Document Title

Summary of the toxicological and metabolism studies for Methiocarb

Data Requirements

EU Regulation 1107/2009 & EU Regulation 283/2013
Document MCA
Section 5: Toxicological and metabolism studies

According to the guidance document, SANCO/10181/2013, for preparing dossiers for the approval of a chemical active substance

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Author(s)

[redacted]

[redacted]

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Version history

Date	Data points containing amendments or additions[1] and brief description	Document identifier and version number
2015-12-09	Orginal Tier II summary	M-540717-01-1
2017-07-18	Update of document considering - CA 5.1: Study summary copied from dRAR when additional tables that were provided to CRD on 2017-01-13 were included. - CA 5.5: Study summary copied from dRAR when additional tables that were provided to CRD on 2017-01-23 were included.	M-540717-02-1

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

INTRODUCTION

Methiocarb is an insecticide and repellent active substance and was included into Annex I of Directive 91/414 on 1st October 2007 (Directive 2007/5/EC).

This Supplementary Dossier contains only data which were not submitted at the time of the Annex I inclusion of methiocarb under Directive 91/414/EEC and which were therefore not evaluated during the first EU review. All data which were already submitted by Bayer for the Annex I inclusion under Directive 91/414/EEC are contained in the DAR, its Addenda and are included in the Baseline Dossier provided by Bayer. These data are only mentioned in the Supplementary Dossier for the sake of completeness and only general information (e.g. author, reference etc.) is available for these data. In order to facilitate discrimination between new data and data submitted during the Annex I inclusion process under Directive 91/414/EEC, the old data are written in grey typeface. For all new studies, detailed summaries are provided within this Supplementary Dossier.

The presented and submitted studies used different synonyms and codes for the active substance Methiocarb.

CA 5.1 Studies on absorption, distribution, metabolism and excretion in mammals

Studies on absorption, distribution, metabolism and excretion in rats were evaluated and summarized by the former RMS United Kingdom in Volume 3, Annex B, B.6 of the DAR, July 2005 (public version). A copy of this summary is given below.

"**Summary of mammalian metabolism**

The toxicokinetics and metabolism in rats was investigated after oral administration of methiocarb using dose levels of 1 and 10 mg [phenyl-1-^{14}C]methiocarb/kg bw followed by a quantitative autoradiography study using radioluminography technology ([redacted], 2001a & b) [Ed: M-032676-01-1 or M-032508-01-1] . In addition, the metabolism of methiocarb and methiocarb phenol (M02) in the rat was investigated at dose levels of 0.25 and 20 mg [phenyl-1-^{14}C]methiocarb/kg bw ([redacted], 1976) [Ed: M-013382-01-1] and 0.19 mg [phenyl-1-^{14}C]methiocarb phenol/kg bw. ([redacted], 1985) [Ed: M-013546-01-1].

Methiocarb was rapidly and extensively absorbed from the gastrointestinal tract into the plasma after an oral dose. Maximum concentration in plasma was achieved within 0.5 h. The extent of oral absorption was approximately 84 – 90% of the administered dose. Absorption commenced immediately after oral administration and was marked by short lag times of absorption (0.03 h to 0.10 h) and short half times of absorption (0.12 h to 0.21 h).

Methiocarb was rapidly and well distributed into organs and tissues. At termination, radiolabel in organs and tissues ranged from 0.243 - 0.310 % of the administered dose, and was rapidly distributed into the body tissues as shown by qualitative and quantitative whole body autoradiography and pharmacokinetics of methiocarb in plasma.

