloerection. Macroscopic examination revealed a distended stomach. Male No. 13 treated at 100 mg/kg/day was killed for welfare reasons approximately 12 hours after administration of the first dose due to poor clinical condition. Last in-life signs included underactivity, reduced body temperature, partially closed eyelids and piloerection. Macroscopic examination revealed a distended stomach. No cause of death could be established for these four animals from the tissues examined at gross or microscopic level. Since half of the animals in the high dose 3-(4-tert-butylphenyl)propionaldehyde group had been killed, the remaining animals were killed on the morning of Day 2 of study prior to dosing.

Signs

Signs of underactivity, reduced body temperature, irregular breathing, piloerection, loose faeces and partially closed eyelids were recorded after dosing at 250 or 100 mg/kg/day; all of the signs in the 100 mg/kg/day group with the exception of underactivity were only recorded after administration of the first dose. In addition, a low incidence of prostrate or hunched posture was recorded after dosing at 250 mg/kg/day.

Signs of piloerection and loose faeces were recorded after administration the first dose of 25 mg/kg/day.

Physical examination revealed four males in the 100 mg/kg/day group with yellow faecal staining on the body.

Bodyweight

All of the three males treated at 250 mg/kg/day and killed on Day 2 of study showed bodyweight loss of 15-30 g between the start of treatment and Day 2 of study. Treatment at 100 mg/kg/day was associated with mean bodyweight loss of 14 g following administration of the first dose. This was followed by mean bodyweight stasis during Days 2-3 of study and mean bodyweight loss during Days 3-4 and 4-5 of study.

Treatment at 25 mg/kg/day was associated with mean bodyweight loss of 10 g following administration of the first dose. Thereafter, there was no clear effect of treatment on bodyweight.

As animals were deprived of food from Day 5 to Day 6, data for this period whilst included, are not commented on.

Food consumption

The males treated at 250 mg/kg/day and killed on Day 2 of study showed low food consumption between the start of treatment and Day 2 of study.

There was no clear effect of treatment at 100 or 25 mg/kg/day on mean food consumption.

Organ weights

Among animals treated at 100 mg/kg/day the mean absolute epididymal weight was marginally high and the mean testes weight was low.n There was no effect of treatment at 25 mg/kg/day.

Macropathology

Males receiving 3-(4-tert-butylphenyl)propionaldehyde at 100 mg/kg/day had enlarged epididymides (3/6), kidney depressions (2/6), thickened fore-stomach (2/6) and pale livers (5/6). Pale livers were also observed in males receiving 3-(4-tert-butylphenyl)propionaldehyde at 25 mg/kg/day (6/6).

Histopathology

Findings considered related to treatment with 3-(4-tert-butylphenyl)propionaldehyde or Lilial were seen in liver, kidney, stomach, testes and epididymides. Generalized hepatocyte vacuolation was observed in animals treated with 3-(4-tert-butylphenyl)propionaldehyde at 100mg/kg/day (5/6 males), 3-(4-tert-butylphenyl)propionaldehyde at 25mg/kg/day (2/6 males) and with Lilial at 250mg/kg/day (6/6 males). In

the kidneys, cortical tubular vacuolation occurred in 5/6 males treated with 100mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde. Seminiferous tubules were evaluated with respect to their stage in the spermatogenic cycle and the integrity of the various cell types present within the different stages. 5/6 animals treated with 100mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde showed treatment-related effects in the testes and epididymides.

Sperm parameters (parental animals):Seminiferous tubular degeneration/atrophy, Sertoli cell vacuolation, multinucleate giant cell and luminal sloughing of spermatogenic cells in the testes and, reduced numbers of spermatozoa, sloughed germ cells in lumen and inflammation in the epididymides - 100 mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde (mean urine TBBA concentration 275 μg/ml). The changes produced in the epididymides are considered secondary to their effects on the testes.

Results and discussion

Results: P0 (first parental generation)

Clinical signs:effects observed, treatment-related

Body weight and weight changes:effects observed, treatment-related

Food consumption and compound intake (if feeding study):effects observed, treatment-related

Organ weight findings including organ / body weight ratios:effects observed, treatment-related

Histopathological findings: non-neoplastic:effects observed, treatment-related

Reproductive function: sperm measures:effects observed, treatment-related

Treatment with 3-(4-tert-butylphenyl)propionaldehyde was associated with marked systemic toxicity and testicular/epididymal toxicity, and the urine contained TBBA, a known metabolite biomarker of testicular toxicity in rats. Treatment at 250 or 100 mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde was not tolerated: 3/6 males and 1/6 males respectively were killed for welfare reasons after administration of the first dose due to poor clinical condition. The remaining animals in the 250 mg/kg/day group showed bodyweight loss and low mean food consumption after administration of the first dose and were killed prior to dosing on Day 2 of study. In the 250 mg/kg/day Lilial group, the marked systemic toxicity was manifest as progressive mean bodyweight loss throughout Days 1-5 of study and low mean food consumption.

Microscopic examination of tissues revealed a similar spectrum of changes following treatment with 3-(4-tert-butylphenyl)propionaldehyde or Lilial as follows:

• Generalised hepatocyte vacuolation in the liver - 100 and 25 mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde and 250 mg/kg/day Lilial - Oil Red O staining of selected liver tissue sections confirmed the vacuoles contained fat. The microscopic finding of fatty vacuolation is consistent with the gross observation of pale liver tissue.

• Cortical tubular vacuolation in the kidneys - 100 mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde and 250 mg/kg/day Lilial - this finding appeared to be localised to the distal tubules within the cortex.

• Epithelial hyperplasia and hyperkeratosis in the forestomach - 100 mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde and 250 mg/kg/day Lilial.

• Seminiferous tubular degeneration/atrophy, Sertoli cell vacuolation, multinucleate giant cell and luminal sloughing of spermatogenic cells in the testes and, reduced numbers of spermatozoa, sloughed germ cells in lumen and inflammation in the epididymides - 100 mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde (mean urine TBBA concentration 275 μg/ml).

The changes produced by both compounds in the epididymides are considered secondary to their effects on the testes. The urine of animals treated at 25 mg/kg/day 3-(4-tert-butylphenyl)propionaldehyde also contained TBBA but at a much lower concentration (mean TBBA concentration 35.8 μg/ml) and this did not result in any testicular or epididymal toxicity.

Conclusions:

Based on the results of this study it was concluded that treatment of sexually mature male CD rats with Bourgeonal was associated with marked systemic toxicity at 250 and 100 mg/kg/day. Treatment at 100 mg/kg/day was also associated with testicular and epididymal toxicity and the urine contained TBBA.

3.10.1.3 Study 3: 4-*tert*-butyltoluene

Reference: Unnamed 2007a

Test guideline: OECD Guideline 421 (Reproduction / Developmental Toxicity Screening Test)

GLP compliance: yes

Test material: 4-tert-butyltoluene

EC Number: 202-675-9

Cas Number:98-51-1

- Name of test material (as cited in study report): p-tert-butyltoluene; supplier: Fuso Chemical Co. Ltd., Osaka, Japan

- Physical state: colourless transparent liquid

- Analytical purity: 96.94%

- Impurities (identity and concentrations): no data

- Lot/batch No.: Lot No. 08008

- Storage condition of test material: room temperature; dark.

Test animals

Species:rat

Strain: Sprague-Dawley

Sex: male/female

male and female Crj:CD(SD)IGS, SPF rats

- Source: Charles River Laboratories, Inc

- Age at study initiation: 8 weeks upon arrival at the testing facility; 10 weeks at the starting day of administration

- Weight at study initiation: 336 - 368 g (males); 221 - 252 (females)

- Fasting period before study: no

- Housing: 5 per cage (during acclimatization), individually after assignment to experimental groups; stainless steel cages; animals were mated in males' cages . Dams were individually housed in a plastic cage with autoclaved beddings from day 18 of gestational period, and were allowed to deliver (spontaneous delivery) and rear the offspring.

- Diet: pellet diet (CRF-1; ORIENTAL YEAST Co., Ltd.), ad libitum

- Water: tap water, ad libitum

- Acclimation period: 5 days quarantine, 7 days acclimatization

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 20 - 26 °C

- Humidity (%): 40 - 70 %

- Air changes (per hr): 12

- Photoperiod (hrs dark / hrs light): 12/12

Administration/exposure

Route of administration: oral: gavage

Vehicle: corn oil

PREPARATION OF DOSING SOLUTIONS:

p-tert-Butyltoluene was adjusted by dissolving and diluting it in corn oil. The amount of the test substance was calculated using its purity in preparing the test substance. It is approved that prepared solutions at 0.2, 2, 20, and 200 mg/mL can be kept stable if stored at room temperature under a shaded condition for 7 days. Therefore, the prepared solution at each concentration was stored at room temperature under a shaded condition, and was used within 7 days after preparation. Concentrations of the test substance in administration samples used on the day of start of administration and the day of the termination of administration were measured. The measurement result showed no problems with concentrations of the test substance.

VEHICLE: corn oil

- Justification for use and choice of vehicle (if other than water): no data

- Concentration in vehicle: 0.2, 2, 20, and 200 mg/mL

- Amount of vehicle (if gavage): 5 ml/kg bw

Details on mating procedure:

- M/F ratio per cage: 1/1

- Length of cohabitation: up to 14 days

The animals were continuously housed together during the mating period until copulation was observed. One male in the 50 mg/kg group could not be mated due to the death of the paired female. Therefore, the male rat was not used for mating.

- Proof of pregnancy: vaginal plug and/or sperm in vaginal smear referred to as day 0 of pregnancy

The females that did not achieve successful copulation were sacrificed by exsanguination via the abdominal aorta under ether anesthesia after the termination of mating period and were subsequently necropsied.

Duration of treatment / exposure: males: 50-52 days (males); premating exposure period: 14 days

females: from 14 days prior to mating until day 3 post partum (females); premating exposure period: 14 days

Frequency of treatment: daily

Details on study schedule:

The administration period for males was total 50 to 52 days including 14 days before mating and subsequent 36 to 38 days (necropsy of males was separately conducted in 3 days since the observation of sperm requires 3 days). The administration period for females was total 41 to 45 days including 14 days before mating, mating period (14 days at the longest), gestational period, and first 3 days in lactation period. The starting day of administration was set as the day 1.

Doses / Concentrations: 1.5, 5, 15, 50 mg/kg bw/d

Basis: actual ingested

No. of animals per sex per dose: 12 males and 12 females per group

Control animals: yes, concurrent vehicle

- Dose selection rationale: Dose levels were selected based on two preliminary oral toxicity studies.

First, a single oral dose toxicity test of p-tert-butyltoluene was conducted in rats (dose levels, 0, 250, 500, 1000, and 2000 mg/kg; number of animals, 5 male rats and 5 female rats).

In regard to the results, a pretest was conducted for the preliminary reproduction toxicity screening test of p-tert-butyltoluene by oral administration in rats (dose levels, 0, 125, 250, 500, and 750 mg/kg; number of animals, 5 male rats and 5 female rats; administration period, 14 days). The dose levels of this study were determined based on the results. There were 3 deaths in the 125 mg/kg group and 5 deaths in each of the 250, 500, and 700 mg/kg groups in the pretest for the preliminary reproduction toxicity of p-tert-butyltoluene by oral administration in rats. Further, there were observed decreases in body weight and food consumption and atrophy of the testis and epididymis in the 125 mg/kg group. Therefore, the highest dose level was set to 50 mg/kg that corresponds to an approximately half amount of 125 mg/kg at which dead animals had been observed in the pretest, and lower dose levels were set at 15, 5, and 1.5 mg/kg by a common ratio of approximately 3. The control group was administered with the vehicle (corn oil) alone at the same volume.

Observations and examinations:

Parental animals

1) MALE RATS

CAGE SIDE OBSERVATIONS and DETAILED CLINICAL OBSERVATIONS:

- Time schedule: Observations about clinical signs and death were conducted twice a day, before and after administration. Necropsy of dead animals was conducted immediately after they were found.

BODY WEIGHT:

- Time schedule for examinations: Body weight was measured twice a week.

FOOD CONSUMPTION:

- Time schedule: Food consumption was measured twice a week during the 14-day period before mating was started and during the period after the termination of mating period

WATER CONSUMPTION: No

2) FEMALE RATS

CAGE SIDE OBSERVATIONS and DETAILED CLINICAL OBSERVATIONS:

- Time schedule: Observations about clinical signs and death were conducted twice a day, before and after administration. Necropsy of dead animals was conducted immediately after they were found.

BODY WEIGHT:

- Time schedule for examinations: Body weight was measured twice a week during the 14-day period before mating was started and during the mating period, at days 0, 7, 14, and 21 during the gestational period, and at days 0 and 4 in the lactational period.

FOOD CONSUMPTION: Yes

- Time schedule: Food consumption was measured twice a week during the 14-day period before mating was started, at days 2, 9, 16, and 21 during the gestational period, and at day 4 during the lactation period.

WATER CONSUMPTION: No

OTHER:

- Females that did not achieve successful copulation

The females that did not achieve successful copulation were sacrificed by exsanguination via the abdominal aorta under ether anesthesia after the termination of mating period and were subsequently necropsied.

- Observation of Delivery Status

The females with successful copulation were allowed to deliver (spontaneous delivery) their litters. An observation of abnormality of delivery status and checks on completion of delivery were conducted from day 21 of the gestational period to 10 AM on day 25 of the gestational period. If delivery had been completed at 10 AM, the day was set as day 0 of the lactation period.

- Females that did not deliver by 10 AM on day 25 of gestational period

The females that did not deliver by 10 AM on day 25 of the gestational period were sacrificed by exsanguination via the abdominal aorta under ether anesthesia and subsequently necropsied. It was determined whether or not the female was pregnant by examining the presence of implantation.

- Observation of Lactation and Necropsy

An observation of lactation of the dams was conducted every day to day 4 of the lactation period. On day 4 of lactation, dams were sacrificed.

Oestrous cyclicity (parental animals):

An observation of estrous cycle was conducted once a day from the starting day of the administration period to the day at which copulation was observed or to the day of the termination of mating period. In the case that estrus was observed for continuous 2 days, it was counted as one estrus.

Sperm parameters (parental animals):

Parameters examined in all male parental generations:

testis weight, epididymis weight, daily sperm production, sperm count in testes, sperm count in epididymides, enumeration of cauda epididymal sperm reserve, sperm motility, sperm morphology

After sacrifice, the right cauda epididymis was minced in a sperm incubation medium (medium 199 supplemented with 0.5% bovine serum albumin) warmed to 37°C and settled for 5 minutes. Subsequently, sperm stock solution was produced. The examinations on motility, viability, and morphology of sperm were conducted with this sperm stock solution.

Litter observations:

PARAMETERS EXAMINED

The following parameters were examined in F1 offspring: number and sex of pups, stillbirths, live births, postnatal mortality, presence of gross anomalies, weight gain, physical or behavioural abnormalities

GROSS EXAMINATION OF DEAD PUPS:yes, for external and internal abnormalities; no further data

- Observation at Delivery

The following data were recorded: number of offspring born, sex ratio, number of stillbirths, number of newborn offspring, and external abnormality.

- Observation of Newborn Offspring

Clinical signs and death were recorded once every day.

- Measurement of Body Weight

Body weight was measured at days 0 (day of birth) and 4 of the lactational period.

Necropsy

The live offspring were sacrificed by exsanguination via the abdominal aorta under ether anesthesia at day 4 of the lactation period and were subsequently necropsied.

Postmortem examinations (parental animals):

SACRIFICE

- Male animals: All surviving animals were sacrificed on days 51 through 53 of the study.

Animals were sacrificed by exsanguination via the abdominal aorta under ether anesthesia and were necropsied at the next day of the last administration (days 51 to 53 of the administration period). The weights of the testis, epididymis and cauda epididymis were measured. The testes and the caput epididymides were fixed in Bouin’s solution for 2 to 3 hours and subsequently refixed in 90% alcohol. The prostate and seminal vesicles were fixed in 20% neutral buffered formalin.

- Maternal animals: All surviving animals were sacrificed on day 4 of lactation.

The dams were sacrificed by exsanguination via the abdominal aorta under ether anesthesia and necropsied at the day that all newborn offspring were dead or at day 4 of the lactation period. The numbers of implantations and pregnant corpora lutea were counted. The weights of ovaries were measured. The ovaries, uterus, and vagina were fixed in 20% neutral buffered formalin.

GROSS NECROPSY

- Gross necropsy consisted of external and internal examinations

HISTOPATHOLOGY / ORGAN WEIGHTS

1) MALE RATS:

Paraffin embedded samples of the testes, caput epididymides, seminal vesicles, and prostate were prepared and evaluated by a conventional method.

HE-stained tissue samples we produced of the testes, caput epididymides, seminal vesicles, and prostate of the animals of the control and 50 mg/kg groups, and histopathological examinations were conducted on the samples. The authors/investigators considered that there was a difference in the number of animals that showed abnormality in the testis and caput epididymis between the 50 mg/kg group and the control group. Therefore, they produced HE-stained tissue samples of these organs of the animals in the 1.5, 5, and 15 mg/kg groups, and conducted the histopathological examinations.

2) MATERNAL RATS

Paraffin embedded samples of the ovaries were prepared using a conventional method. HE-stained tissue sample of the ovaries were prepared from the animals of the control and 50 mg/kg groups and histopathological examinations were conducted.

Postmortem examinations (offspring):

SACRIFICE

- The F1 offspring were sacrificed at 4 days of age.

- These animals were subjected to postmortem examinations (macroscopic and/or microscopic examination) as follows:

GROSS NECROPSY

- Gross necropsy consisted of external and internal examinations; no further data.

HISTOPATHOLOGY / ORGAN WEIGTHS no data

Statistics: statistical methods are presented in the freetext

Results and discussion

Results: P0 (first parental generation)

1) MALE RATS

CLINICAL SIGNS AND MORTALITY (PARENTAL ANIMALS)

There was a death in the 50 mg/kg group. In the dead case, the animal showed transient salivation, a decrease in locomotor activity, soiled fur, reddish urine, and hypothermia.

In the observation of clinical signs on the live animals, no abnormality was observed in the control group. There was transient salivation in the 1.5 mg/kg group and soiled fur in one animal of the 50 mg/kg group.

BODY WEIGHT AND FOOD CONSUMPTION (PARENTAL ANIMALS)

There were no significant differences in body weight at any day of measurement in the 1.5 and 5 mg/kg groups compared with the control group. There were significant decreases in body weight from day 18 to day 49 of the administration period in the 15 mg/kg group compared with the control group. There were significant decreases in body weight from day 4 to day 49 of the administration period in the 50 mg/kg group compared with the control group. See Fig. 1, tert-butyltoluene study TG 421 in Confidential Annex.

There were no significant differences in food consumption at any day of measurement in the 1.5, 5, and 15 mg/kg groups compared with the control group. There was a significant decrease in food consumption at day 48 of the administration period in the 50 mg/kg group compared with the control group. There was a significant increase in food consumption at day 13 of the administration period in the same group compared with the control group. However, this was a transient change, and was not considered to be attributed to the administration. See Fig. 2, tert-butyltoluene study TG 421 in Confidential Annex.

REPRODUCTIVE FUNCTION: SPERM MEASURES (PARENTAL ANIMALS)

There were no significant differences in sperm motility ratio, path velocity, straight line velocity, curvilinear velocity, beat cross frequency, ratios of morphological abnormality of sperms (ratios of abnormality in the head, tail and the total of those), sperm viability, sperm survivability, sperm count, and sperm count per one gram of the left cauda epididymis in the 1.5 and 5 mg/kg groups, compared with the control group.

There were significant decreases in sperm motility ratio, path velocity, straight line velocity, curvilinear velocity, sperm viability, sperm survivability, sperm count, and sperm count per one gram of the left cauda epididymis in the 15 mg/kg groups compared with the control group. There were significant increases in beat cross frequency and ratios of morphological abnormality of sperms (ratios of abnormality in the head, tail and the total of those) in the same group.

There were significant decreases in sperm motility ratio, sperm count, sperm count per one gram of the cauda epididymis in the 50 mg/kg group compared with the control group. Among the animals with low numbers of motile sperms, there was only one animal that could be used for measurements of path velocity, straight line velocity, curvilinear velocity, beat cross frequency, sperm viability, and sperm survivability. However, there were decreases in all the parameters. The authors could conduct the observation of sperm morphology on only 5 animals in the 50 mg/kg group. However, there were significant increases in the ratios of morphological abnormality of sperms (ratios of abnormality in the head, tail and the total of those). See Table 2, tert-butyltoluene study TG 421 in Confidential Annex.

ORGAN WEIGHTS (PARENTAL ANIMALS)

There was no significant difference in the body weight at the day of necropsy in the 1.5 and 5 mg/kg groups compared with the control group. There were significant decreases in body weight at the day of necropsy in the 15 and 50 mg/kg groups compared with the control group.

In the organ weight measurement, there were no significant differences in absolute and relative weights of the testis and epididymis in the 1.5 and 5 mg/kg groups compared with the control group. There were significant decreases in absolute weight of the epididymis and a decreasing tendency of absolute weight of the testis in the 15 mg/kg group compared with the control group. There were significant decreases in absolute and relative weights of the testis and epididymis in the 50 mg/kg group compared with the control group. See Table 1, tert-butyltoluene study TG 421 in Confidential Annex.

GROSS PATHOLOGY (PARENTAL ANIMALS)

In the live animals, there was no abnormality in the control, 1.5, and 5 mg/kg groups. There was atrophy of the testes and the epididymides in one animal of the 15 mg/kg group. There was atrophy of the testes and the epididymides in all 11 animals of the 50 mg/kg group.

There was atrophy of the thymus, ulcer on the anterior gastric mucosa, and atrophy of the testes, epididymides, seminal vesicles, and prostate in the dead animals of the 50 mg/kg group.

HISTOPATHOLOGY (PARENTAL ANIMALS)

Testis: There was no abnormality in the control, 1.5, and 5 mg/kg groups. There was atrophy of the seminiferous tubules in 4 animals, hyperplasia of Leydig Cells in 2 animals, and remaining spermatids at step 19 in the seminiferous tubules of groups 3 and 4 in one animal of the 15 mg/kg group. There was atrophy of the seminiferous tubules and hyperplasia of Leydig cells in 11 animals in the 50 mg/kg group.

Epididymis (Caput Epididymis): There was no abnormality in the control, 1.5, and 5 mg/kg groups. There was a decrease in sperm count in 4 animals in the 15 mg/kg group. There was a decrease in sperm count in 11 animals in the 50 mg/kg group.

There was atrophy of the seminiferous tubules in the testis and a decrease in sperm count in the epididymis in the 15 and 50 mg/kg group. In addition, there was hyperplasia of Leydig cells in the testis in the 50 mg/kg group. Significant differences were observed with all these changes compared with the control group. However, the remaining spermatids in the seminiferous tubules of groups 3 and 4 were observed in only one animal of the 15 mg/kg group. Further, this change was not observed in the 50 mg/kg group. Therefore, this change is considered to be an accidental case.

Besides, there was lymphoid cell infiltration in the prostate. However, it was determined that this was an accidental change. See Table 3, tert-butyltoluene study TG 421 in Confidential Annex.

2) FEMALE RATS

CLINICAL SIGNS AND MORTALITY (PARENTAL ANIMALS)

There were one death in the 15 mg/kg group and 6 deaths in the 50 mg/kg group. There was hypothermia and a decrease in locomotor activity, and transient salivation in the 15 mg/kg group. There was hypothermia, prone position, a decrease in locomotor activity, piloerection, soiled fur, bradypnea, and transient salivation in the 50 mg/kg group.

In the observation of clinical signs on the living animals, there was no abnormality in the control, 1.5, and 5 mg/kg groups. There was transient salivation in the 15 mg/kg group. There was transient salivation, hypothermia, a decrease in locomotor activity, staggering gait, lacrimation, diarrhea, and muscle relaxation in the 50 mg/kg group.

BODY WEIGHT AND FOOD CONSUMPTION (PARENTAL ANIMALS)

There was no significant difference in body weight at any day of measurement in the 1.5, 5, and 15 mg/kg groups before mating was started, compared with the control group. There were significant decreases in body weight from day 4 to day 15 of the administration period in the 50 mg/kg group compared with the control group.

There was no significant difference in body weight at any day of measurement during the gestational period in the 1.5 mg/kg group, compared with the control group. There were significant decreases in body weight at days 7 and 14 of the gestational period in the 5 mg/kg group compared with the control group. There were significant decreases in body weight from day 7 to day 21 of the gestational period in the 15 mg/kg group compared with the control group.

There was no significant difference in body weight at any day of measurement during the lactation period in the 1.5 mg/kg group, compared with the control group. There was a significant decrease in body weight at day 4 of the lactation period in the 5 mg/kg group compared with the control group. There was a decreasing tendency of body weight in one animal of the 15 mg/kg group at day 4 of the lactation period. See Fig. 3, tert-butyltoluene study TG 421 in Confidential Annex.

There was no significant difference in food consumption at any day of measurement before mating was started in the 1.5 and 5 mg/kg groups, compared with the control group. There were significant increases in food consumption at day 6 of the administration period in the 15 and 50 mg/kg groups compared with the control group.

There was no significant difference in food consumption at any day of measurement during the gestational period in the 1.5, 5, and 15 mg/kg groups, compared with the control group.

There was no significant difference in food consumption in the 1.5 mg/kg group during the lactation period, compared with the control group. There was a significant decrease in food consumption at day 4 of the lactation period in the 5 mg/kg group compared with the control group. There was a decreasing tendency of food consumption in one animal of the 15 mg/kg group at day 4 of the lactation period. See Fig.4, tert-butyltoluene study TG 421 in Confidential Annex.

REPRODUCTIVE FUNCTION: ESTROUS CYCLE (PARENTAL ANIMALS)

No changes attributable to administration of the test substance were noted in the numbers of estrous cases.

ORGAN WEIGHTS (PARENTAL ANIMALS)

There was no significant difference in the body weight at the day of necropsy in the 1.5 mg/kg group compared with the control group. There were significant decreases in body weight at the day of necropsy in the 5, 15, and 50 mg/kg groups compared with the control group.

In the organ weight measurement, there were no significant differences in absolute and relative weights of the ovary in the 1.5, 5, 15 and 50 mg/kg groups compared with the control group. See Table 4, tert-butyltoluene study TG 421 in Confidential Annex.

GROSS PATHOLOGY (PARENTAL ANIMALS)

There was no abnormality in the live animals of any group.

Among the dead animals, there was a dark red spot on the glandular gastric mucosa in one animal of the 15 mg/kg group. There was pale coloor of the liver in one animal and atrophy of the thymus in one animal of the 50 mg/kg group. All the 3 animals that died after copulation were infertile.

HISTOPATHOLOGY (PARENTAL ANIMALS)

Ovary: there was no abnormality in the control and 50 mg/kg groups.

REPRODUCTIVE PERFORMANCE (PARENTAL ANIMALS); MALES AND FEMALES

(1) Frequency of Estrus, Copulation Index, and Fertility Index

There was no significant difference in frequency of estrus during the administration period (14 days) before mating between each group and the control group.

There was no significant difference in days required for copulation between each group and the control group.

One pair of animals did not achieve copulation in the 50 mg/kg group. There was no significant difference in copulation index between each group and the control group.

There were 8 non-pregnant females in the 15 mg/kg group. There was no pregnant female in the 50 mg/kg group. There were significant decreases in fertility index in the 15 and 50 mg/kg group compared with the control group. See Table 5, tert-butyltoluene study TG 421 in Confidential Annex.

(2) Gestational Period, Delivery Status, Number of Pregnant Corpora Lutea, Implantation Index, and Gestation Index

There was no significant difference in gestational period in the 1.5, 5, and 15 mg/kg groups compared with the control group.

There was no abnormality of delivery status in the control, 1.5, and 5 mg/kg groups. No newborn offspring were obtained with one dam of the 15 mg/kg group since the litters were all dead.

There were no significant differences in number of pregnant corpora lutea, number of implantations, and implantation index in the 1.5, 5, and 15 mg/kg groups compared with the control group.

The gestation index was 100% in the 1.5 and 5 mg/kg groups. The gestation index of the 15 mg/kg group was 66.7% since one dam did not deliver live offspring.

In the observation of lactation status, there was no abnormality in the control, 1.5, and 5 mg/kg groups. All newborn pups of one dam of in the 15 mg/kg group died by day 1 of the lactation period. See Table 6, tert-butyltoluene study TG 421 in Confidential Annex.

(3) Delivery Index and Live Birth Index

There was no significant difference in number of offspring born, number of stillbirths, number of newborn offspring at day 0 of the lactation period, sex ratio, delivery index, birth index, and live birth index in the 1.5 and 5 mg/kg groups, compared with the control group. There were significant decreases in number of offspring born and number of newborn offspring at day 0 of the lactation period, decreasing tendency of delivery index, birth index, and live birth index, and an increase in number of stillbirths in the 15 mg/kg group, compared with the control group. See Table 6, tert-butyltoluene study TG 421 in Confidential Annex.

Results: F1 generation

VIABILITY and CLINICAL SIGNS (OFFSPRING)

There were no significant differences in number of live offspring at day 4 of the lactation period and viability index at day 4 of the lactation period in the 1.5 and 5 mg/kg group compared with the control group. The newborn offspring of one dam died by day 1 of the lactation period in the 15 mg/kg group, and there were decreasing tendencies of number of live offspring at day 4 of the lactational period and viability index at day 4 of the lactation period.

There was no abnormality in the observation of external abnormality of newborn offspring in any group. In the observation of clinical signs on the newborn offspring.

There was no abnormality in the control, 5, and 15 mg/kg groups. There was necrosis of the tail in one offspring of the 1.5 mg/kg group.

See Table 6, tert-butyltoluene study TG 421 in Confidential Annex.

BODY WEIGHT (OFFSPRING)

There were no significant difference in body weights of males and females at days 0 and 4 of the lactation period at 1.5 mg/kg bw/day compared with the control group. There were significant decreases in body weights of males and females at days 0 and 4 of the lactation period in the 5 mg/kg group compared with the control group. There were decreasing tendencies of body weights of males and females at days 0 and 4 of the lactation period in the 15 mg/kg group compared with the control group. See Table 6, tert-butyltoluene study TG 421 in Confidential Annex.

NECROPSY OF OFFSPRING

There was no abnormality in the control, 5, and 15 mg/kg groups. There was necrosis of the tail in one male in the 1.5 mg/kg group in the observation of clinical signs. However, there was no significant difference compared with the control group.

Conclusions:

According to the authors, the NOELs for repeated dose toxicity are considered to be 5 mg/kg bw/d for males and 1.5 mg/kg bw/d for females. The NOELs for reproductive performance are considered to be at 5 mg/kg bw/d for both sexes. The NOEL for pups is considered to be at 1.5 mg/kg/day.

3.10.1.4 Study 4: 4-*tert*-butyltoluene

Reference: Unnamed 1982a

Study period: The experimental work was done during June 1981. Histologic evaluation was made during August 1981.

Test guideline: Screening study (only one dose level tested, examinations focussed on evaluation of testes), no GLP. Tables are mentioned in text of the report section, but were not included in the data submitted to the U.S. Office of Toxic Substances.

The study was performed to assess the acute toxicity of the test substance to the testes of rats. No further information is given on the guideline/standard procedure followed.

GLP compliance:no

Limit test:yes

Test material:

Reference substance name:4-tert-butyltoluene

EC Number: 202-675-9

Cas Number: 98-51-1

- Name of test material (as cited in study report): Benzene, (1,1,-dimethylethyl)-4-methyl- (i.e. p.-tert. butyl toluene); supplied by the sponsor, internal substance code: 94-0522

- Physical state: pale yellowish coloured liquid

- Analytical purity: no data

- Storage condition of test material: in a metal bottle

Test animals:

Species:rat

Strain:albino

Sex:male

male albino SPF rats

- Source: Institute of Biological and Medical Research, Füllinsdorf, Switzerland

- Age at study initiation: no data

- Weight at study initiation: 144 - 164 g

- Housing: during the dosing period: individually or 2 per cage; no further data

- Diet (ad libitum): NAFAG No. 850, pulverized

- Water (ad libitum): tap water

- Acclimation period: 7 days

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 19 - 23°C

- Humidity (%): 45 - 65%

- Air changes (per hr): no data

- Photoperiod (hrs dark / hrs light): 12/12

Administration/exposure:

Route of administration:

oral: gavage

Vehicle: rape oil

The test substance was suspended in rape oil

VEHICLE: rape oil (Rüböl DAB 6, supplier: R.P. Scherer GmbH, Eberbach, Germany)

- Amount of vehicle: 10 ml/kg bw

No information is given on the final concentration of the test substance in the vehicle. Furthermore, no information is given on the density of both test substance and vehicle; therefore, the final concentration of the test substance in the test suspension cannot be estimated.

- Purity: food grade (DAB 6)

Analytical verification of doses or concentrations:no

Duration of treatment / exposure: 5 consecutive days

Frequency of treatment: once daily

Doses / Concentrations: 200 mg/kg bw/d

Basis: actual ingested

No. of animals per sex per dose:7 males per group

Control animals:yes, concurrent vehicle

The dose was selected based on the mortality results obtained from a dose-range-finding study (data not presented).

Examinations:

Parental animals: Observations and examinations:

CAGE SIDE OBSERVATIONS and DETAILED CLINICAL OBSERVATIONS: Yes

- Time schedule: The rats were observed for overt signs of toxicity and behavioural changes once daily during the treatment period and on the day after treatment.

BODY WEIGHT: Yes

- Time schedule for examinations: Individual body weights were recorded once daily during the treatment period and on the day after treatment.

Postmortem examinations (parental animals):

SACRIFICE

All rats were euthanized by the use of carbon dioxide and exsanguinated by incision of the neck on the day after the 5th dose.

GROSS NECROPSY

A gross necropsy was performed on all rats.

HISTOPATHOLOGY / ORGAN WEIGHTS

Liver, kidneys and testes were weighed (wet weight, paired organs were weighed together).

The following organs were examined histologically: liver, kidneys, testicles

Results and discussion

Results: P0 (first parental generation)

CLINICAL SIGNS AND MORTALITY:

No deaths occurred. All rats appeared normal.

BODY WEIGHT:

Slight body weight loss was apparent in treated males up to 3 days following treatment. A tendency to return to normal was noted at the end of treatment. No further details.

ORGAN WEIGHTS:

There was a treatment-related decrease in the testes weight of treated rats when compared with controls. No further details.

GROSS PATHOLOGY:

An inflammatory reaction was observed in the liver of one treated rat. No further details.

HISTOPATHOLOGY:

The histological evaluation of liver and kidneys of the dosed rats revealed no substance-related pathological alterations when compared with control rats. However, an acute hepatitis and acute interstitial nephritis occurred in all dosed animals. According to the authors, these histological findings were commonly seen in the performing laboratory and were possibly caused by parasitic infestation.

The effect of the test substance on the testes was very evident. The seminiferous tubules of all dosed rats were changed. Lesions seen in the epithelium comprised degeneration of spermatocytes and spermatids,

reduction of spermatozoa as well as appearance of giant cells. Sertoli cells and interstitial cells of Leydig were unaffected. No further details.

Conclusions:

Administration of the test substance at 200 mg/kg bw/d for 5 days to male rats resulted in decreased testes weight and produced injury in the seminiferous tubules.

3.10.1.5 Study 5: 4-*tert*-butyltoluene

Reference:Unnamed 1982b

The experimental work was done during October 1981. Histologic evaluation of the testes was made during January - February 1982.

Test guideline: no guideline available. The study was performed to assess the acute toxicity of the test substance to the testes of rats. No further information is given on the guideline/standard procedure followed.