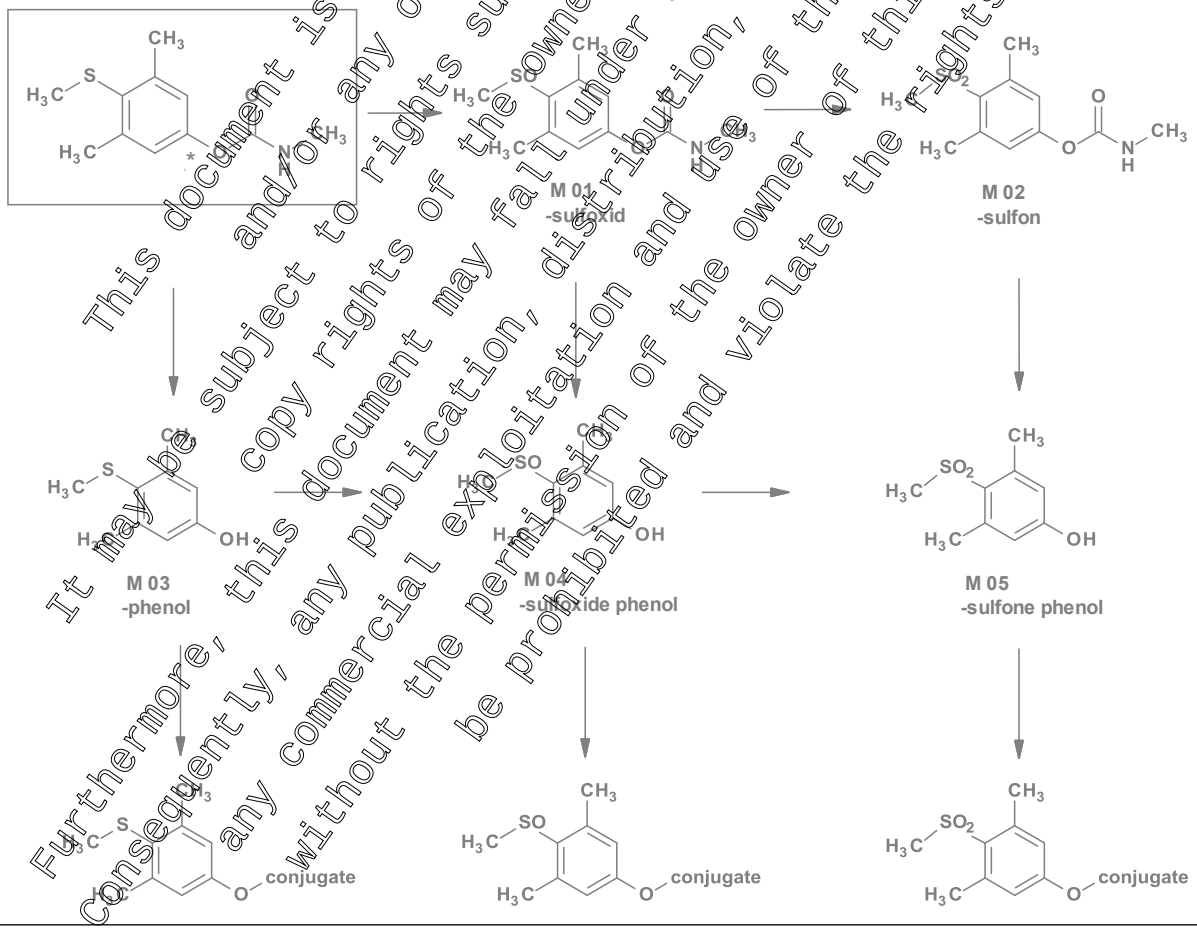
The excretion of radioactivity was nearly complete 48 hours after dosing. Renal excretion was the major route of elimination (84 – 90% of the dose) and only 5 - 10 % was excreted via faeces. No more than 0.243 – 0.301 % of the dose remained in the whole body after 48 hours.

Metabolism studies showed that in rat urine, the major metabolites were conjugated phenol metabolites (conjugates of M03, M04 and M05). Most abundant were conjugates of methiocarb sulfoxide phenol (M04), followed by methiocarb phenol (M03) conjugates and methiocarb sulfone phenol conjugates. Methiocarb sulfoxide phenol (M04) and methiocarb sulfone phenol (M05) were conjugated with sulfate or glucuronic acid. The only conjugate of methiocarb phenol (M03) was a glucuronic acid conjugate. Minor metabolites were the unconjugated methiocarb phenol and methiocarb sulfoxide phenol. Low amounts of the parent compound were detected in the urine from rats dosed with 0.25, 1, and 10 mg methiocarb/kg b.w., while no methiocarb, but low amounts of methiocarb sulfoxide (M01) were found in urine from rats dosed with 20 mg/kg b.w. In faeces from rats, traces of methiocarb were detected. In addition, methiocarb phenol (M03) and methiocarb sulfoxide phenol (M04) were present in the range of 1- 6 % of the administered dose.