GLP compliance: no

Test material:

Reference substance name:4-tert-butyltoluene

EC Number: 202-675-9 EC

Cas Number: 98-51-1

- Name of test material (as cited in study report): p.-tert. butyl toluene; supplied by the sponsor, internal substance code: Ro 94-0522

- Physical state: pale yellowish coloured liquid

- Analytical purity: no data

- Storage condition of test material: in a metal bottle

Test animals:

Species: rat

Strain: albino

Sex:male

male albino SPF rats; no further data

- Source: Institute of Biological and Medical Research, Füllinsdorf, Switzerland

- Age at study initiation: no data

- Weight at study initiation: 186 - 225 g

- Housing: during the dosing period: 2 per cage; after treatment: individually in metabolic cages for 24 h; no further data

- Diet (ad libitum): NAFAG No. 850, pulverized

- Water (ad libitum): tap water

- Acclimation period: 6 days

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 19 - 23°C

- Humidity (%): 45 - 65%

- Air changes (per hr): no data

- Photoperiod (hrs dark / hrs light): 12/12

Administration / exposure

Route of administration:oral:gavage

Vehicle:rape oil

PREPARATION OF DOSING SOLUTIONS:

The test substance was suspended in rape oil; no further data.

VEHICLE

rape oil (Rüböl DAB 6, supplier: R.P. Scherer GmbH, Eberbach, Germany)

- Justification for use and choice of vehicle: no data

- Concentration in vehicle / Amount of vehicle:

-- 10 ml/kg bw rape oil,

-- (12.5 mg test substance + 10 ml rape oil)/kg bw,

-- (25 mg test substance + 10 ml rape oil)/kg bw,

-- (50 mg test substance + 10 ml rape oil)/kg bw,

-- (100 mg test substance + 10 ml rape oil)/kg bw.

No information is given on the final concentration of the test substance in the vehicle. Furthermore, no information is given on the density of both test substance and vehicle; therefore, the final concentration of the test substance in the respective test suspension cannot be estimated either.

- Purity: food grade (DAB 6)

Duration of treatment / exposure:5 consecutive days

Frequency of treatment:once daily

Doses / Concentrations: 12.5, 25, 50, 100 mg/kg bw/d

Basis: actual ingested

No. of animals per sex per dose: 8 males per dose group; 4 males in the vehicle control group

Control animals: yes, concurrent vehicle

Examinations:

Parental animals: Observations and examinations:

CAGE SIDE OBSERVATIONS / DETAILED CLINICAL OBSERVATIONS:

- Time schedule: The rats were observed for overt signs of toxicity or behaviour change once daily during the dosing period and on the 6th test day.

BODY WEIGHT:

- Time schedule for examinations: Individual body weights were recorded once daily during the dosing period and on the 6th test day.

Postmortem examinations (parental animals):

SACRIFICE

All rats were euthanized by the use of carbon dioxide and exsanguinated by incision of the neck after the 6th test day.

GROSS NECROPSY

A gross necropsy was performed on all rats.

HISTOPATHOLOGY / ORGAN WEIGHTS

Liver, kidneys and testes were weighed (wet weight, paired organs were weighed together).

The following organs were examined histologically: liver, kidneys, testes, epididymides, seminiferous tubules.

Results and discussion

Results: P0 (first parental generation)

CLINICAL SIGNS AND MORTALITY:

No deaths occurred.

Clinical signs of toxicity were observed in rats treated with 50 and 100 mg/kg bw and comprised loss of hair, shaggy fur, hunched posture, lethargy, and diarrhea.

BODY WEIGHT:

The mean body weights of rats receiving 12.5 mg/kg bw/d were similar to those of control rats.

In the group receiving 25 mg/kg bw/d, a slight, transient reduction of mean body weights was seen in the beginning of the study. Body weight gain returned to normal in the middle of the study.

Mean body weight of rats receiving 50 mg/kg bw/d most decreased on day 4 of the study and increased to the initial value at the end of the study.

A marked progressive loss of body weight was seen in the majority of rats dosed with 100 mg/kg bw/d throughout the study. For details, see below, tables 1 and 2.

ORGAN WEIGHTS:

The mean testes weight of rats treated with 100 mg/kg bw/d was approximately 23% lower than those of control rats. For details, see below, table 2.

GROSS PATHOLOGY:

The main finding was a delineation of hepatic lobules in the majority of rats treated with 50 and 100 mg/kg bw/d. For details, see below, table 3.

HISTOPATHOLOGY:

The seminiferous epithelium of control rats as well as of rats receiving 12.5 and 25 mg/kg bw/d did not exhibit any histological changes.

In rats treated with 50 and 100 mg/kg bw/d, the germinal epithelium showed severe cell-deformations. Spermatids and spermatocytes were mainly degenerated. Spermatozoa were reduced. Giant cells were observed sporadicallly.

Overall reproductive toxicity

Table 1: mean body weights at each study day (adapted from registration dossier).

Dose (mg/kg bw/d)	**No of rats**	**Test day**					
		1	**2**	**3**	**4**	**5**	**6**
0	4	212	217	226	229	234	237
12.5	8	210	213	215	220	225	231

25	8	213	209	209	219	226	228
50	8	209	199	204	197	201	208
100	8	207	200	197	182	179	181

Table 2: mean body weights and mean weights of testes, kidneys and liver at necropsy (adapted from registration dossier).

Dose (mg/kg bw/d)	**No of rats**	**Body weight (g)**	**Testis weight (g)**	**Kidney weight (g)**	**Liver weight (g)**
0	4	238±7	2.504±0.144	2.059±0.148	12.14±0.87
12.5	8	231±11	2.595±0.346	2.020±0.175	12.61±1.03
25	8	228±12	2.703±0.203	2.061±0.109	12.20±1.03
50	8	208±13	2.647±0.148	1.838±0.134	12.13±0.58
100	8	181±13	1.038±0.288	1.852±0.219	11.93±1.42

Table 3: incidence of gross pathology findings in the two highest dose groups (adapted from registration dossier).

Finding	50 mg/kg bw/day	100 mg/kg bw/day
Slight delineation of heaptic lobules	6/8	2/8
Severe delineation of heaptic lobules	-	4/8
Pale liver	1/8	3/8
Whitish spots in the liver	1/8	-
Pale kidneys	-	3/8

No findings reported for 0, 12.5 and 25 mg/kg groups.

Table 4: Testes evaluation (adapted from registration dossier).

Concentration (mg/kg bw)	Grading mean %*			
	0	1	2	3
Control	85.6	14.4	0	0
12.5	86.1	13.9	0	0
25.0	84.7	15.3	0	0
50.0	10.3	40.4	29.1	20.2
100.0	0	1.3	12.4	86.3

*examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

Conclusions:

The authors conclude that, under the condition of this study, the maximum oral dose level producing no effect in rat testes was considered to be 25 mg/kg bw/d of p-tert-butyl toluene.

3.10.1.6 Study 6: 4-*tert*-butyltoluene

Reference: Unnamed 1984a

The experimental work was done during May 1984 (first day of dosing: 1984-05-24). Histopathologic evaluation of the testes was made during September 1984.

Test guideline: Principles of method if other than guideline:The study was performed to assess the acute toxicity of the test substance to the testes of guinea pigs. No further information is given on the guideline/standard procedure followed.

GLP compliance:no

Limit test:yes

Test material:

Reference substance name: 4-tert-butyltoluene

EC Number: 202-675-9

Cas Number: 98-51-1

- Name of test material (as cited in study report): p.-tert. butyl toluene; supplied by the sponsor, internal substance code: Ro 94-0522

- Physical state: colourless liquid

- Analytical purity: no data

- Storage condition of test material: in the dark, at room temperature, under an atmosphere of dry nitrogen

Test animals:

Species:guinea pig

Strain: Himalayan

Sex: male

male Himalayan spotted SPF guinea pigs

- Source: Institute of Biological and Medical Research, Füllinsdorf, Switzerland

- Age at study initiation: no data

- Weight at study initiation: 493 - 613 g at start of treatment (day 0)

- Housing: individually in metal cages

- Diet (ad libitum): pellet diet (Nafag No. 814)

- Water (ad libitum): tap water from drinking bottles

- Acclimation period: 6 days

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 19 - 23°C

- Humidity (%): 45 - 65%

- Air changes (per hr): no data

- Photoperiod (hrs dark / hrs light): 12/12

IN-LIFE DATES: From: 1984-05-18 To: 1984-05-29

Administration/exposure

Route of administration:oral: gavage

Vehicle:other: rape oil

PREPARATION OF DOSING SOLUTIONS:

The test substance, as a suspension in rape oil, was formulated freshly each day.

VEHICLE

rape oil (Rüböl DAB 6, supplier: R.P. Scherer GmbH, Eberbach, Germany)

- Justification for use and choice of vehicle: no data

- Amount of vehicle: 0.4 ml/kg bw/d

- Purity: food grade (DAB 6)

Analytical verification of doses or concentrations:no

Duration of treatment / exposure:5 consecutive days

Frequency of treatment:once daily

Doses / Concentrations: 100 mg/kg bw/d

Basis: actual ingested

No. of animals per sex per dose: 5 males per group

Control animals: yes, concurrent vehicle

- Dose selection rationale:The dose level was chosen with the intention to demonstrate toxicological effects and to avoid unscheduled deaths. The dose level of 100 mg/kg bw/d was selected based on pilot studies. A constant dose volume of 0.4 ml/kg bw was used for both groups. The dosage was adjusted daily, according to body weight.

- Rationale for animal assignment: random

Examinations:

Parental animals: Observations and examinations:

CAGE SIDE OBSERVATIONS / DETAILED CLINICAL OBSERVATIONS:

- Time schedule: All animals were observed once daily during the treatment period and on the day of necropsy. Changes in condition or behaviour were recorded.

BODY WEIGHT:

- Time schedule for examinations: Individual body weights were recorded once daily during the dosing period and on the day of necropsy.

OTHER:

24-hour urine was collected at study day 5.

Postmortem examinations (parental animals):

SACRIFICE

All animals were euthanized by the use of carbon dioxide and exsanguinated by incision of the neck.

GROSS NECROPSY

A gross necropsy was performed on all animals.

HISTOPATHOLOGY / ORGAN WEIGHTS

Testes (without epididymides) were weighed before fixation.

Testes and epididymides were fixed, sectioned (5 µm) and stained. A semiquantitative evaluation of the conditions of the seminiferous tubules was carried out.

Results and discussion

Results: P0 (first parental generation)

CLINICAL SIGNS AND MORTALITY:

No deaths were observed.

No signs of toxicity were noted.

BODY WEIGHT:

Body weight gain was similar in both dosed and control animals. For details, see tables 1 and 2.

ORGAN WEIGHTS:

Mean testes weights were similar in both dosed and control animals. For details, see table 2.

GROSS PATHOLOGY:

Greyish-white areas on the surface of the liver were found in 1/5 control animal (control animal no. 2). No autopsy findings related to treatment with the test substance were reported.

HISTOPATHOLOGY incl. semiquantitative evaluation of the condition of the seminiferous tubules:

A petty damage of germinal epithelium was seen in testes of 2/5 control animals and in 1/5 treated animal. Furthermore, 1/5 dosed animal exhibited a moderate damage of germinal epithelium; this finding was considered to be treatment-related. There were no other testicular or epididymal changes.

The histopathological evaluation of the liver of the above-mentioned control animal no. 2 discovered severe parenchymal necrosis (both focal and subcapsular) which was supposed to be caused by a cholestasis because bile pigment was seen in hepatocytes. However, canicular bile casts were not found.

Table 1: mean body weights at each dosing day and at the day of necropsy (adapted from registrion dossier).

Concentration (mg/kg bw/day)	No. of animals	Day					
		0	1	2	3	4	5
0	5	554±48	553±46	545±39	550±40	556±49	552±50
100	5	554±39	542±10	539±30	550±28	562±34	555±25

Table 2: mean body weights and mean testes weights at necropsy (adapted from registrion dossier).

Concentration (mg/kg bw/day)	No. of animals	Body weight (day 5) (g)	Testes weight absolute (g)	Testes weight adjusted (g/100 g bw)
0	5	552±50	2.35±0.27	0.43±0.06
100	5	555±25	2.29±0.38	0.41±0.06

Table 3: Histological assessment of seminiferous tubules per testis (adapted from registration dossier).

Concentration (mg/kg bw/day)	Grading mean %*			
	0	1	2	3
Control	97.5	2.3	0	0.2
100	91.0	4.5	1.8	2.7

*examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

Conclusions:

The authors conclude that, under the conditions of this study, the oral administration of 100 mg/kg bw/d p-*tert*-butyl toluene for 5 consecutive days had a harmful effect on germinal epithelium of guinea pigs.

3.10.1.7 Study 7: 4-*tert*-butyltoluene

Reference: Unnamed 1984b

Dosing started on 1984-06-17. Histopathologic evaluation was made during November 1984.

Test guideline: no guideline available. The study was performed to assess the acute toxicity of the test substance to the testes of Beagle dogs. No further information is given on the guideline/standard procedure followed.

GLP compliance: no

Limit test: yes

Test material:

Reference substance name: 4-tert-butyltoluene

EC Number: 202-675-9

Cas Number: 98-51-1

- Name of test material (as cited in study report): p.-tert. butyl toluene; supplied by the sponsor, internal substance code: Ro 94-0522

- Physical state: colourless liquid

- Analytical purity: no data

- Storage condition of test material: in the dark, at room temperature, under an atmosphere of dry nitrogen

Test animals:

Species:dog

Strain:Beagle

Sex:male

three male Beagle dogs

- Source: Kleintierfarm Madörin AG, Füllinsdorf, Switzerland

- Age at study initiation: ca. 10 months (all animals were born in August 1983)

- Weight at study initiation: 9.3 kg (dog no. 1; control); 9.2 kg (dog no. 2; 100 mg/kg bw/d); 10.0 kg (dog no. 3; 100 mg/kg bw/d)

- Fasting period before study: no data

- Housing: by threes in a conventional, air-conditioned room. After the 5th day of treatment, the animals were individually kept in metabolic cages for collection of urine (18 h)

- Diet (ad libitum): pelled complete diet (Nafag-Dog cubes no. 939/930)

- Water (ad libitum): tap water provided and dispensed from automatic drinking valves

- Acclimation period: 6 weeks

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 19 - 23°C

- Humidity (%): 45 - 65%

- Air changes (per hr): no data

- Photoperiod (hrs dark / hrs light): 12/12

IN-LIFE DATES: From: approximately 6 weeks prior to dosing To: no data (no sacrifice)

Administration / exposure

Route of administration: oral: capsule

Vehicle: unchanged (no vehicle)

Duration of treatment / exposure: 5 consecutive days

Frequency of treatment: once daily

Doses / Concentrations: 100 mg/kg bw/d

Basis:actual ingested

No. of animals per sex per dose: 1 control male (dog no. 1); 2 dosed males (dog no. 2 and dog no. 3)

Control animals: yes, sham-exposed

- Dose selection rationale: The dose level was chosen with the intention of demonstrating toxicological effects but to avoid a decline in health. The dose level of 100 mg/kg bw/d was selected on the basis of similar studies with rodents. One male Beagle dog received the empty capsule only (control animal). The dose was adjusted daily, according to body weight.

The test substance/control item was applied once daily until the day before orchiectomy.

Examinations:

Parental animals: Observations and examinations:

CAGE SIDE OBSERVATIONS and DETAILED CLINICAL OBSERVATIONS:

- Time schedule: The dogs were observed daily during the treatment period and on the day of orchiectomy. Changes in conditions and behaviour of the animals were recorded.

BODY WEIGHT:

- Time schedule for examinations: Individual body weights of the dogs were recorded once daily during the treatment period and on the day of orchiectomy.

OTHER:

Urine was collected for 18 hours after the 5th dose.

Removal of testes: The testes were removed under barbiturate anesthesia (Nembutal).

HISTOLOGY:

Testes and epididymides of the dogs were fixed, sectioned (5 µm) and stained. Section of testes and epididymides were examined microscopically.

Results and discussion

Results: P0 (first parental generation)

CLINICAL SIGNS AND MORTALITY:

No clinical symptoms were noted. No dog died.

BODY WEIGHT:

Body weight was not affected in both dosed dogs. For details, see table 1.

HISTOPATHOLOGY of the testes and epididymides:

A few multinucleated giant cells were seen in the lumen of seminiferous tubules of the control dog (dog no. 1).

There was a small quantity of seminiferous tubules with nearly total depopulation of germinal epithelium in both testes of dog no. 2. The concerned seminiferous tubules (ca. 20 in testis 1 and 10 in testis 2) showed early stages of spermatogenesis and Sertoli cells.

No changes were found in testes of dog no. 3 and in epididymides of all dogs.

For details, see table 2.

Table 1: individual body weights at each study day (adapted from registration dossier).

Dog	Dose level (mg/kg bw/d)	Body weight (kg) at day					
		1	2	3	4	5	6
1	0	9.3	9.9	9.6	9.6	9.5	9.2
2	100	9.2	9.6	9.6	9.2	9.3	9.2
3	100	10.0	10.3	10.3	10.1	10.1	9.6

Table 2: grading of histological findings (adapted from registration dossier).

Organ	Finding	Dog 1 (control)	Dog 2 (treated)	Dog 3 (treated)
Testes	Occurrence of multinucleated giant cells in the lumen of seminiferous tubules (disseminated at random throughout the testis section)	none to minimal	none	none
Testes	Occurrence of seminiferous tubules with severe depopulation of germinal epithelium (disseminated at random throughout the testis section)	none	none to minimal	none
Epididymides		No change in any dog		

Conclusions:

The authors conclude that, under the conditions of this study, the oral administration of the test substance at a dose level of 100 mg/kg bw/d for 5 consecutive days was well tolerated by male Beagle dogs. The observed, very slight atrophy of seminiferous tubules of one dosed dog could not be clearly attributed to treatment with the test substance because this finding had also been observed in testes of the concurrent control dog and, according to the authors, in testes of untreated dogs used in other studies.

3.10.1.8 Study 8: 4-*tert*-butyltoluene

Reference: Unnamed 1984c

The experimental work was done during June 1984 (first day of dosing: 1984-06-24). Histopathologic evaluation of the testes was made during August 1984.