From the above mentioned metabolite pattern the following metabolic reactions were proposed for methiocarb in animals: the oxidation of the sulfide group, the removal of the carbamate group by hydrolysis with formation of phenol metabolites and the conjugation of the phenolic hydroxy group with sulfate or glucuronic acid. Methiocarb was almost completely metabolised to conjugates of the phenol moiety. The sulfoxide was the preferred oxidation state of the metabolic products. Conjugation with sulfate rather than with glucuronic acid was the preferred metabolic reaction.

A proposed metabolic pathway in the rat is shown in Figure 5.1- 1.

Figure 5.1- 1: Proposed metabolic pathway of methiocarb in the rat



M 03 - conjugates [1] M 04 - conjugates [2] M 05 - conjugates [2]

* position of ^{14}C label

1 = glucuronic acid conjugate identified in the rat
2 = glucuronic acid and sulfate conjugates identified in the rat

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

The following study on toxicokinetics and metabolism of methiocarb in the blood of rats was conducted in addition to the prior, already evaluated ADME studies to aid the risk assessment of occupational, non-dietary exposure (e.g. during spray application). This study complements a similar study with dermal application.

Report:	KCA 5.1.1/05 [redacted].; 2013; M-348264-02-1
Title:	Amendment No. 1 to [phenyl-1-14C]methiocarb: Mechanistic study in male rats after oral administration
Report No.:	MEF-09/114
Document No.:	M-348264-02-1
Guideline(s):	US EPA OPPTS 870.7485; EU 91/414/EC amended by 94/79/EC; OECD 417
Guideline deviation(s):	not specified in the report; short-term study (8 hours) at one dose level using only male rats to analyse for metabolism in blood
GLP/GEP:	**yes**

Executive Summary

Toxicokinetics and the metabolism of the insecticide and molluscide methiocarb were investigated in blood and urine of male rats. [Phenyl-1-^{14}C]methiocarb was orally administered to seven groups of each four male rats per gavage at a dose level of approx. 1 mg/kg bw (actually 0.91 mg kg/bw). The dose groups were sacrificed 0.25, 0.5, 1, 2, 3, 4 and 8 hours after administration. Blood, urine, skin, carcass (plus blood debris) and GIT (plus faeces) were collected and pooled within a group. The samples were radioassayed by LSC. Blood (at sacrifice) was immediately mixed with acetonitrile to stop potential enzymatic reactions, to haemolyse the blood cells and to precipitate protein and cell debris. The GIT, skin and residual carcass were minced in half-frozen state, freeze dried, homogenized and radioassayed. The urine and the supernatant of processed blood were analysed for the metabolic profile by radio-HPLC with authentic reference standards, by LC-MS and, following LC-MS isolation of certain metabolites, by ^{1}H-NMR.

The radioactivity balance of all tests was excellent (recovery 96.6 - 102.2% of administered dose).
The total radioactive residues (TRR) in pooled blood samples showed a very quick peak level (t_{max} = 15 min after oral dose, C_{max} = 0.816 mg eq/kg) followed by a rapid decline to nearly a tenth of the peak level already 3 hours after administration, indicating a quick absorption and very rapid elimination.

TRR in skin and carcass reached also a very early maximum at the first time point (15 min post dose) following by a continuous decrease until study end. Excretion of radioactive residues happened predominantly with the urine. The renal excretion was very quick and already complete. Within the experimental period of 8 hours post dose 82% of the dose was already excreted with the urine.

In blood, the parent substance methiocarb and its toxicologically relevant metabolite methiocarb-sulfoxide (M01) reached a maximum 30 min post dose, with a dose-normalized concentration (CN) of 0.012 and 0.017. [Ed.: A CN value can be roughly estimated by division of the actual concentration by the dose level and is equivalent to the concentration in the respective sample at a dose of 1 mg/kg bw.] The concentrations steadily decreased until study end (8 hours post dose) to CN levels of 0.001.

The predominant metabolites in blood were methiocarb-sulfoxide-phenol (M04) and its sulfate and glucuronic acid conjugates. They peaked together at 15 min after administration (CN = 0.667) followed by a steady decrease until the study end (CN = 0.006). The cleavage product methiocarb-

phenol (M03) and its conjugates also peaked at 15 min (CN = 0.111) indicating a high enzymatic activity for esterase, glucuronosyltransferase, and sulfotransferase. The lowest level of these metabolites was also measured at test end (CN = 0.020). Methiocarb-sulfone-phenol (M05) and its conjugates peaked at 30 min (CN = 0.023) and declined to CN ≤ 0.001 at test end. Methiocarb-sulfone (M02) was not detected in blood.

In urine (0 – 8 hours) the sulfate and glucuronide conjugates of methiocarb sulfoxide phenol (M04) represented also the predominant metabolites amounting to approx. 66% of the dose. Methiocarb-phenol and its glucuronate accounted for approx. 10% of the dose. Free methiocarb-sulfoxide and Methiocarb-sulfone were not found. The parent substance methiocarb was only detected in the 0 – 15 min urine at a very low level (0.02% of the dose).

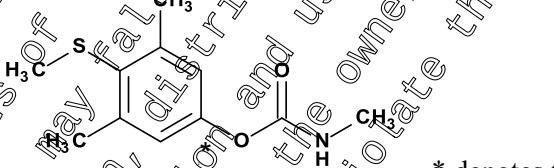
The metabolic profiles in blood and urine were similar. The metabolites were completely identified. From these investigations the following four metabolic reactions were derived:

- Ester hydrolysis of the carbamate group (major metabolic step)
- Hydroxylation of the methyl group of carbamate group (minor reaction)
- Oxidation of the thioether group to form the sulfoxide and the sulfone
- Conjugation of the phenolic hydroxy group with sulfate or glucuronic acid

The proposed metabolic pathway of methiocarb in the rat is shown in Figure 5.1.-2. The radioactive residues could be identified completely suggesting that the metabolism of methiocarb in the rat is well understood after oral administration

Material and Methods

Test Material

Structural formula	 * denotes the ^{14}C label
Chemical name	4-methylthio-3,5-xylyl methylcarbamate (ISO) Phenol, 3,5-dimethyl-4-(methylthio)-, methylcarbamate (CAS, 9CI)
Common name	Methiocarb, Mercaptodimethur (ISO)
CAS RN	2032-65-7
Empirical formula	C_{11} H_{15} N O_2 S
Company code	BAY 37344
Molar mass (non-labelled)	225.3 g/mol
Label	Phenyl-1-^{14}C (see structural formula)
Specific radioactivity	9.43 MBq/mg = 5.658 x 10^5 dpm/µg = 254.86 µCi/mg = 57.42 Ci/mol
Radiochemical purity	> 99% by HPLC and TLC
Chemical purity	> 99% by HPLC
Water solubility	0.027 g/L (20°C)
Log P o/w	3.18 (20°C, pH 7)
Use	Non-systemic insecticide and molluscide with contact and stomach action to control slugs, snails and pest insects (including soil insects)

Test Animals

Test animals	Rat (*Rattus norvegicus domesticus*)
Breed	Wistar, Hsd/Cpd: WU
Number, sex	28 male animals (4 per test)
Age	Approx. 7 weeks at delivery
Body weight	200 – 213 g
Identification	Cage cards, additionally by water-insoluble spots at tail
Accommodation period	Approx. 7 – 9 days prior to administration
Housing during test	Individually in Makrolon® metabolism cages allowing separate collection of urine and faeces
Feed and water	Rat/mice maintenance long life diet, *ad libitum*, tap water *ad libitum* Starvation: 16 hours prior to administration
Housing conditions	Temperature: 20 – 24°C Relative humidity: 45 – 63% Photoperiod: alternating 12- to 12-hours light / dark cycles Air change: 10 – 15 times per hour