Test guideline: The study was performed to assess the acute toxicity of the test substance to the testes of mice. No further information is given on the guideline/standard procedure followed.

GLP compliance: no

Limit test: yes

Test material:

Reference substance name: 4-tert-butyltoluene

EC Number: 202-675-9

Cas Number: 98-51-1

- Name of test material (as cited in study report): p.-tert. butyl toluene; supplied by the sponsor, internal substance code: Ro 94-0522

- Physical state: colourless liquid

- Analytical purity: no data

- Storage condition of test material: in the dark, at room temperature, under an atmosphere of dry nitrogen

Test animals:

Species:mouse

Strain:albino

Sex:male

male albino SPF mice; no further data

- Source: Institute of Biological and Medical Research, Füllinsdorf, Switzerland

- Age at study initiation: no data

- Weight at study initiation: 45 - 69 g at start of treatment (day 0)

- Housing: individually in macrolon cages

- Diet (ad libitum): pelled complete diet (Nafag No. 850)

- Water (ad libitum): tap water from drinking bottles

- Acclimation period: 6 days

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 19 - 23°C

- Humidity (%): 45 - 65%

- Air changes (per hr): no data

- Photoperiod (hrs dark / hrs light): 12/12

IN-LIFE DATES: From: 1984-06-18 To: 1984-06-29

Administration: exposure:

Route of administration: oral: gavage

Vehicle:rape oil

PREPARATION OF DOSING SOLUTIONS:

The test substance, as a suspension in rape oil, was formulated freshly each day; therefore the stability of the test substance in the vehicle was not determined.

VEHICLE

rape oil (Rüböl DAB 6, supplier: R.P. Scherer GmbH, Eberbach, Germany)

- Justification for use and choice of vehicle: no data

- Amount of vehicle: 5 ml/kg bw/d

- Purity: food grade (DAB 6)

Analytical verification of doses or concentrations:no

Duration of treatment / exposure:5 consecutive days

Frequency of treatment:once daily

Doses / Concentrations:100 mg/kg bw/d

Basis:actual ingested

No. of animals per sex per dose:6 males per group

Control animals:yes, concurrent vehicle

- Dose selection rationale:

The dose level was chosen with the intention to demonstrate toxicological effects and to avoid unscheduled deaths. The dose level of 100 mg/kg bw/d was selected based on pilot studies. A constant dose volume of 5 ml/kg bw was used for both groups. The dosage was adjusted daily, according to body weight.

- Rationale for animal assignment: random

Examinations:

Parental animals: Observations and examinations:

Examinations during the treatment period:

CAGE SIDE OBSERVATIONS / DETAILED CLINICAL OBSERVATIONS:

- Time schedule: All animals were observed once daily during the treatment period and on the day of necropsy. Changes in condition or behaviour were recorded.

BODY WEIGHT:

- Time schedule for examinations: Individual body weights were recorded once daily during the dosing period and on the day of necropsy.

OTHER:

24-hour urine was collected at study day 5.

Postmortem examinations (parental animals):

SACRIFICE

All animals were euthanized by the use of carbon dioxide and exsanguinated by incision of the neck.

GROSS NECROPSY

A gross necropsy was performed on all animals.

HISTOPATHOLOGY / ORGAN WEIGHTS

Testes (without epididymides) were weighed before fixation.

Testes and epididymides were fixed, sectioned (5 µm) and stained. A semiquantitative evaluation of the conditions of the seminiferous tubules was carried out.

Results and discussion

Results: P0 (first parental generation)

CLINICAL SIGNS AND MORTALITY:

No treatment-related deaths were observed.

No signs of toxicity were noted.

BODY WEIGHT:

Body weight gain was similar in both dosed and control animals. For details, see tables 1 and 2.

ORGAN WEIGHTS:

Mean testes weights of the dosed animals were slightly increased when compared with controls. For details, see table 2.

GROSS PATHOLOGY:

No autopsy findings were reported.

HISTOPATHOLOGY incl. semiquantitative evaluation of the condition of the seminiferous tubules:

A slight damage of germinal epithelium was seen in testes of 1/6 control animals and in 3/6 treated animals. There were no other testicular or epididymal changes.

Table 1: mean body weights at each dosing day and at the day of necropsy (adaped from registration dossier).

Dose level (mg/kg bw/d)	No of animals	Body weight (kg) at day					
		0	1	2	3	4	5
0	6	57±4	54±5	56±5	56±5	56±5	55±5
100	6	61±9	59±8	59±8	59±8	59±8	57±8

Table 2: mean body weights (at day 5) and testes weights (adapted from registration dossier)

Concentration (mg/kg bw/day)	No. of animals	Body weight (day 5) (g)	Testes weight absolute (g)	Testes weight adjusted (g/100 g bw)
0	6	55±5	0.29±0.02	0.68±0.07
100	6	57±8	0.34±0.03	0.60±0.13

Table 3: Histological assessment of seminiferous tubules per testis (adapted from registration dossier).

Concentration (mg/kg bw/day)	Grading mean %*			
	0	1	2	3
Control	95.75	4.08	0	0.17
100	94.83	4.25	0.25	0.67

*examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

Conclusions:

The authors conclude that, under the conditions of this study, the oral administration of 100 mg/kg bw/day p-tert.butyl toluene for 5 consecutive days had a petty effect on germinal epithelium of mice.

3.10.1.9 Study 9: 4-*tert*-butyltoluene

Reference: Long-lasting learning and memory impairments induced by prenatal exposure to 4-tert-butyltoluene in rats. Author: Hass U et al. Year: 1996

Teratology 53: 22A, abstract no. F15.

GLP compliance: not specified

Limit test: yes

Test material:

Reference substance name: 4-tert-butyltoluene

EC Number: 202-675-9

Cas Number: 98-51-1

- Name of test material (as cited in study report): 4-tert-butyltoluene

- Physical state: liquid

- Analytical purity: no data

Test animals. Species:rat

Strain:Wistar

female Mol:WIST rats. no further data

ENVIRONMENTAL CONDITIONS: no data

IN-LIFE DATES: no data

Route of administration:inhalation

Type of inhalation exposure (if applicable):not specified

Vehicle:not specified

Details on exposure:No details given.

Analytical verification of doses or concentrations:not specified

Details on mating procedure:No details given.

Duration of treatment / exposure:days 7 through 20 of gestation

Frequency of treatment:6 hours/day

Duration of test:until 22 months after delivery

Doses / concentrationsca. 0.12 mg/l (20 ppm)

Basis:nominal conc.

No. of animals per sex per dose:no data

Control animals:yes

Details on study design:No further details given.

Maternal examinations:No details given.

Statistics:No data.

Results and discussion

Details on maternal toxic effects:

The dose level used, ca. 0.12 mg/l (20 ppm), did not induce maternal toxicity. No further data.

Results (fetuses)

Details on embryotoxic / teratogenic effects:

The dose level used, ca. 0.12 mg/l (20 ppm), did not induce decreased viability of offspring.

Lowered pup body weight until day 10 and delayed ontogeny of reflexes - also after correction for body weight - was recorded.

At the age of 3 months, increased latencies and swim length were observed in the learning period of treated female offspring (p = 0.6%). Three weeks later, indications of memory impairments were noted. However, these increases were not statistically significant (p = 8.7%). No substance-related effects were observed at 17

months. At the age of 22 months, increases in latencies and swim length indicating memory impairments were observed in the first 3 trials and in the trials following a 4-days break in testing (p = 5.5%).

Conclusion:

According to the authors, the impairment in exposed female offspring was not considered to be related to poorer swimming capability since swim lengths were increased in proportion to the increased latencies; swim speed was similar to control. The results indicated that substance-related neurobehavioral impairments could interact with the consequences of aging.

3.10.1.10 Study 10: 4-*tert*-butyltoluene

Reference: Unnamed 2007b

Test guideline:OECD Guideline 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents)

Guideline for 28-Day Repeated Dose Toxicity Test in Mammalian Species (Chemical Substances Control Law of Japan)

GLP compliance:yes

Test material:

Reference substance name: 4-tert-butyltoluene

EC Number: 202-675-9

Cas Number: 98-51-1

- Name of test material (as cited in study report): p-tert-butyltoluene; supplier: Fuso Chemical Co. Ltd., Osaka, Japan

- Physical state: colourless transparent liquid

- Analytical purity: 95.93%

- Impurities (identity and concentrations): no data

- Lot/batch No.: Lot No. 09006

- Storage condition of test material: room temperature; dark.

Test animals.

Species: rat

Strain: Sprague-Dawley

Sex: male/female

male and female Crj:CD(SD)IGS, SPF rats

- Source: Charles River Laboratories, Inc

- Age at study initiation: 4 weeks upon arrival at the testing facility; 6 weeks at beginning of dosing

- Weight at study initiation: 161 - 188 g (males); 128 - 151 (females)

- Fasting period before study: no

- Housing: 5 per cage (during acclimatization), individually after assignment to experimental groups; stainless steel cages

- Diet: pellet diet (CRF-1; ORIENTAL YEAST Co., Ltd.), ad libitum

- Water: tap water, ad libitum

- Acclimation period: 5 days quarantine, 7 days acclimatization

ENVIRONMENTAL CONDITIONS

- Temperature (°C): 20 - 26 °C
- Humidity (%): 40 - 70 %
- Air changes (per hr): 12
- Photoperiod (hrs dark / hrs light): 12/12

IN-LIFE DATES: no data

Administration/exposure:

Route of administration:oral: gavage

Vehicle: corn oil

PREPARATION OF DOSING SOLUTIONS:

p-tert-Butyltoluene was adjusted by dissolving and diluting it in corn oil. The amount of the test substance was calculated using its purity in preparing the test substance. It is approved that prepared solutions at 0.2, 2, 20, and 200 mg/mL can be kept stable if stored at room temperature under a shaded condition for 7 days. Therefore, the prepared solution at each concentration was stored at room temperature under a shaded condition, and was used within 7 days after preparation. Concentrations of the test substance in administration samples used on the day of start of administration and the day of the termination of administration were measured. The measurement result showed no problems with concentrations of the test substance.

VEHICLE: corn oil

- Justification for use and choice of vehicle (if other than water): no data
- Concentration in vehicle: 0.2, 2, 20, and 200 mg/mL
- Amount of vehicle (if gavage): 5 ml/kg bw

Analytical verification of doses or concentrations: not specified

Duration of treatment / exposure: 28 days

Frequency of treatment: daily

Doses / Concentrations: 1.5, 5, 15, 50 mg/kg bw/d

Basis:actual ingested

No. of animals per sex per dose:12 males and 12 females per group

Control animals:yes, concurrent vehicle

- Dose selection rationale:

The doses were determined according to the results of “Preliminary Reproduction Toxicity Screening Test of p-tert-Butyltoluene by Oral Administration in Rats" (dose levels: 0, 1.5, 5, 15, and 50 mg/kg; 12 males and 12 females in each group). There was one death in females of the 15 mg/kg group, and one death in males and 6 deaths in females of 50 mg/kg group. There were decreases in body weight in females of 5 mg/kg group and males and females of the 15 mg/kg and higher dose groups, and atrophy of the testis and epididymis in males of the 15 mg/kg and higher dose groups. Hence, the dose levels of this study were set at

the highest 50, lower 15, 5, and 1.5 mg/kg by a common ratio of approximately 3. The control group was administered with the vehicle (corn oil) alone at the same volume.

Grouping was carried out by random sampling after body weights were stratified by a computer so that the mean body weight and standard deviation are equivalent between each group.

- Rationale for selecting satellite groups:

12 male and 12 female animals were used in each group. Six animals of each sex were used for necropsy on the termination of administration period, and 6 animals of each sex were used for necropsy on the termination of recovery period.

- Post-exposure recovery period in satellite groups: 14 days

Animals were treated for 28 days and were sacrificed at day 29 or 43 after beginning of treatment.

- Section schedule rationale: random

Examinations:

CAGE SIDE OBSERVATIONS and DETAILED CLINICAL OBSERVATIONS

- Time schedule: Clinical signs and death were observed twice a day, before and after administration, during the administration period and once a day during the recovery period.

BODY WEIGHT:

- Time schedule for examinations: The body weight was measured twice a week during both the administration and recovery periods.

FOOD CONSUMPTION:

- Time schedule for examinations: The food consumption was measured once a week during both the administration and recovery periods.

WATER CONSUMPTION:

- Time schedule for examinations: The water consumption was measured once a week during both the administration and recovery periods.

HAEMATOLOGY:

- Time schedule for collection of blood: at the next day of the last administration and after the termination of recovery period
- Anaesthetic used for blood collection: Yes (sodium pentobarbital)
- Animals fasted: Yes
- How many animals: all
- Parameters checked in tables 5 and 6 (see attached study report) were examined.

Parameters examined: erythrocyte count (RBC), hemoglobin, hematocrit, platelet count, and leukocyte count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count, leukocyte percentage, prothrombine time (PT), activated partial thromboplastin time (APTT), plasma fibrinogen

CLINICAL CHEMISTRY:

- Time schedule for collection of blood: at the next day of the last administration and after the termination of recovery period
- Animals fasted: Yes
- How many animals: all
- Parameters checked in tables 7 and 8 (see attached study report) were examined.

Parameters examined: AST, ALT, ALP, gamma-GTP, total protein, albumin A/G ratio, total bilirubin, BUN, creatinine, glucose, total cholesterol, triglycerides, sodium, potassium, chloride, calcium, inorganic phosphate.

URINALYSIS:

- Time schedule for collection of urine: before the termination of administration period and before the termination of recovery period
- Metabolism cages used for collection of urine:
- Animals fasted: Yes
- Parameters checked in tables 1-4 (~~see attached study report~~) were examined.

Urine was collected using diuresis cages before the termination of administration period and before the termination of recovery period. The following examinations were conducted for the urine collected in 3 hours (3-hour urine) under a condition that the animals were fasted and supplied with water, the urine collected in the subsequent 21 hours (21-hour urine) under a condition that the animals were supplied with food and water, and the urine that the above urines were totaled (24-hour urine).

Parameters examined: volume, specific gravity, colour, pH, protein, glucose, ketone body, bilirubin, occult blood, urobilinogen, sediments

GROSS PATHOLOGY:

The animals were sacrificed by exsanguination after collecting blood samples, and subsequently necropsy was conducted. The following organs were weighed: brain (cerebrum, cerebellum, and medulla oblongata), pituitary, thyroid, thymus, heart, liver, spleen, kidneys, adrenal glands, testes, epididymides, and ovaries (the pituitary and thyroid were weighed after fixed in 20% neutral buffer formalin for one night). These organs were fixed in 20% neutral buffer formalin together with the lungs, trachea, pancreas, salivary glands (sublingual gland and submandibular gland), esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, lymph nodes (mandibular and mesenteric), urinary bladder, seminal vesicles, prostate, uterus, vagina, parathyroid, spinal cord, sciatic nerve, eyeballs, harderian glands, sternum, femurs, and mammary glands. However, the testes and epididymides were fixed in Bouin's solution from 2 to 3 hours and subsequently refixed in 90% alcohol. The eyeball was fixed in glutaraldehyde-formalin mixture for one night and subsequently refixed in 20% neutral buffer formalin.

HISTOPATHOLOGY:

The investigators/authors produced HE-stained tissue sample of each organ and tissue of the animals of the control and 50 mg/kg groups that were necropsied on the termination of administration period and, conducted the histopathological examination. For the organs and tissues that the number of animals that

showed abnormalities was different compared with the control group in the examination of the 50 mg/kg group, the examination was conducted in the same manner for the 1.5, 5, and 15 mg/kg groups on the termination of administration period, and for the control group and the 1.5, 5, 15, and 50 mg/kg groups on the termination of recovery period.

Statistics:

Statistical analyses were carried out between the control group and each administration group with a significance level of 5% as explained in the following.

The means and standard deviations were calculated for the body weight, food consumption, water consumption, urine volume, urine specific gravity, results on the hematological and blood chemical examinations, and absolute and relative organ weights for each group. Subsequently, Bartlett's test was performed to assess the homogeneity of the variances. If the variances were homogenous, one-way ANOVA was conducted. Further, if there was statistical significance, Dunnett's test was conducted. On the other hand, if the variance was not homogenous, one-way ANOVA by ranks (Kruskal-Wallis test) were conducted. If there was statistical significance, a Dunnett-type rank test was performed.

There was a toxicological effect in the 50 mg/kg group in the histopathological examination. The Dunnett-type rank test was conducted on the findings on the organs and tissues that the examination was conducted, for comparison between the control group and groups of the other dose levels.

Results and discussion

CLINICAL SIGNS AND MORTALITY

1) Administration Period

There was no death or mobidity in males and females in any group.

In the observation of clinical signs, no abnormality was found in males and females of the control, 1.5, and 5 mg/kg groups. Transient salivation was found in males and females of the 15 and 50 mg/kg groups.

2) Recovery Period

There was no death or mobidity in males and females in any group.

In the observation of clinical signs, there was no abnormality in males and females in any group.

BODY WEIGHT AND WEIGHT GAIN

1) Administration Period

There was no significant difference in body weight at any day of measurement in males and females of the 1.5, 5, 15, and 50 mg/kg groups compared with the control group.

2) Recovery Period

There was no significant difference in body weight at any day of measurement in males and females of the 1.5, 5, 15, and 50 mg/kg groups compared with the control group.

FOOD CONSUMPTION

1) Administration Period

In males, there was no significant difference in food consumption at any day of measurement in the 1.5 and 5 mg/kg groups compared with the control group. There were significant decreases in food consumption at day 3 of the administration period in the 15 and 50 mg/kg groups compared with the control group.

In females, there was no significant difference in food consumption at any day of measurement in the 1.5 and 5 mg/kg groups compared with the control group. There was a significant decrease in food consumption at day 3 of the administration period in the 50 mg/kg group compared with the control group. There were significant increases in food consumption at day 17 of the administration period in the 15 and 50 mg/kg groups compared with the control group. However, these were transient changes, and were not considered to be a toxicological effect.

2) Recovery Period

There was no significant difference in food consumption at any day of measurement in males and females of the 1.5, 5, 15, and 50 mg/kg groups compared with the control group.