Study regimen
The animals were separated in seven groups of each four individual rats. They were sacrificed at different intervals after administration as shown in the following table

Test No.	Time of sacrifice [hrs p. admin.]	Samples collected	Collection interval of urine and faeces [hrs p. admin.]
A	0.25	Blood, urine, skin, carcass plus blood debris, GIT plus faeces	0 – 0.25
B	0.5		0 – 0.5
C	1		0 – 1
D	2		0 – 2
E	3		0 – 3
F	4		0 – 4
I	8		0 – 8

Preparation of the application mixture and administration
The solid radiolabelled test substance was dissolved in 25 mL acetonitrile. Small aliquots were taken for an identity and purity check using spectroscopic methods and radio-chromatography. Seven aliquots containing 1.36 – 2.72 mg test substance were pipetted into individual glass flasks, concentrated to near dryness in a nitrogen stream at room temperature and reconstituted in 0.5% aqueous Tragacanth® (pH 6.0) using a ultrasonic bath for 10 – 15 min at concentration of 0.1 mg test substance/mL suspension.

The rats were orally dosed with a 2 mL of test substance suspension using a syringe attached to an animal-feeding knob cannula (gavage). The target dose was 1.0 mg/kg bw. The mean actual dose was 0.91 mg/kg bw.

Collection and processing of excreta
Urine and faeces were collected separately. The cage and urine collecting funnels were rinsed with water and the rinsing water (cage wash) added to the urine samples. The faeces samples were added to the respective GIT samples. Urine and GIT/faeces samples (following freeze-drying and homogenization) were radioassayed. The individual urine samples were pooled within one animal group.

Sacrifice and dissection

The animals were narcotized with Pentobarbital-Na at the end of the scheduled in-life period and sacrificed by transection of the cervical vessels and exsanguination.

The oozed out blood was collected in heparinized test tubes and immediately diluted with acetonitrile in a ratio of approx. 1/1 (v/v) in order to stop any possible enzyme activity and precipitate crude protein as well as haemolyse blood cells. Following centrifugation the supernatant and the precipitated protein and blood cells debris fraction were radioassayed. The individual supernatants of an animal group were pooled to provide one plasma sample per sacrifice time point.

The gastrointestinal tract (GIT) including contents was dissected from the body weighed, freeze dried, homogenized and radioassayed. The residual carcass (including precipitated protein and blood cells debris) was passed up to five times through a mincing machine in half-frozen state. From this tissue pulp, an aliquot was freeze-dried, homogenised and also radioassayed. The skin was dissected from the body and weighed. A small piece of the neck/dorsal skin was shaved, freeze-dried, and radioassayed.

Radioassaying and rendering of the results

Radioassaying (measurement of the radioactivity) of liquid samples was carried out by liquid scintillation counting with automatic quench correction (LSC). The counting time was stopped after reaching a 2σ error of 0.7% or after maximum 20 min. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC. All LSC measurements were conducted repeatedly (≥ 2 for liquid and 2-5 for solid samples) and averaged.

The results of radioassaying were given as percentage of radioactivity in the blood/urine/tissue sample per dose administered and/or in a dose-normalized concentration (CN, assuming this sample fills up the whole body.) A CN-value of 1 is equivalent to the so-called equidistribution concentration. Its calculation is based upon the assumption that the dose is evenly distributed within the body volume for organs and tissues. CN can be also roughly estimated by division of the actual concentration by the dose and is equivalent to the concentration in the respective sample at a dose of 1 mg/kg body weight. It easily allows comparison of organ concentrations obtained from experiments with different doses.

Radio-HPLC of blood (supernatant after precipitation) and urine

Radio-HPLC was conducted on a Phenomenex C18 column (250 x 2 mm, 5 µm particle size) operated with a gradient mixture of 1% formic acid in water and 1% formic acid in acetonitrile at 40°C. The system was equipped with an autosampler, precolumn (also C18), an UV detector (254 nm) and a radiomonitor with a glass scintillator. The flow was adjusted to 0.2 mL/min. The column recovery of approx. 100% was proven by comparison of the radioactivity of an injected blood sample and the eluted radioactivity. A quantifiable radioactive peak was regarded as relevant giving a signal approx. 2.5 times higher as the background noise.

For identification of peaks radiolabelled and non-labelled reference standards were co-injected.

LC-MS of blood (supernatant after precipitation) and urine

LC-MS was conducted by a combination of a LTQ Orbitrap mass spectrometer (FT-MS) and a HPLC system with a C18 or Phenyl-hexyl columns (250 or 150 x 2 mm, 5 µm particle size) that was operated with gradient mixtures of 0.1% formic acid in water and 0.1% formic acid in acetonitrile or aqueous 5 mM ammonium carbonate and methanol. The HPLC effluent was split into a line connected with the mass spectrometer and a line connected with a UV detector and a radiomonitor. The flow was adjusted of 0.2 mL/min. MS ionisation was performed by electro-spray ionisation (ESI).

LC-MS for isolation of peaks further analysed by NMR

LC-MS for isolation of an NMR sample was conducted by a combination of a Esquire HCT mass spectrometer and a HPLC system with a Nucleolus C18 column (250 x 2 mm, 5 µm particle size) that was operated with a gradient mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The effluent of the column was split into two lines, one entering the mass spectrometer, the other one was led to a SPE cartridge (solid phase extraction with a resin polymer as absorbing phase). The absorption in the SPE unit was stirred by its quasi molecular ion detected in the mass spectrometer. After trapping the HPLC peak the SPE cartridge was dried and transferred to an NMR probe head using deuterated methanol.

^{1}H-NMR spectroscopy was performed using a 600 MHz spectrometer. Deuterated methanol was used as solvent.

Identification of parent substance and metabolites in blood and urine

For identification of the residue components of methiocarb in blood and urine the following strategy was applied.

- Co-chromatography of composite blood samples with authentic reference standards of methiocarb-sulfoxide, methiocarb-sulfoxide-phenol, methiocarb-sulfone, methiocarb-sulfone-phenol, methiocarb-phenol and the parent substance methiocarb
- Comparison of the HPLC profiles of different samples (e.g. blood vs. urine)
- Comparison of the HPLC retention times of individual components in the samples with a characteristic urine profile of the former methiocarb rat ADME study (already evaluated, see above) and the methiocarb mechanistic study in male rats after dermal application (see below). In these studies, the metabolites were identified by chromatographic and spectroscopic methods.
- The metabolites methiocarb-hydroxy-methyl and Methiocarb-phenol-sulfate (conjugate) were isolated from the 0.25 h and 0.5 h composite blood samples, respectively, by semipreparative HPLC and identified by LC-MS/MS. The original 0.25 h blood sample was analysed afterwards by HPLC co-chromatography together with these isolated metabolites.
- The HPLC profile of urine samples of this study was compared with that taken from rats after dermal application of ^{14}C-methiocarb (see below).

Sample storage

Freeze dried samples were stored at room temperature or at approx. +4 °C in a refrigerator. All liquid samples were kept frozen at -18 °C at all times except during aliquotation for analysis.

Findings

Recovery of radioactivity

The radioactivity balances of all tests ranged between 96.57% and 102.20% of the administered dose as measured from radioassaying of urine, blood and organs and tissues at sacrifice.