WATER CONSUMPTION

1) Administration Period

In males, there was no significant difference in water consumption at any day of measurement in the 1.5 and 5 mg/kg groups compared with the control group. There was a significant increase in water consumption at day 17 of the administration period in the 15 mg/kg group compared with the control group. There were significant increases in water consumption from day 3 to day 24 of the administration period in the 50 mg/kg group compared with the control group.

In females, there was no significant difference in water consumption at any day of measurement in the 1.5, 5, and 15 mg/kg groups compared with the control group. There was a significant increase in water consumption at day 10 of the administration period in the 50 mg/kg group compared with the control group.

2) Recovery Period

In males, there was no significant difference in water consumption at any day of measurement in the 1.5, 5, 15, and 50 mg/kg groups compared with the control group.

In females, there was no significant difference in water consumption at any day of measurement in the 1.5, 5, and 50 mg/kg groups compared with the control group. There was a significant decrease in water consumption at day 10 of the recovery period in the 15 mg/kg group compared with the control group. However, this change was not due to the dose, and therefore not considered to be a toxicological effect.

HAEMATOLOGY

1) On Termination of Administration Period

In males, there were no significant differences in any measurement subjects in the 1.5 mg/kg group compared with the control group. There were significant decreases in APTT and fibrinogen in the 5, 15, and 50 mg/kg groups compared with the control group. There were significant decreases in MCH in the 15 and 50 mg/kg groups compared with the control group. However, since there were no differences in erythrocyte count, hemoglobin concentration, and hematocrit value, it is not considered to be a toxicological effect.

In females, there were no significant differences in any measurement subjects in the 1.5 mg/kg group compared with the control group. There was a significant increase in fibrinogen in the 15 mg/kg group compared with the control group. There was a significant decrease in fibrinogen and a significant increase in PT in the 50 mg/kg group compared with the control group. Further, there was a significant decrease in platelet in the 5 mg/kg group compared with the control group. There were significant decreases in MCHC and platelet in the 15 mg/kg group compared with the control group. There were significant decreases in MCH and MCHC in the 50 mg/kg group compared with the control group. The significant differences in platelets found in the 5 and 15 mg/kg groups did not represent changes due to the administration since the differences from the control group were small and there were no significant differences in the high dose groups. Further, it is not considered that the significant decrease in MCHC found in the 15 mg/kg group and the significance decreases in MCH and MCHC found in the 50 mg/kg group are not toxicological effects since there were no differences in hemoglobin and hematocrit.

2) On Termination of Recovery Period

In males, there were no significant differences in any measurement subjects in the 1.5 and 5 mg/kg groups compared with the control group. There were significant decreases in erythrocyte count, hemoglobin concentration, and hematocrit value in the 15 and 50 mg/kg groups compared with the control group.

In females, there were no significant differences in any measurement subjects in the 1.5, 5, and 15 mg/kg groups compared with the control group. There were significant decreases in hemoglobin and hematocrit in the 50 mg/kg group compared with the control group.

CLINICAL CHEMISTRY

1) On Termination of Administration Period

In males, there were no significant differences in any measurement subjects in the 1.5 mg/kg group compared with the control group. There were significant decreases in total protein and triglyceride and significant increases in AST, blood urea nitrogen, and inorganic phosphorus in the 5 mg/kg group compared with the control group. There were significant decreases in total protein, albumin, and triglyceride and significant increases in AST, A/G, total bilirubin, blood urea nitrogen, and inorganic phosphorus in the 15 mg/kg group compared with the control group.

There were significant decreases in total protein, albumin, total cholesterol, triglyceride, and Na, and significant increases in AST, A/G, total bilirubin, blood urea nitrogen, creatinine, and inorganic phosphorus in the 50 mg/kg group compared with the control group. Further, there was a significant increase in ALT in the 5 mg/kg group. However, since the difference from the control group was small and there were no significant differences in the high dose groups, this was not considered to be a change due to the administration.

In females, there were no significant differences in any measurement subjects in the 1.5 and 5 mg/kg groups compared with the control group. There were significant decreases in total protein, albumin, total cholesterol, triglyceride, and Ca, and a significant increase in gamma-GTP in the 15 mg/kg group compared with the control group. There were significant decreases in total protein, albumin, triglyceride, K, and Ca, a

decreasing tendency of total cholesterol, and significant increases in gamma-GTP and total bilirubin in the 50 mg/kg group compared with the control group.

2) On Termination of Recovery Period

In males, there were no significant differences in any measurement subjects in the 1.5, 5, 15, and 50 mg/kg groups compared with the control group.

In females, there were no significant differences in any measurement subjects in the 5, 15, and 50 mg/kg groups compared with the control group. There was a significant decrease in glucose in 1.5 mg/kg group compared with the control group. However, this did not represent a change due to the administration since the difference from the control group was small and there were no significant differences in the high dose groups.

URINALYSIS

1) Before Termination of Administration Period

In males, there were no significant differences in urine volume and urine specific gravity in the 1.5 and 5 mg/kg groups compared with the control group. There was a significant increase in urine volume in the 15 mg/kg group compared with the control group. There was a significant increase in urine volume and a significant decrease in urine specific gravity in the 50 mg/kg group compared with the control group. Colour, pH, protein, glucose, ketone body, bilirubin, occult blood, urobilinogen, and urine sediments were at almost the same level as the control group in the 1.5, 5, and 15 mg/kg groups. There were decreasing tendencies in pH and protein in the 50 mg/kg group compared with the control group.

In females, there were no significant differences in urine volume and urine specific gravity in the 1.5 and 5 mg/kg groups compared with the control group. There was a significant increase in urine volume in the 50 mg/kg group compared with the control group. There was a significant increase in urine specific gravity in the 15 mg/kg group compared with the control group. However, this change was not due to the dose. Colour, pH, protein, glucose, ketone body, bilirubin, occult blood, urobilinogen, and urine sediments were at almost the same level as the control group in the 1.5, 5, and 15 mg/kg groups. There was a decreasing tendency in pH in the 50 mg/kg group compared with the control group.

2) Before Termination of Recovery Period

In males, there were no significant differences in urine volume and urine specific gravity in the 1.5, 5, and 15 mg/kg groups compared with the control group. There was a significant increase in urine volume and a significant decrease in urine specific gravity in the 50 mg/kg group compared with the control group. Colour, pH, protein, glucose, ketone body, bilirubin, occult blood, urobilinogen, and urine sediments were at almost the same level as the control group in the 1.5, 5, 15, and 50 mg/kg groups.

In females, there were no significant differences in urine volume and urine specific gravity in the 1.5, 5, 15, and 50 mg/kg groups compared with the control group. Colour, pH, protein, glucose, ketone body, bilirubin, occult blood, urobilinogen, and urine sediments were at almost the same level as the control group in the 1.5, 5, 15, and 50 mg/kg groups.

ORGAN WEIGHTS and GROSS PATHOLOGY

1) On Termination of Administration Period

In males, there were no significant differences in absolute and relative weights of each organ in the 1.5 and 5 mg/kg groups compared with the control group. There was a significant increase in relative weight of the liver in the 15 mg/kg group compared with the control group. There were significant decreases in absolute weights of the testis and epididymis and relative weight of the testis and significant increases in **absolute and** relative weights of the liver in the 50 mg/kg group compared with the control group. Further, there was a significant decrease in absolute weight of the heart in the 50 mg/kg group compared with the control group. However, since there was no significant difference in relative weight, this was considered to be a change based on the difference in body weight, and that the change was not due to the administration.

In females, there was a significant decrease in body weight at the day of necropsy in the 50 mg/kg group compared with the control group. In the organ weight examination, there were no significant differences in absolute and relative weights of each organ in the 1.5 and 5 mg/kg groups compared with the control group. There were significant increases in absolute and relative weights of the liver in the 15 mg/kg group compared with the control group. There was a significant decrease in absolute weight of the ovary and significant increases in absolute weight of the liver and relative weights of the liver, kidney, and adrenal gland in the 50 mg/kg group compared with the control group. Further, there was a significant decrease in absolute weight of the thymus in the 50 mg/kg group compared with the control group. However, since there was no significant difference in relative weight, it was considered to be a change based on the difference in body weight, and that the change was not due to the administration.

2) On Termination of Recovery Period

In males, There were no significant differences in absolute and relative weights of each organ in the 1.5, 5, and 15 mg/kg groups compared with the control group. There were significant decreases in absolute and relative weights of the epididymis and decreasing tendencies of absolute and relative weight of the testis in the 50 mg/kg group compared with the control group.

In females, there were no significant differences in absolute and relative weights of each organ in the 1.5 and 5 mg/kg groups compared with the control group. There was a significant increase in relative weight of the liver in the 15 mg/kg group compared with the control group. There were significant increases in absolute and relative weights of the liver in the 50 mg/kg group compared with the control group.

HISTOPATHOLOGY

1) On Termination of Administration Period

(a) Male Rats

Liver: There was periportal hepatocyte hypertrophy in 4 animals in the 50 mg/kg group.

Testis: There was atrophy of the seminiferous tubules and hyperplasia of Leydig cells in 6 animals in the 50 mg/kg group.

Epididymis: There was a decrease in sperm count in the lumen of the ductus epididymis in 6 animals in the 50 mg/kg group.

Statistical significance was observed in periportal hepatocyte hypertrophy in the liver, atrophy of seminiferous tubules and hyperplasia of Leydig cells in the testis, the decrease in sperm count in the lumen of the ductus epididymis in the epididymis compared with the control group. The other observed changes are normally observed in the control group. Hence, those were considered accidental changes.

(b) Female Rats

Liver: There was periportal hepatocyte hypertrophy in one animal in the 50 mg/kg group.

The other observed changes are normally observed in the control group. Hence, those were considered accidental changes.

2) On Termination of Recovery Period

(a) Male Rats

Testis: There was atrophy of the seminiferous tubules in 6 animals in the 50 mg/kg group. There was atrophy of the seminiferous tubules in one animal of the 5 mg/kg group. However, this was considered an accidental change since there was no such change in the 15 mg/kg group.

Epididymis: There was a decrease in sperm count in the lumen of the ductus epididymis in 6 animals in the 50 mg/kg group.

Statistical significance was observed in atrophy of the seminiferous tubules in the testis and the decrease in sperm count in the lumen of the ductus epididymis in the epididymis in the 50 mg/kg group compared with the control group. The other observed changes are normally observed in the control group. Hence, those were considered accidental changes.

(b) Female Rats

There were microgranulomas and vacuolation of periportal hepatocytes in the liver. However, these changes are normally observed in the control group. Hence, these were considered accidental changes.

Conclusions:

According to the authors, the NOELs for repeated dose toxicity are considered to be at 1.5 mg/kg bw/d for males and at 5 mg/kg bw/d for females.

3.10.1.11 Study 11: 4-*tert*-butylbenzaldehyde

Reference: Unnamed 1981

Test guideline: Testicular toxicity screening test: The test material was administered orally to 4 groups of rats at doses from 6.5 to 50 mg/kg bw for 5 consecutive days.

GLP compliance: not specified

Limit test: no

Test material:

Reference substance name:

4-tert-butylbenzaldehyde

EC Number: 213-367-9

Cas Number: 939-97-9

- Name of test material (as cited in study report): p-tert-butylbenzaldehyde

- Analytical purity: no data given

Test animals:

Species: rat

Strain: SPF albino

Sex: male

- Weight at study initiation: 156-220 g

- Housing: two animals per cage during dosing period, after treatment in a metabolic cage for 24 h

- Diet: not specified, ad libitum

- Water: tap water, ad libitum

- Acclimation period: 7 days

ENVIRONMENTAL CONDITIONS

- Temperature: 19-23 °C

- Humidity: 45-65 %

- Air changes: air-conditioned room

- Photoperiod: 12 hrs dark / 12 hrs light

Administration/exposure:

Route of administration: oral: gavage

Vehicle:rape oil

Animals were randomly allocated to test groups by means of a random table.

The test material and the respective control was administered by gavage using a metal stomach tube once daily for 5 consecutive days at a dose volume of 10 ml/kg bw.

Analytical verification of doses or concentrations: no

Duration of treatment / exposure: 5 days Frequency of treatment: once daily

Details on study schedule: only male animals were used and there was no mating

Doses / Concentrations: 6.5, 12.5, 25 and 50 mg/kg bw/d

Basis: nominal conc.

No. of animals per sex per dose: test group: 8 males control group: 4 males

Control animals: yes, concurrent vehicle

Details on study design: Post-exposure period: 1 day

After the 5th treatment, urine was collected for 24 hours.

Examinations:

Parental animals: Observations and examinations:

Mortality, clinical symptoms and body weight were observed and recorded daily during the treatment period and on the 6th test day.

Sperm parameters (parental animals):not assessed

Postmortem examinations (parental animals):

Gross necropsy was performed after the 6th day on all rats. Liver, kidneys and testes were weighed. Liver/kidneys were fixed in 4% formol and embedded in PARAPLAST PLUS (no furhter assessment). Testes were fixed in mixture of Bouin, embedded in PARAPLAST PLUS, sectioned at a nominal thickness of 5µm and stained with haematoxylin/eosin

The testes of all rats were microscopically examined.

The condition of the seminiferous tubules was evaluated semiquantitative by examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

Results and discussion:

Results: P0 (first parental generation)

Mortality and general observation:

All animals survived the experimental period. 3 rats treated with 12.5 mg/kg bw/d showed slight aggressiveness on test days 3 and 4. From days 3 to 6, a slight loss of hair was seen in one animal of the 50 mg/kg bw/d group.

Body weight development:

The test material did not affect the weight development of those animals treated with 6.5 and 12.5 mg/kg bw/d. Rats treated with 25 mg/kg bw/d initially showed a slight weight loss and returned to normal at the end of the treatment. The animals of the highest dose group had a severe weight loss throughout the study.

Necropsy and organ weights:

During the dissection, a marbled liver was recorded in 2 rats treated with 50 mg/kg bw/d. In one rat treated with 25 mg/kg bw/d a small dell was seen in the right kidney.

Testes weights of rats treated with 50 mg/kg bw/d were significantly lower than these recorded for the controls.

Histology:

The changes of testes caused by the treatment were circumscribed on the seminiferous epithelium. Interstitial cells and Sertoli cells were unaffected.

The following changes were seen: disorganisation of the epithelial structure, degeneration of cells, and reduction of the spermatozoa. A testis of a control rat showed about 80 % convoluted tubules with a normal epithelium (graduation 0) and about 20 % convoluted tubules with a normal epithelium but with degenerated cells or detritus in the lumina (graduation 1). This ratio occurred in the 6.5 and 12.5 mg/kg bw/d group, too. An alteration of this ratio was seen from the 25 mg/kg bw/d group on. Moderate to severe injuries were discovered in the seminiferous epithelia of all rats treated with 50 mg/kg bw/d.

Table 1: Mean organ weights (g) of the rats from day 1 to day 6 (adapted from registration dossier).

Dose (mg/kg bw)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0	212	216	220	225	227	227
6.5	206	210	215	218	220	222
12.5	207	208	211	214	215	218
25	211	208	209	214	214	217
50	206	198	195	196	198	197

Table 2: mean organ weights (g) of the rats (adapted from registration dossier).

Dose (mg/kg bw)	Testis	Kidney	Liver
0	2.576 ±0.232	1.897±0.113	10.69±0.57
6.5	2.522±0.101	1.974±0.075	10.82±0.49
12.5	2.570±0.191	1.800±0.152	10.34±0.92
25	2.485±0.203	1.807±0.152	10.86±0.76
50	2.206±0.199	1.749±0.076	10.11±0.63

Table 3: Histological assessment of seminiferous tubules per testis (adapted from registration dossier).

Concentration (mg/kg bw)	Grading mean %*			
	0	1	2	3
Control	78.1	21.9	0	0
6.5	82.5	17.5	0	0
12.5	83.0	17.0	0	0
25.0	53.6	34.6	5.7	6.1
50.0	1.5	27.6	43.8	27.1

*examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

3.10.1.12 Study 12: 4-*tert*-butylbenzaldehyde

Reference: Unnamed 1984d

Test guideline: Testicular toxicity screening test: One dose of the test material was administered orally to male mice for 5 consecutive days.

GLP compliance: not specified

Limit test: no

Test material:

Reference substance name: 4-tert-butylbenzaldehyde

EC Number: 213-367-9

Cas Number: 939-97-9

- Name of test material (as cited in study report): p-tert-butylbenzaldehyde, TBB

- Analytical purity: no data given

Test animals:

Species: mouse

Strain:SPF albino

Sex: male

- Weight at study initiation: 52 - 63 g

- Housing: individually in macrolon cages

- Diet: pelled complete diet (Nafag No. 850), ad libitum

- Water: tap water, ad libitum

- Acclimation period: 6 days

ENVIRONMENTAL CONDITIONS

- Temperature: 19-23 °C

- Humidity: 45-65 %

- Photoperiod: 12 hrs dark / 12 hrs light

Administration / exposure:

Route of administration: oral: gavage

Vehicle:rape oil

Animals were randomly allocated to test groups by means of a random table.

The test material and the respective control was administered by gavage using a metal stomach tube once daily for 5 consecutive days at a dose volume of 5 ml/kg bw.

The test material was formulated as suspension in rape oil freshly each day; surplus test material was discarded.

When not in use, the test material was stored in the dark under the atmosphere of dry nitrogen to ensure its stability.

Analytical verification of doses or concentrations:no

Duration of treatment / exposure:5 days Frequency of treatment: once daily

Doses / Concentrations: 100 mg/kg bw/d Basis: nominal conc.

No. of animals per sex per dose: 6 male animals per dose group

Control animals: yes, concurrent vehicle

Details on study design:

Post-exposure period: 1 day

After the 5th treatment, urine was collected for 24 hours.

Parental animals: Observations and examinations:

All animals were observed once daily during the treatment period and on the day of necropsy. Changes in condition or behaviour of the animals were recorded.

Individual body weights were recorded once daily during the treatment period and on the day of necropsy.

Sperm parameters (parental animals):not determined

Postmortem examinations (parental animals):

All animals were sacrificed by use of CO2 and exsanguinated by incision of the neck.

A gross necropsy examination was performed on all animals.

Testes (without epididymides) of all animals were weighed before fixation.

Testes and epididymides of all animals were fixed in the mixture of Bouin and embedded in Paraplast Plus. Tissues were sectioned at a normal thickness of 5 µm and stained with haematoxilin/eosin.

The condition of the seminiferous tubules was evaluated semiquantitative by examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

Results and discussion:

Results: P0 (first parental generation)

Symptoms of incompatibility were not seen.

Body weight:The body weight gain of controls and treated animals was comparable.

Necropsy: A turbid pericardiopleural region with deposits was seen in 2 test substance treated mice. These findings, i.e. pleurisy and pericarditis, resulted from an aspiration pneumonia and were not considered to be related to treatment with the test substance.

Testes weight: The testes weights of the treated animals showed no effect when compared to controls.

Histology: A slight damage of germinal epithelium was seen in testes of 1 control and 4 treated animals. Other testicular changes and changes of epididymides were not observed (no further information given).

Table 1: Mean body weights (g) of treated animals and controls (adapted from registration dossier).

Body weight (g) on day						
	0	1	2	3	4	5
Control	57	54	56	56	56	55
Treated	60	56	57	57	58	56

Table 2: Mean organ weights (g) of treated animals and controls on day 5 (adapted from registration dossier).

	Testes weights (g) absolute ± SD	Testes weights (g) adjusted (100g) ± SD
Control	0.29±0.02	0.53±0.07
Treated	0.31±0.03	0.55±0.07

Table 3: Histological assessment of seminiferous tubules per testis (adapted from registration dossier).

Concentration (mg/kg bw/day)	Grading mean %±SD*			
	0	1	2	3
0	95.75±1.29	4.08±1.00	0	0.17±0.58
100	94.42±2.39	4.08±1.73	0.33±0.65	1.17±2.12

*examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

3.10.1.13 Study 13: 4-*tert*-butylbenzaldehyde

Reference: Unnamed 1984e

Test guideline: Testicular toxicity screening test: One dose of the test material was administered orally to male guinea pigs for 5 consecutive days.

GLP compliance:not specified

Limit test:no

Test Material:

Reference substance name:4-tert-butylbenzaldehyde

EC Number:213-367-9

Cas Number:939-97-9

- Name of test material (as cited in study report): p-tert-butylbenzaldehyde, TBB

- Analytical purity: no data given

- When not in use, the test material was stored in the dark under the atmosphere of dry nitrogen to ensure its stability.

Test animals:

Species:guinea pig

Strain:Himalayan

Sex:male

- Weight at study initiation: 493 - 613 g

- Housing: individually in metal cages

- Diet: pelled complete diet (Nafag No. 814), ad libitum

- Water: tap water, ad libitum

- Acclimation period: 6 days

ENVIRONMENTAL CONDITIONS

- Temperature: 19-23 °C

- Humidity: 45-65 %

- Photoperiod: 12 hrs dark / 12 hrs light

Administration / exposure:

Route of administration:oral: gavage

Vehicle:rape oil

Animals were randomly allocated to test groups by means of a random table.

The test material and the respective control was administered by gavage using a metal stomach tube once daily for 5 consecutive days at a dose volume of 0.4 ml/kg bw/d.

The test material was formulated as suspension in rape oil freshly each day; surplus test material was discarded.

Analytical verification of doses or concentrations:no

Duration of treatment / exposure:5 consecutive days

Frequency of treatment:once daily

Doses / Concentrations:100 mg/kg bw/d

Basis:nominal conc.

No. of animals per sex per dose:5 male animals per dose group

Control animals:yes, concurrent vehicle

Details on study design:

Post-exposure period: 1 day

After the 5th treatment, urine was collected for 24 hours.

Parental animals: Observations and examinations:

All animals were observed once daily during the treatment period and on the day of necropsy.

Changes in condition or behaviour of the animals were recorded.

Individual body weights were recorded once daily during the treatment period and on the day of necropsy.

Postmortem examinations (parental animals):

All animals were sacrificed by use of CO2 and exsanguinated by incision of the neck.

A gross necropsy examination was performed on all animals.

Testes (without epididymides) of all animals were weighed before fixation.

Testes and epididymides of all animals were fixed in the mixture of Bouin and embedded in Paraplast Plus. Tissues were sectioned at a normal thickness of 5 µm and stained with haematoxilin/eosin.

The condition of the seminiferous tubules was evaluated semiquantitative by examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

Results and discussion:

Results: P0 (first parental generation)

Mortality:No death occurred

General observation:Symptoms of incompatibility were not observed.

Body weight:No effect was seen.

Necropsy:No treatment-related necropsy findings were seen.

Testes weights:No significant difference has been observed between treated and control group

Histology:A petty damage of germinal epithelium was seen in 2 control animals and in 1 treated animal, however the lumen of the seminiferous tubules of the test substance treated animals showed more detritus than those of the control animals. Other testicular changes and changes of epididymides were not observed.

Table 1: mena body weights (g) of treated animals and controls (adapted from registration dossier).

	Body weight (g) on day					
	0	1	2	3	4	5
Controls	554	553	545	550	556	552
Treated	544	540	537	541	555	534

Table 2: mean organ weights (g) of treated animals and controls on day 5 (adapted from registration dossier).

	Testes weights (g) absolute ± SD	Testes weights (g) adjusted (100g) ± SD
Control	2.35±0.27	0.43±0.06
Treated	2.25±0.21	0.42±0.03

Table 3: Histological assessment of seminiferous tubules per testis (adapted from registration dossier).

Concentration (mg/kg bw/day)	Grading mean %±SD*			
	0	1	2	3
Control	97.5±1.18	2.3±1.06	0	0.2±0.42
100	89.7±2.41	10.1±2.51	0.1±0.32	0.1±0.32

*examination/grading of 100 randomly selected, cross-sectioned tubules in a respective testis section.

3.10.1.14 Study 14: 4-*tert*-butylbenzaldehyde

Reference: Unnamed 1984f

Test guideline: Testicular toxicity screening test: One dose of the test material was administered orally to male beagle dogs for 5 consecutive days.

GLP compliance:not specified

Limit test:no

Test Material:

Reference substance name:4-tert-butylbenzaldehyde

EC Number:213-367-9

Cas Number:939-97-9

- Name of test material (as cited in study report): p-tert-butylbenzaldehyde, TBB

- Physical state: yellowish liquid

- Analytical purity: no data given

- Storage condition of test material: in the dark at room temperature under an atmosphere of dry nitrogen to ensure stability

Test animals:

Species:dog

Strain:Beagle

Sex:male

- Source: Marshall Research Animals, North Rose, USA

- Weight at study initiation: 11.1 and 12.2 kg

- Housing: two animals per box; after the 5th treatment the animals were housed individually in a metabolism cage.

- Diet: pelled complete diet (Nafag-Dog cubes No. 939/930), ad libitum

- Water: tap water, ad libitum from automatic drinking valves

- Approx. presence time at animal house before experimental period: 10 and 26 weeks

ENVIRONMENTAL CONDITIONS

- Temperature: 19-23 °C

- Humidity: 45-65 %

- Air changes: air-conditioned rooms

- Photoperiod: by daylight

Route of administration: oral: capsule Vehicle:in gelatine capsules

Analytical verification of doses or concentrations:not specified

Duration of treatment / exposure:5 days

Frequency of treatment:once daily

Doses / Concentrations:100 mg/kg bw/d

Basis:nominal conc.

No. of animals per sex per dose:two male animals in the treated group, one male animal as control

Control animals:yes, concurrent vehicle

Post-exposure period: 1 day

After the 5th treatment, urine was collected for 18 h, For that purpose, the dogs were individually kept in a metabolism cage.

Examinations

Parental animals: Observations and examinations:

The dogs were observed daily during the treatment period and on the day of orchiectomy. Changes in condition or behaviour of the dogs were recorded

Individual body weights of the dogs were recorded once daily during the treatment period and an the day of orchiectomy.

However, the testes were removed under barbiturate anaesthesia for histopathological examination.

Testes and epididymides of the dogs were fixed in a mixture of Bouin and embedded in Paraplast Plus. Tissues were sectioned at a normal thickness of 5 µm and stained with haematoxilin/eosin. Testis weight was not determined. Sections of the testes and epididymides were microscopically examined.

Results and discussion

Results: P0 (first parental generation)

Symptoms of incompatibility were not observed.

Body weight: Both dosed dogs showed a slight weight loss up to day 6:

Dog 1 (test substance treated): 12.2 kg on day 1, 11.6 kg on day 6;

Dog 2, (test substance treated): 11.1 kg on day 1, 10.0 kg on day 6

No information given about the control dog.

Histopathology:There were about 60 cross sectioned seminiferous tubules with nearly total depopulation of germinal epithelium in both testes of one dog. In these seminiferous tubules, early stages of spermatogenesis and Sertoli cells were preserved only. With the exception of the occurrence of multinucleated giant cells - a background finding seen also in the control animal - no abnormalities were discovered in the testes of the other treated dog. No changes were seen in epididymides of both dogs. The slight damage of germinal epithelium of one dog was considered to be related to treatment.

Table 1: Graduation of histological findings in the testes of the animals (adapted from registration dossier)

Testes	control animal*	treated animal 1	treated animal 2
Occurrence of multinucleated giant cells in the lumen of seminiferous tubules	0-1	0	0-1
Occurrence of seminiferous tubules with severe depopulation of germinal epithelium	0	1	0

0 = no change

1 = minimal change

* The untreated dog acted as control in a similar study. In this study, sections of testes and epididymides of that dog served as control sections.

3.10.1.15 Study 15: 4-*tert*-butylbenzaldehyde

Reference: TSCATS, NTIS/OTS0505405, New Doc. I.D. 88-8100336, 1982

Test guideline: Testicular toxicity screening test: One dose of the test material was administered orally to male rats for 5 consecutive days.

GLP compliance:not specified

Limit test:no

Test material:

Reference substance name:4-tert-butylbenzaldehyde

EC Number:213-367-9

Cas Number:939-97-9

- Name of test material (as cited in study report): p-tert-butylbenzaldehyde

- Analytical purity: no data given

Test animals:

Species:rat

Strain:SPF albino

Sex:male

- Housing: individually or in groups of 2 animals

- Acclimatization period: 7 days

- Diet: NAFAG No. 850, pulverized, ad libitum

- Water: tap water, ad libitum

ENVIRONMENTAL CONDITIONS

- Temperature: 19-23 °C

- Humidity: 45-65 %

- Air changes per hr: air conditioned rooms

- Photoperiod: 12 hrs dark / 12 hrs light

Administration / exposure

Route of administration:oral: gavageVehicle:rape oil

The test material and the respective control was administered by gavage using a metal stomach tube once daily for 5 consecutive days at a dose volume of 10 ml/kg bw.

Analytical verification of doses or concentrations:no

Duration of treatment / exposure:5 days

Frequency of treatment:once daily

Doses / Concentrations:100 mg/kg bw/d

Basis:nominal conc.

No. of animals per sex per dose:7 male animals per dose group

Control animals:yes, concurrent vehicle

Post-exposure period: 1 day

Examinations:

Parental animals: Observations and examinations:

Mortality, clinical symptoms, signs of behavioural change and body weight were observed and recorded daily during the treatment period and on the day after treatment.

Sperm parameters (parental animals):not assessed

Postmortem examinations (parental animals):

All rats were sacrificed by the use of CO2 and exanguinated by incision of the neck.

A gross necropsy examination was performed on the day after the 5th application on all rats. Liver, kidneys and testicles were weighed. Sections of theses organs were fixed in 4 % formol, embedded in paraplast plus and microscopally examined.

Results and discussion

Results: P0 (first parental generation)

CLINICAL SIGNS AND MORTALITY

No mortality occurred throughout the study and all animals appeared normal.

BODY WEIGHT AND FOOD CONSUMPTION

During the first two days an initial body weight loss was observed, but these rats showed subsequent weight gain at the end of the treatment period.

ORGAN WEIGHTS

Testes weights of the treated animals were lower than those of the controls, weight of liver and kidney showed no difference in comparison with the control.

GROSS PATHOLOGY

One of the 7 tested animals had an agenesia of the left kidney and testis.

HISTOPATHOLOGY

Liver:One treated and 4 control animals showed moderate to severe inflammatory infiltration of the portal tract by mainly mononuclear cells associated with alterated bile ducts; one treated and 3 control animals showed multifocal necrosis in the parenchym (treated: minimal to moderate, control: moderate to severe) and two treated and 5 control animals showed minimal to moderate accumulation of lymphocytes in the parenchyma.

Kidneys:2 treated and 4 control animals showed minimal to moderate lymphocytic interstitial inflammation, one control animal showed minimal subcapsular lymphocytic infiltration. The authors stated, that the histological evaluation of liver and kidney sections revealed no sign of any compound related pathological alteration when compared to the control males. Indeed an acute hepatitis and acute interstitial nephritis occurred in males of all test groups. These histological findings commonly seen in their laboratory rats were possibly caused by parasitic infestation.

Testes:The treated animals showed injuries in the seminiferous epithelium. Five treated animals showed minimal to moderate degeneration of spermatids and spermatocytes. One treated animal showed a minimal reduction of spermatozoa and all treated animals showed minimal to moderate appearance of multinucleate giant cells. Sertoli cells and Leydig cells were unaffected.

No other treatment related effects were found by general observation, necropsy and histological examinations.

Table 1: mean body weights (g) of treated animals and controls (adapted from registration dossier).

	Mean body weight (g) on day					
	1	2	3	4	5	6
Control	155	158	168	174	177	185
Treated	156	145	148	156	161	170

Table 2: organs weights (g) of treated animals and controls (adapted from registration dossier).

	Mean organ weights (g) on day 6 ± SD		
	liver	kidneys	testes
Control animals	10.52±1.19	1.86 ±0.17	2.07 ±0.07
Treated animals	10.56 ±0.8	1.67 ±0.19	1.75 ±0.29

Table 3: scores of histological findings (adapted from registrion dossier). Seven treated animals.

Degeneration of spermatids and spermatocytes							
	0	0	0-1	0-1	1-2	0-1	0-1
Reduction of spermatozoa							
	0	0	0	0	1	0	0
Appearance of multinucleate giant cells							
	0-1	0-1	0-1	0-1	2	0-1	0-1

Seven control anmials: no findings

0 = no change, 1 = minimal change, 2 = moderate change, 3 = severe change

3.10.2 Other data

3.10.2.1 Study 1: *tert*-butylbenzoic acid

Type of information:Experimental data for relevant metabolite

Materials and methods

Test guideline: no guideline available

Principles of method if other than guideline: Ex vivo study using a 3D cell culture with primary seminiferous tubules from juvenile Sprague Dawley rats (Bio-Alter®). Cytotoxicity, blood-testis barrier functionality via trans-epithelial electrical resistance (TEER) measurements and cell numbers of different somatic and germ cell populations have been quantified. In addition, the content of TBBA conjugated with CoA and the metabolome has been assessed in cell culture lysates.

GLP compliance: no

Type of method: other: ex vivo

Test material

Reference substance name: 4-tert-butylbenzoic acid

EC Number:202-696-3

EC Name:4-tert-butylbenzoic acid

Cas Number:98-73-7

Molecular formula:C11H14O2

IUPAC Name:4-tert-butylbenzoic acid

Details on test material:

- Supplier: Sigma

- Batch number: STBC2750V

- Storage conditions: room temperature

Specific details on test material used for the study: Two strucural isomers were tested, i.e. para-4-tert-butylbenzoic acid (p-TBBA) and meta-3-tert-butylbenzoic acid (m-TBBA).

p-TBBA CAS 98-73-7:

- Supplier: Sigma

- Batch number: STBC2750V

- Storage conditions: room temperature

m-TBBA CAS 7498-54-6:

- Supplier: Sigma

- Batch number: CDS021677

- Storage conditions: room temperature

Positive control: Methoxyacetic acid

- Supplier: Sigma

- Batch number: STBC9332V

- Storage conditions: room temperature

Test animals

Species:rat

Strain:Sprague-Dawley

Sex:male

Details on test animals or test system and environmental conditions:

- Number of animals: 9

- Supplier: Charles River, Saint-Germain-sur l'Arbresle, France

- Age: 22-28 days

- Housing, Animals feed and water: Weaned animals delivered the day before the experiment in NovoPack with food and water ad libitum

- Animal sacrifice: Decapitation after anesthesia with chloroform

- Tissue harvesting: Testes are quickly removed and collected in Ham's F12/Dulbecco's Modified Eagle's Medium (F12/DMEM)

Administration / exposure

Details on study design:Preparation of seminiferous tubule segments and culturing:

The tunica albuginea of testes was removed and seminiferous tubules were carefully dissociated by collagenase enzymatic digestions at 33°C and mechanical dissociation. Between each step of this process, seminiferous tubule fragments were washed with F12/DMEM supplemented by 10 µg/ml gentamycin and 10µU/ml nystatin

Cell culture from rat seminiferous tubules was performed according to the Bio-AlteR® technology.

The cells from pooled seminiferous tubules were seeded (Day 0 of the experiment) in bicameral chambers in the culture medium. The culture medium consists of 15mM Hepes-buffered F12/DMEM supplemented with 10µg/ml gentamicin, 10µU/ml nystatin, 1.2g/L sodium bicarbonate, 10µg/ml insulin, 10µg/ml human

transferrin, 10^-4M vitamin C, 10µg/ml vitamin E, 10^-7M testosterone, 3.3x10^-7M retinoic acid, 3.3x10^-7M retinol, 10^-3M pyruvate (all from Sigma), and 1ng/ml of FSH (NIH, Bethesda, MD). Incubation was carried out at 33°C in a water-saturated atmosphere of 95% air and 5% CO2. The compounds or vehicle were added in the basal compartment of the bicameral chambers of the culture from day 2 onward. Only basal media (+/- compounds) was renewed every 2±1 days. On day 7 and 14, cells were detached from the permeable membrane of the bicameral chamber by trypsinization and the cells from 3-4 wells were pooled. An aliquot of the cell suspensions was used to determine the number of cells and to assess cell viability by trypan blue exclusion. Aliquots of cells were fixed with ice-cold ethanol for flow cytometric analysis.