Toxicokinetics of radioactivity in blood (Table 5.1.1- 1)

The total radioactive residues (TRR) in pooled blood samples showed a very quick peak level (t_{max} = 15 min after oral dose, C_{max} = 0.816 mg eq/kg) followed by a rapid decline to nearly a tenth of the peak level after only 3 hours after administration. This curve progression indicates a rapid elimination of methiocarb related radioactivity from the rat body

Toxicokinetics of radioactivity in the urine (Table 5.1.1- 1)

As known from the ADME rat study already evaluated by the former RMS the urinary excretion is the preferred path for the excretion of methiocarb and its possible metabolites (84 – 90% of the administered dose within 48 hrs). During the experimental period of eight hours a steady increase up to 82% of the administered dose was measured in this study which confirmed the results obtained in the former rat study.

TRR in carcass, GIT (including faeces) and skin (Table 5.1.1- 2)
The total radiolabelled residues in carcass and skin reached a maximum at the first time point at sacrifice (15 min) followed by steady decrease to less than a tenth after 8 hours. In the GIT (plus faeces) the maximum residue level was measured 0.5 hours after administration following by continuous decrease.

Parent substance and metabolites in blood (Table 5.1.1- 3)
For methiocarb and methiocarb-sulfoxide (M01), the highest dose normalised concentrations (CN) amounted to 0.012 and 0.017 in blood, respectively measured at 30 min after administration. The CN-values declined steadily to values lower or equal to CN ≤ 0.001 until the test end (8 hours post dose). Methiocarb-sulfone (M02) was not detected.

The cleavage product methiocarb-phenol (M03) and its conjugates also peaked at 15 min (CN = 0.111) indicating a high enzymatic activity for esterase, glucuronosyltransferase, and sulfotransferase. The lowest level of these metabolites was also measured at the test end (CN = 0.020).

The predominant metabolites were methiocarb-sulfoxide-phenol (M04) and its sulfate and glucuronic acid conjugates which peaked together at 15 min after administration (CN = 0.667). For these metabolites, the lowest value was reached again at the test end (CN = 0.006).

Methiocarb-sulfone-phenol (M05) and its conjugates peaked at 30 min (CN = 0.023) and declined to CN ≤ 0.001at the test end.

Renally excreted metabolites (Table 5.1.1- 4)
Methiocarb was only detected in the sample of test A (0 – 15 min), however on a rather low level (0.02% of the dose).

The most prominent residue components in the 0 – 8 hours urine were the sulfate and glucuronate conjugates of methiocarb-sulfoxide phenol amounting to approx. 66% of the dose. Methiocarb-phenol and its glucuronate accounted for approx. 10% of the dose. Free methiocarb-sulfoxide and Methiocarb-sulfone were not found.

Conclusion

In this mechanistic study toxicokinetics of TRR in blood and urine and metabolic conversions was investigated in male rats at different time-points of sacrifice 0 – 8 hours after oral administration of approx. 1 mg/kg bw. In blood, the residue maximum was already detected at the first sampling point, 15 min post dose. The blood levels rapidly declined.

Methiocarb was readily absorbed and metabolised extensively by the rat following oral administration. The radioactive residues in blood and urine were completely identified. The metabolic profiles were similar in blood and urine. Eleven metabolites and very low levels of the parent compound were identified in rat blood and urine. The following key points were concluded from this study:

- Four types of metabolic reactions were observed:
  - Ester hydrolysis of the carbamate group (major metabolic step)
  - Hydroxylation of the methyl group of carbamate group (minor reaction)
  - Oxidation of the thioether group to form the sulfoxide and the sulfone
  - Conjugation of the phenolic hydroxy group with sulfate or glucuronic acid

- The predominant metabolites in blood were the cleavage products methiocarb-sulfoxide-phenol (M04) and its sulfate and glucuronic acid conjugates which peaked together at 15 min after administration followed by a steady decrease until the study end. This early phenol peak indicated that the enzymatic degradation of methiocarb to non-toxic metabolites is a rapid process.

- The unchanged methiocarb was observed in the blood samples of all time points. However, the concentrations were low and reached a maximum level of 0.011 mg/kg 0.5 hours after oral administration.

- The toxic metabolite methiocarb-sulfoxide was primarily detected in the blood samples of the early time points until 30 minutes after dosing followed by an even quicker decrease to non-toxic metabolites than that of parent methiocarb.