For CoA-TBBA conjugate determination, cells were detached from the permeable membrane of the bicameral chamber and the cells from the 3 wells of the same replicate pooled (day 8 and day 15). Hence 3 replicates were recovered per condition and per day of study.

For metabolome analyses 9 wells of culture were used per day of study (day 8 and day 15), per condition (i.e. vehicle, mid and high concentration of p-TBBA and m-TBBA, respectively).

Trans-Epithelial Electrical Resistance (TEER):

The integrity of the blood-testis barrier is assessed by trans-epithelial electrical resistance (TEER) measurements in control and in treated cultures at day 2 (before the addition of the compounds or vehicle) and at days 5, 7, 9, 12, 14, 16, 18-19 and 21. Trans-epithelial electrical resistance (TEER) measurement is performed with an EVOM2 (World Precision Instruments, Florida, USA). The culture is equilibrated at room temperature before assay. The TEER is calculated according to the following equation: TEER=(R total−R control)•A (Ω cm2), where R total is the resistance measured, R control is the resistance of the control insert (insert alone with the culture medium), and A is the surface area of the insert. Six wells of culture were used per condition.

Total Cell number & viability (on total cells):

The total cell number and viability is assessed by microscopic counting and trypan blue exclusion on day 7 and on 14.

Immunolabeling of Cultured Cells for Flow Cytometric Analysis:

The number of each cell type (somatic cells, spermatogonia, young spermatocyte I, middle to late pachytene spermatocytes I, secondary spermatocytes and round spermatids) is measured by flow cytometric analysis and compared to control at days 7 and 14. To distinguish germ cells from somatic cells, fixed cultured cells are immunolabeled with a monoclonal antibody against vimentin. Hoechst is added to the labelled cell suspension to assess their DNA content. After immunolabeling, cells is analysed using a Attune Acoustic Focusing cytometer (Life Technologies). The vimentin-positive somatic cells and the vimentin-negative 4C, 2C and 1C germ cells are separated with the bivariate analysis of DNA content/vimentin. Then the bivariate liner forward light scatter and liner side angle light scatter analyses allow the identification of each germ cell population. Four wells of culture were used at D7 and 5 wells at D14 per condition.

Quantification of TBBA-CoA conjugates:

On day 8 and 15, cells were detached from the permeable membrane of the bicameral chamber by trypsinisation (100 µL of trypsin solution per well of culture) and cell metabolism was stopped by addition of citric acid (100 µL, 0.5 M) and cold acetonitrile (200 µL) containing 2 µM decanoyl CoA as internal standard. Cells from three wells were pooled and transferred to 2 ml tubes (final volume 1.2 ml) and frozen at -80°C. Samples were thawed, and centrifuged (21,000 x g, 5 min, RT). 500 µL of the supernatant (from total~1.2 mL) was diluted with 6.8 ml Tris-buffer (4 mM) containing KCl (6 mM), MgCl2 (0.3 mM) and n-heptadecanoyl CoA as internal standard (0.2 µM). Samples were neutralized by addition of NaOH (150 µL of 1M NaOH) and loaded onto solid phase extraction cartridges (OASIS HLB µElution plate). Columns were washed with 200 µL of ammonium solution (1 M), and samples eluted sequentially with 50 µL acetonitrile and 50 µL water. Samples were then further concentrated to 50 µL under a N2 stream.

CoA conjugates were analysed with high resolution LC-MS (LC-HRMS) on a Dionex UltiMate 3000 RS HPLC system coupled to a Q-Exactive orbitrap mass spectrometer (Thermo Scientific, Reinach, Switzerland) with electrospray ionization (ESI) in both positive and negative ionization mode. For liquid chromatography separation an Agilent Zorbax 300Extend-C18 column with dimensions 2.1 mm x 50 mm

and particle size of 3.5 µm with a 2.1 mm x 10 mm pre-column of the same material was used. The flow rate was 0.4 mL/min. Eluent A consists of water containing 0.0025% ammonia (pH around 9.8) and eluent B consists of acetonitrile containing 0.0025% ammonia. A linear gradient was run from 95% eluent A (hold for 1 min) to 100% eluent B within 6 min (hold for 1 min), back to 95% eluent A within 0.5 min followed by 1.5 min equilibration time. The injection volume of the sample was 10 µl. The mass resolution of the HR-MS spectra was set to 70,000. The mass accuracy was <5 ppm. Datadependent high resolution product ion spectra (HR-MS/MS) were recorded at a resolution of 17,500. Ion source parameters adjusted were as follows: sheath gas flow (30 arbitrary unit), auxiliary gas flow (10 arbitrary unit), capillary temperature (270°C), and source voltage (4kV in positive mode, -3kV in negative mode). Fragmentation was obtained from dissociation in an octopole collision cell using higher energy collision dissociation settings at 35 and 45 (arbitrary unit). The mass scan range was set from 120 to 1800 m/z. Calibration standards were prepared with tBBA-CoA.

Decanoyl-CoA and heptadecanoyl-CoA were used as internal standards. CoA conjugates are identified by comparison to the synthetic reference and by their exact mass. The analytical method does not discriminate between position isomers, with p-tBBA-CoA and m-tBBA-CoA having the same exact mass of 927.2040. The limit of detection with this method is around 0.0025 µM for CoA conjugates. Spiking experiments were performed to prove analytical proficiency in this experimental system and to estimate recovery.

Metabolome analysis

The membrane with the adherent cells of each well of culture was recovered. Each membrane was washed 3-times by a warm solution of NaCl 0.9%. Then, the membrane was transferred into an Eppendorf vial cooled in dry ice, in which the cold dichloromethane/ethanol (11:9) was pipetted before. 3 membranes were recovered per tube. Each cut membrane was placed with a tweezer directly into the cooled quenching solution. Samples were stored at -80°C.

For each analytical sample three combined assay membranes, quenched with 600 µl DCM/Eth-anol (11/9, v/v), were extracted using a ball mill and centrifugated. The supernatant was transferred into an empty vial. Subsequently, the pellet was extracted twice with 300 µl of the same DCM/Ethanol mixture and all extracts of a sample were combined. After evaporation metabolites were extracted with 1600 µl of a mixture of methanol, dichloromethane, water and toluene (93:47:16.5:1, v/v) containing formic acid. Internal standards were added to the extraction mixture to confirm reproducible analysis. After centrifugation an aliquot of the extract was subjected to LC-MSMS analysis using reversed phase and hydrophilic interaction liquid chromatography (HILIC) followed by MS/MS detection (AB Sciex QTrap 6500+) using the positive and negative ionization mode. RP-HPLC gradient elution (0 to 0.1 min 100% A, 0.5 min 75% A, 5.9 min 10% A, 6.0 min 0% A, 600 µl/min) was performed with water/ methanol/ 0.1 M ammonium formate (1:1:0.02 w/w) (A) and methyl-tert-butylether/ 2-propanol/ methanol/ formic acid/ 0.1 M ammo-nium formate (2:1:0.5:0.0175:0.035 w/w) (B). HILIC gradient elution (0 to 0.1 min 100% A, 5 min 10% A, 600 µl/min) was performed with acetonitrile containing 1 vol-% water and 0.2 vol-% acetic acid (A) and 0.007 M ammonium acetate containing 0.2 vol-% acetic acid (B).

A second aliquot of the extract was mixed with water (3.75:1, v/v) resulting in a phase separation. Both phases were analyzed separately with GC-MS after derivatization as described in Gross-mann et al., 2010. Briefly, the non-polar fraction was treated with methanol under acidic condi-tions to give fatty acid methyl esters that were derived from both free fatty acids and hydrolyzed complex lipids. The polar and non-polar fractions were further derivatized with O-methyl-hydrox-ylamine hydrochloride to convert oxo-groups to O-methyloximes, and subsequently with a silylat-ing agent (N-methyl-N-(trimethylsilyl) trifluoroacetamide).

Extracts from a separate set of control samples were pooled and aliquots were measured in parallel throughout the entire analytical process. All of the analytical samples were analyzed once in a randomized analytical sequence design to avoid artificial results with respect to analytical shifts. Data were corrected to internal standards and normalized against the median of the pool reference samples to give pool-normalized ratios (performed for each sample per metabolite). This accounts for inter- and intra-instrumental variation.

To check for potential differences in cell numbers within and between different treatment groups, the data for intracellular metabolite levels were also normalized to the within sample median. The median normalization produced a new set of values xij. (see Ramirez, T., et al. (2018). "Prediction of liver toxicity and mode of action using metabolomics in vitro in HepG2 cells." Archives of Toxicology 92(2): 893-906). For each of the

32 biological samples, the median xij of the pool-normalized ratios was calculated based on the pool-normalized ratios of all 347 evaluated metabolites.

Metabolite values were log10-transformed for the entire statistical analysis to better approximate a normal distribution. For principal component analysis, the metabolite data was centered and scaled to unit variance using the commercial software Simca 15 (Umetrics, Sartorius Stedim Biotech). For all multivariate analyses, only samples and analytes with at least 50% data cover-age were included (missing value imputation is handled by the software).

Results and discussion

Observed effects

MAA

The positive control MAA at 2500µM had a strong effect on spermatogenesis. MAA lowered the TEER at D5, D7, D9, D12, D14, D19 and D21. MAA strongly decreased the number of cells in all the studied germ cell populations at D7 and D14: spermatogonia, young pachytene spermatocytes, middle to late pachytene spermatocytes, secondary spermatocytes and round spermatids.MAA also increased the number of somatic cells at D7 and D14.

The results with the positive control validate the sensitivity and the responsiveness of the cell cultures.

p-TBBA

- The effect of p-TBBA on the blood-testis barrier was low. A transient and slight decrease versus control was observed. This result could be related to a transient disturbance of the blood-testis barrier and with an alteration of the tight junctions. However, compared to the changes observed in controls, the relation between p-TBBA treatment and the variations in TEER is questionable.

- p-TBBA had no effect on cell viability.

- Incubation with p-TBBA at the 3 tested concentrations increased the number of somatic cells in a rather dose dependent manner at D7. No such effect was observed at D14.

- p-TBBA increased the number of spermatogonia at D7 (50µM) and at D14 (3 tested concentrations). However, the increase at D14 was not confirmed in the former study 1501-03 under comparable testing conditions.

- Incubation with p-TBBA had an adverse effect on the meiotic process of germ cells, starting at the stage of middle to late pachytene spermatocytes. At D7, p-TBBA at 10µM and 50µM decreased in a rather dose-dependent manner:

o the number of middle to late pachytene spermatocytes,

o the number of secondary spermatocytes,

o the number and of round spermatids.

At D14, p-TBBA decreased:

o the number of middle to late pachytene spermatocytes at 50µM,

o the number of secondary spermatocytes at 10µM and 50µM,

o the number of round spermatids at the 3 tested concentrations.

m-TBBA

- The effect of m-TBBA on the blood-testis barrier was low. Compared to the changes observed in controls, the relation between m-TBBA treatment and the variations in TEER is questionable.

- The test substance m-TBBA had no effect on cell viability.

- No effect on the number of somatic cells was observed for m-TBBA at all tested concentrations.

- m-TBBA had only a slight effect on the meiotic process of germ cells. Indeed, m-TBBA

decreased:

o the number of middle to late pachytene spermatocytes and of secondary spermatocytes at D7 (2µM and 10µM); not confirmed in the former study 1501-03 under comparable testing conditions.

o the number of round spermatids at D7 (2µM) and at D14 (50µM).

Further, an increase of the number of middle to late pachytene spermatocytes observed at D14 at the 3 tested concentrations was found. However, due to the slight and dose independent changes, the relevance of this finding for m-TBBA is questionable.

In samples dosed with 10 and 50 µM of p-tBBA, the corresponding CoA-conjugate p-tert-butyl-benzoyl-CoA was detectable both at 8 and 15 days, while trace amounts around the detection limit were detected in the samples dosed with 2 µM.

The average levels of the detected CoA conjugate were in the range of 0.005 – 0.00065 µM and 0.012 - 0.021 µM in the analytical samples dosed with 10 and 50 µM p-tBBA, respectively. In contrast, no respective CoA-conjugate meta-tert-butyl-benzoyl-CoA (meta-TBBA-CoA) was detectable in the samples dosed with either 2, 10 or 50 µM of meta-TBBA collected after 8 or 15 days of exposure.

Metabolom analysis via PCA

The impact of the chemical treatment with the two different TBBA isomers (p-TBBA and m-TBBA) at 10µM and 50µM on the metabolome of rat seminiferous tubule culture cells at two timepoints (8d and 15d) was compared. In a Principal Component Analysis (PCA), the first two principal components explain 50% of the overall metabolic variability and the main driver for sample separation is the timepoint (pc1: 37%) as well as isomer and concentration used (pc1 and pc2). The datapoints (representing results from single samples, i.e. wells) are found in high proximity, forming cluster according to their respective treatment groups, which demonstrated a high sample homogeneity and comparability in the metabolic changes observed. The samples of the two different timepoints are clearly separated based on pc1. For both timepoints investigated, m-TBBA treated samples were found in close proximity to control samples with a marginal separation of the 8 day samples at the highest concentration tested (50 µM m-TBBA) only, indicating a marginal concentration response. No such concentration response was observed for samples incubated for 15 days with m-TBBA. In contrast, a clear separation from control clusters and a concentration response was found for p-TBBA at both timepoints assessed. Para-TBBA incubation was found to result in lower levels of several lipids as well as some amino acids (glutamate, glutamine, 5-oxoproline).

3.10.2.2 Study 2: *tert*-butylbenzoic acid

Type of information:other: Experimental data for relevant metabolite

Title:Unnamed

Year:2020

Report date:2020

Materials and methods

Test guideline:no guideline available

Principles of method if other than guideline: Ex vivo study using a 3D cell culture with primary seminiferous tubules from juvenile Sprague Dawley rats (Bio-Alter®). Cytotoxicity and cell numbers of different somatic and germ cell populations have been quantified.

GLP compliance: no

Type of method:other: ex vivo

Test material

Reference substance name:4-tert-butylbenzoic acid

EC Number:202-696-3

EC Name:4-tert-butylbenzoic acid

Cas Number:98-73-7

Molecular formula:C11H14O2

IUPAC Name:4-tert-butylbenzoic acid

Details on test material:

- Supplier: Sigma

- Batch number: STBC2750V

- Storage conditions: room temperature

Specific details on test material used for the study: Two strucural isomers were tested, i.e. para-4-tert-butylbenzoic acid (p-TBBA) and meta-3-tert-butylbenzoic acid (m-TBBA).

p-TBBA CAS 98-73-7:

- Supplier: Sigma

- Batch number: STBC2750V

- Storage conditions: room temperature

m-TBBA CAS 7498-54-6:

- Supplier: Combi-Blocks

- Batch number: QH-0813

- Storage conditions: room temperature

Positive control: Methoxyacetic acid

- Supplier: Sigma

- Batch number: STBD2084V

- Storage conditions: room temperature

Test animals

Species:rat

Strain:Sprague-Dawley

Sex:male

Details on test animals or test system and environmental conditions:

- Number of animals: 3

- Supplier: Charles River, Saint-Germain-sur l'Arbresle, France

- Age: 22-28 days

- Housing, Animals feed and water: Weaned animals delivered the day before the experiment in NovoPack with food and water ad libitum

- Animal sacrifice: Decapitation after anesthesia with chloroform

- Tissue harvesting: Testes are quickly removed and collected in Ham's F12/Dulbecco's Modified Eagle's Medium (F12/DMEM)

Administration / exposure

Details on study design:Preparation of seminiferous tubule segments and culturing:

The tunica albuginea of testes was removed, and seminiferous tubules were carefully dissociated by collagenase enzymatic digestions at 33°C and mechanical dissociation. Between each step of this process,

seminiferous tubule fragments were washed with F12/DMEM supplemented by 10 µg/ml gentamycin and 10µU/ml nystatin.

Cell culture from rat seminiferous tubules was performed according to the Bio-AlteR® technology.

The cells from pooled seminiferous tubules were seeded (Day 0 of the experiment) in bicameral chambers in the culture medium. The culture medium consists of 15mM Hepes-buffered F12/DMEM supplemented with 10µg/ml gentamicin, 10µU/ml nystatin, 1.2g/L sodium bicarbonate, 10µg/ml insulin, 10µg/ml human transferrin, 10^-4M vitamin C, 10µg/ml vitamin E, 10^-7M testosterone, 3.3x10^-7M retinoic acid, 3.3x10^-7M retinol, 10^-3M pyruvate (all from Sigma), and 1ng/ml of FSH (NIH, Bethesda, MD). Incubation was carried out at 33°C in a water-saturated atmosphere of 95% air and 5% CO2. The compounds or vehicle were added in the basal compartment of the bicameral chambers of the culture from day 2 onward. Only basal media (+/- compounds) was renewed every 2±1 days.

On day 8 and 15, cells were detached from the permeable membrane of the bicameral chamber by trypsinization and the cells from 4-5 wells were pooled. An aliquot of the cell suspensions was used to determine the number of cells and to assess cell viability by trypan blue exclusion. Aliquots of cells were fixed with ice-cold ethanol for flow cytometric analysis.

Total Cell number & viability (on total cells):

The total cell number and viability is assessed by microscopic counting and trypan blue exclusion on day 8 and day 15.

Immunolabeling of Cultured Cells for Flow Cytometric Analysis:

The number of each cell type (somatic cells, spermatogonia, young spermatocyte I, middle to late pachytene spermatocytes I, secondary spermatocytes and round spermatids) is measured by flow cytometric analysis and compared to control at days 8 and 15. To distinguish germ cells from somatic cells, fixed cultured cells are immunolabeled with a monoclonal antibody against vimentin. Hoechst is added to the labelled cell suspension to assess their DNA content. After immunolabeling, cells was analyzed using a Attune Acoustic Focusing cytometer (Life Technologies). The vimentin-positive somatic cells and the vimentin-negative 4C, 2C and 1C germ cells are separated with the bivariate analysis of DNA content/vimentin. Then the bivariate liner forward light scatter and liner side angle light scatter analyses allow the identification of each germ cell population. Four wells of culture were used at D8 and 5 wells at D15 per condition.

Results and discussion

Observed effects

MAA

The positive control MAA at 2.5 mM had a strong effect on spermatogenesis. MAA decreased the number of cells in all the studied germ cell populations (spermatogonia, young pachytene spermatocytes, middle to late pachytene spermatocytes, secondary spermatocytes and round spermatids) at D8 and D15. MAA also increased the number of somatic cells at D8 and D15. These results validate the sensitivity and the responsiveness of the cell cultures.

p-TBBA

- p-TBBA had no effect on cell viability.

- p-TBBA at 3 tested concentrations increased the number of somatic cells in a dose dependent manner at D8. No such effect was observed at D15.

- p-TBBA increased the number of spermatogonia at D8 (10µM and 50µM) but not at D15.

- Incubation with p-TBBA had an adverse effect on the meiotic process of germ cells, starting at the stage of middle to late pachytene spermatocytes.

At D8, p-TBBA decreased the number of:

o middle to late pachytene spermatocytes at 50µM,

o secondary spermatocytes at the 3 tested concentrations in a dose dependent manner,

o round spermatids at the 3 tested concentrations in a rather dose dependent manner.

At D15, p-TBBA decreased, at the 3 tested concentrations in a rather dose dependent manner, the number of:

o middle to late pachytene spermatocytes,

o secondary spermatocytes

o round spermatids.

m-TBBA

- The test substance m-TBBA had no effect on cell viability.

- At D8 only, m-TBBA slightly increased the number of somatic cells at the 3 tested concentrations.

- Incubation with m-TBBA had only a slight effect on the meiotic process of germ cells. Indeed, m-TBBA only decreased the number of middle to late pachytene spermatocytes and of secondary spermatocytes at 2µM at D15.

Further, m-TBBA increased the number of:

o spermatogonia (at 50µM at D8),

o young and of middle to late pachytene spermatocytes (at 10µM at D8 and D15),

o secondary spermatocytes (at 10µM at D8),

o round spermatids (at 50µM at D15).

However, due to the slight and dose independent changes, the relevance of this finding for m-TBBA is questionable.

3.10.2.3 Study 3: *tert*-butylbenzoic acid

Type of information: Experimental data for relevant metabolite

Restrictions are based on limited number of donors, variability within the dataset and choice of positive control substance

Materials and methods

Test guideline:no guideline available

Principles of method if other than guideline:Ex vivo study using a 3D cell culture with primary seminiferous tubules from a transgender male who underwent castration. Cytotoxicity and cell numbers of different somatic and germ cell populations have been quantified. In addition, the content of TBBA conjugated with CoA has been assessed in cell culture lysates.

GLP compliance:no

Type of method:other: ex vivo

Test material

Reference substance name:4-tert-butylbenzoic acid

EC Number:202-696-3

EC Name:4-tert-butylbenzoic acid

Cas Number:98-73-7

Molecular formula:C11H14O2

IUPAC Name:4-tert-butylbenzoic acid

Details on test material:

- Supplier: Sigma

- Batch number: STBC2750V

- Storage conditions: room temperature

Specific details on test material used for the study: Two strucural isomers were tested, i.e. para-4-tert-butylbenzoic acid (p-TBBA) and meta-3-tert-butylbenzoic acid (m-TBBA).

p-TBBA CAS 98-73-7:

- Supplier: Sigma

- Batch number: STBC2750V

- Storage conditions: room temperature

m-TBBA CAS 7498-54-6:

- Supplier: Synthonix (ref B54768)

- Batch number: 51

- Storage conditions: room temperature

Positive control: Methoxyacetic acid

- Supplier: Sigma

- Batch number: STBC9332V

- Storage conditions: room temperature

Test animals

Species:other: human tissue

Sex:male

Administration / exposure

Details on study design:Preparation of seminiferous tubule segments and culturing:

Culture of seminiferous tubules was performed using the pooled testes from a transgender male who underwent castration. The testes from a young patient who was no longer been treated by antiandrogenic compound were chosen for confident results. Androcur (cyproterone acetate) treatment was stopped and replaced by Finasteride (inhibitor of 5-alpha-reductase) + Provames (oestradiol) since several months.

The tunica albuginea of testes was removed, and seminiferous tubules were carefully dissociated by collagenase enzymatic digestions at 33°C and mechanical dissociation. Between each step of this process, seminiferous tubule fragments were washed with F12/DMEM supplemented by 10 µg/ml gentamycin and 10µU/ml nystatin

Cell culture from rat seminiferous tubules was performed according to the Bio-AlteR® technology.

The cells from pooled seminiferous tubules were seeded (Day 0 of the experiment) in bicameral chambers in the culture medium. The culture medium consists of 15mM Hepes-buffered F12/DMEM supplemented with 10µg/ml gentamicin, 10µU/ml nystatin, 1.2g/L sodium bicarbonate, 10µg/ml insulin, 10µg/ml human transferrin, 10^-4M vitamin C, 10µg/ml vitamin E, 10^-7M testosterone, 3.3 10^-7M retinoic acid, 3.3 10^-7M retinol, 10^-3M pyruvate (all from Sigma), 1ng/ml of FSH (NIH, Bethesda, MD) and FCS 5%. Incubation was carried out at 33°C in a water-saturated atmosphere of 95% air and 5% CO2. The compounds or vehicle were added in the basal compartment of the bicameral chambers of the culture from day 2 onward. Only basal media (+/- compounds) were renewed every 2±1 days. On day 14 and 21, cells were detached from the permeable membrane of the bicameral chamber by trypsinization and the cells from 5-6 wells were pooled (5 wells at D14 and 6 wells at D21 per condition). An aliquot of the cell suspensions was used to determine the number of cells and to assess cell viability by trypan blue exclusion. Aliquots of cells were fixed with ice-cold ethanol for flow cytometric analysis.

For CoA-TBBA conjugate determination, cells were detached from the permeable membrane of the bicameral chamber and the cells from the 3 wells of the same replicate pooled (day 15 and day 22). Hence 3 replicates were recovered per condition and per day of study. Moreover, 9 extra wells of the high concentration of p-TBBA, of the high concentration of m-TBBA and of the control condition (vehicle) were added for both timepoints.

Total Cell number & viability (on total cells):

The total cell number and viability is assessed by microscopic counting and trypan blue exclusion on day 14 and day 21.

Immunolabeling of Cultured Cells for Flow Cytometric Analysis:

The number of each cell type (somatic cells, spermatogonia, young spermatocyte I, middle to late pachytene spermatocytes I, secondary spermatocytes and round spermatids) is measured by flow cytometric analysis and compared to control at days 14 and 21. To distinguish germ cells from somatic cells, fixed cultured cells are immunolabeled with a monoclonal antibody against vimentin. Hoechst is added to the labelled cell suspension to assess their DNA content. After immunolabeling, cells were analysed using a Attune Acoustic Focusing cytometer (Life Technologies). The vimentin-positive somatic cells and the vimentin-negative 4C, 2C and 1C germ cells are separated with the bivariate analysis of DNA content/vimentin. Then the bivariate liner forward light scatter and liner side angle light scatter analyses allow the identification of each germ cell population.

Quantification of TBBA-CoA conjugates:

On day 15 and 22, cells were detached from the permeable membrane of the bicameral chamber by trypsinisation (100 µL of trypsin solution per well of culture) and cell metabolism was stopped by addition of citric acid (100 µL, 0.5 M) and cold acetonitrile (200 µL) containing 2 µM decanoyl CoA as internal standard. Cells from three wells were pooled and transferred to 2 ml tubes (final volume 1.2 ml) and frozen at -80°C. Samples were thawed, and centrifuged (21,000 x g, 5 min, RT). 500 µL of the supernatant (from total~1.2 mL) was diluted with 6.8 ml Tris-buffer (4 mM) containing KCl (6 mM), MgCl2 (0.3 mM) and n-heptadecanoyl CoA as internal standard (0.2 µM). Samples were neutralized by addition of NaOH (150 µL of 1M NaOH) and loaded onto solid phase extraction cartridges (OASIS HLB µElution plate). Columns were washed with 200 µL of ammonium solution (1 M), and samples eluted sequentially with 50 µL acetonitrile and 50 µL water. Samples were then further concentrated to 50 µL under a N2 stream.

CoA conjugates were analysed with high resolution LC-MS (LC-HRMS) on a Dionex UltiMate 3000 RS HPLC system coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, Reinach, Switzerland) with electrospray ionization (ESI) in positive ionization mode. For liquid chromatography separation an Agilent Zorbax 300Extend-C18 column with pore size of 300 Å,

dimensions 2.1 mm x 150 mm and particle size of 3.5 µm with a 2.1 mm x 12.5 mm pre-column with particle size of 5 µm of the same material was used. The flow rate was 0.4 mL/min. Eluent A consists of water containing 0.0025 % ammonia (pH around 9.8) and eluent B consists of acetonitrile containing 0.0025 % ammonia. A linear gradient was run from 95 % eluent A (hold for 1 min) to 100 % eluent B within 6 min (hold for 1 min), back to 95 % eluent A within 0.5 min followed by 1.5 min equilibration time. The injection volume of the sample was 10 µl. The mass resolution of the HR-MS spectra was set to 70,000. The mass accuracy was <5 ppm. Data-dependent high resolution product ion spectra (HR-MS/MS) were recorded at a resolution of 17,500. Ion source parameters adjusted were as follows: sheath gas flow (30 arbitrary unit), auxiliary gas flow (10 arbitrary unit), sweep gas flow (5 arbitrary unit), capillary temperature (270° C), and source voltage (4 kV in positive mode). Fragmentation was obtained from dissociation in an octopole collision cell using higher energy collision dissociation settings at NCE = 35 (arbitrary unit). The mass scan range was set from 120 to 1800 m/z.

Calibration standards were prepared with tBBA-CoA. Decanoyl-CoA and heptadecanoyl-CoA were used as internal standards. CoA conjugates are identified by comparison to the synthetic reference and by their exact mass. The analytical method does not discriminate between position isomers, with p-tBBA-CoA and m-tBBA-CoA having the same exact mass of 927.2040. The limit of detection with this method is around 0.0025 µM for CoA conjugates. Spiking experiments were performed to prove analytical proficiency in this experimental system and to estimate recovery.

Results and discussion

Observed effects

MAA

The positive control MAA at 2500µM had an effect on spermatogenesis. MAA at 2.5 mM decreased the number of somatic cells after 14 culture days (D14) but not after 21 culture days (D21). MAA also decreased the number of germ cells at D21 including spermatogonia and pachytene spermatocytes, which was not observed at D14. Compared to the respective control, MAA had no impact on cell viability at the tested concentration. MAA increased the number of secondary spermatocytes (D14) and of round spermatids (D14 and D21). It is hypothesized, that the presence of cellular debris after incubation with MAA could have distorted the flow cytometry analysis, which might have increased artificially the number of 1C cells (round spermatids).

The results with the positive control MAA indicate, that MAA affects the spermatogenesis process in the human Bio-AlteR® setup, when based on the suppression of early stage spermatogonia and pachytene spermatocyte numbers after 3 weeks of incubation. However, such a decrease could not be established for later stage populations such as secondary spermatocytes and round spermatids.

p-TBBA

- p-TBBA had no effect on cell viability.

- p-TBBA at 10µM and 50µM resulted in decreased numbers of somatic cells at D14, however, the numbers of cells were not changed in a dose related fashion. This effect was also not observed at a later time point, since no evident and dose-related impact on the cell number was observed at culture day 21. Thus, the impact of p-TBBA incubation on the number of somatic cells is questionable.

- The presence of p-TBBA increased the total number of germ cells and the number of spermatogonia on D14 and D21 at the highest tested concentration (50µM).

- Concerning pachytene spermatocytes, secondary spermatocytes and round spermatids, no clear dose-related effect of p-TBBA was observed at both time points investigated when compared to the respective controls. Accordingly, no dose related decrease in the numbers of pachytene spermatocytes, secondary spermatocytes and round spermatids was indicated for both time points investigated.

As for MAA, it could not be excluded that p-TBBA induced the presence of cellular debris, distorting the flow cytometry analysis. Furthermore, the proportion of germ cells was low and a small variation of the proportion of cells in the germ cell populations would result in a high variability.

m-TBBA

- The test substance m-TBBA had no effect on cell viability.

- Incubation with m-TBBA at 10µM and 50µM decreased slightly the number of somatic cells at D14. Only a slight decrease was also observed at D21 (50µM). Overall, no clear dose-related effect of m-TBBA on the number of somatic cells was observed on culture day 21 when compared to the respective controls. Due to the lack of a clear dose dependency and consistency between the two time points examined, the impact of m-TBBA incubation on the number of somatic cells is questionable.

- No dose-related impact on germ cell numbers and spermatogonia were observed for both time points investigated. Thus, the impact of m-TBBA incubation on the number of germ cells and spermatogonia is questionable.

- Concerning pachytene spermatocytes, no clear dose-related effect of m-TBBA was observed on culture day 14 when compared to the respective controls. The decrease in pachytene spermatocytes on culture day 21 was only slight and not confirmed at the earlier time point investigated. Thus, the impact of m-TBBA incubation on the numbers of pachytene spermatocytes is questionable.

- Concerning secondary spermatocytes, no clear dose-related effect was determined after day 14 when compared to the respective controls. After culture day 21, these numbers increased in a dose related fashion when compared to controls.

- Concerning round spermatids, no clear dose-related effects were determined at both time points investigated when compared to the respective controls. Overall, a dose related decreases in the numbers of secondary spermatocytes and round spermatids is not indicated.

As for MAA, it could not be excluded that m-TBBA induced the presence of cellular debris, distorting the flow cytometry analysis. Furthermore, the proportion of germ cell populations were low and a small variation of the proportion of cells in the germ cell populations would result in a high variability.

Analysis of tBBA-CoA using LC- HRMS

In the culture samples dosed with 2 and 10 µM of p-tBBA, the corresponding CoA-conjugate (ptBBA-CoA) was below the limit of quantification in all samples at both time points. Trace amounts around the detection limit were detected in two out of six samples dosed with 50 µM p-tBBA after 22 days of treatment, while p-tBBA-CoA was not detectable in the remaining 4 samples at this time point and not in any sample of the tissues dosed for 15 days. No m-tBBA-CoA was detectable at any concentrations in any sample dosed with either 2, 10 or 50 µM of m-tBBA and collected after 15 or 22 days of exposure. Furthermore, no tBBA-CoA was detectable in the vehicle controls exposed to 0.1% DMSO without test compounds or in the positive control samples exposed to MAA.

3.10.2.4 Study 4: 2-(4-*tert*-butylbenzyl)propionaldehyde

Type of information:experimental study

Title:Oestrogenic activity of benzyl salicylate, benzyl benzoate and butylphenylmethylpropional (Lilial) in MCF7 human breast cancer cells in vitro

Author:A. K. Charles and P. D. Darbre

Year:2009

Bibliographic source:J. Appl. Toxicol. 2009; 29: 422–434

Materials and methods

Test guideliner:no guideline followed

In vitro test using estrogen responsive MCF7 human breast cancer cell line and human recombinant ER alpha and ER beta.

GLP compliance:no

Type of method:in vitro

Test material

Reference substance name:2-(4-tert-butylbenzyl)propionaldehyde

EC Number:201-289-8

EC Name:2-(4-tert-butylbenzyl)propionaldehyde

Cas Number:80-54-6

Molecular formula:C14H20O

IUPAC Name:3-(4-tert-butylphenyl)-2-methylpropanal

Details on test material:Lilial; CAS no. 80-54-6; (≥95% purity), purchased from Sigma (Poole, UK).

Results and discussion

Observed effects

- Ligand Binding to Human ER

At 3 000 000- fold molar excess, lysmeral gave 27% inhibition of [3H]estradiol binding to ERα, and approx 15% inhibition (estimated from graph) of [3H]estradiol binding to ERβ (mean value of triplicate assays

performed in five independent experiments for ERα (n = 15) and three independent experiments for ERβ (n = 9)).

- Competitive Binding Assay to ER of MCF7 Cytosol

The maximal inhibition of [3H]estradiol binding at 3 000 000-fold molar excess of lysmeral was 47% (mean values of triplicate assays each carried out in triplicate and performed on three independent cytosolic preparations (n = 27)).

- Assay of stably transfected ERE-CAT reporter gene in MCF7 cells

Lysmeral induced CAT gene expression, with maximal effects by 5 × 10^-4M lysmeral (1.8-fold), although in no case was the CAT gene expression of the same magnitude as with 10^-8M 17β-oestradiol (2.0-fold in each case).

Cell Proliferation Experiments

Lysmeral increased the growth of the MCF7 cells after 7 days in a dose-dependent manner: MCF7 cells gave an average number of doublings in 7 days of 1.84 ± 0.08 without any addition and 5.23 ± 0.05 with 10^-8M 17β-estradiol. Over the same period, MCF7 cells went through 2.44 ± 0.06 doublings with 10^-4M lysmeral.

Cell density reached near confluence after 14 days with 10^-8M 17β-estradiol and after 35 days with 10^-4M lysmeral.

Stimulatory action of 10^-10M 17β-oestradiol on MCF7 cell growth was slightly inhibited by 10^-4M but not by 10^-5M lysmeral.

- RT-PCR analysis

Following 7 days of estrogen deprivation, a 24 h exposure to lysmeral could increase the expression of the estrogen-regulated gene pS2 mRNA, although in no case to the same extent as with a 24 h exposure to 17β-estradiol.

3.11 Specific target organ toxicity – single exposure

Not evaluated.

3.12 Specific target organ toxicity – repeated exposure

Not evaluated.

3.13 Aspiration hazard

Not evaluated.

4 ENVIRONMENTAL HAZARDS

Not evaluated in this dossier.