- The free methiocarb-sulfone metabolite was not detected in blood or in urine.

- The administered radioactivity was excreted very rapidly and predominantly *via* the urine. The excretion was almost complete within eight hours' time period after oral administration. Consequently, a potential of bioaccumulation is excluded.

- In turn, very low residual radioactivity that was measured in the combined carcass (plus blood cells debris) and skin at sacrifice at the end of the study, 8 hours after oral administration.

The proposed metabolic pathway of methiocarb in the rat is shown in Figure 5.1- 2. The radioactive residues could be identified completely suggesting that the metabolism of methiocarb in the rat is well understood after oral administration.

Table 5.1.1- 1: Time course of radioactivity in blood and urine of rats following oral administration of ^{14}C-methiocarb at dose level of 0.91 mg/kg bw

Animal groups		TRR in Blood			Urine
Test no.	Time [h p. admin.]	% of dose administered	TRR [mg eq/kg]	CN*	% of dose administered (cumulative)
A	0.25	1.24	0.816	0.875	2.88
B	0.5	0.71	0.486	0.529	5.95
C	1	0.89	0.439	0.468	30.08
D	2	0.22	0.158	0.171	32.34
E	3	0.15	0.070	0.076	63.76
F	4	0.18	0.093	0.104	64.64
7	8	0.04	0.027	0.028	81.76

* CN: Dose-normalized concentration (approx. TRR / dose level)

Table 5.1.1- 2: Time course of radioactivity in carcass, GIT and skin of rats following oral administration of ^{14}C-methiocarb at dose level of 0.91 mg/kg bw

Animal groups		Carcass and blood debris	GIT and faeces	Skin
Test no.	Time [h p. admin.]	TRR [mg eq/kg]	TRR [mg eq/kg]	TRR [mg eq/kg]
A	0.25	0.716	3.544	0.507
B	0.5	0.423	4.487	0.303
C	1	0.436	3.250	0.300
D	2	0.270	3.801	0.183
E	3	0.146	2.752	0.045
F	4	0.118	2.219	0.068
7	8	0.032	1.676	0.024

Table 5.1.1- 3: Methiocarb and its metabolites in blood of rats orally administered with ^{14}C-methiocarb at dose level of 0.91 mg/kg bw

Time [h post admin.]	Methiocarb-sulfoxide-phenol (M04) + conjugates*	Methiocarb-sulfone-phenol (M05) + conjugates*	Methiocarb-phenol (M03) + conjugates*	Methiocarb-sulfoxide (M01)	Methiocarb-OH-methyl	Methiocarb (parent substance)
	[% of administered radioactive dose]					
0.25	0.943	0.031	0.157	0.021	0.069	0.014
0.5	0.474	0.037	0.103	0.023	0.057	0.017
1	0.702	0.035	0.106	n.d.	0.031	0.015
2	0.162	0.007	0.038	0.001	0.005	0.006
3	0.089	0.007	0.044	n.d.	0.003	0.003
4	0.120	0.007	0.040	0.002	0.006	0.006
8	0.009	0.001	0.032	n.d.	0.001	0.002
	[mg eq/kg blood]					
0.25	0.622	0.021	0.104	0.014	0.045	0.010
0.5	0.324	0.025	0.071	0.016	0.039	0.011
1	0.346	0.017	0.052	n.d.	0.015	0.008
2	0.117	0.005	0.027	0.001	0.004	0.004
3	0.043	0.003	0.021	n.d.	0.001	0.001
4	0.061	0.004	0.021	0.001	0.003	0.003
8	0.005	<0.001	0.019	n.d.	<0.001	0.001
	Dose normalised concentration CN					
0.25	0.667	0.022	0.111	0.015	0.049	0.010
0.5	0.353	0.028	0.077	0.017	0.042	0.012
1	0.370	0.018	0.056	n.d.	0.016	0.008
2	0.127	0.006	0.029	0.001	0.004	0.005
3	0.046	0.004	0.023	n.d.	0.002	0.002
4	0.069	0.004	0.023	0.001	0.003	0.003
8	0.006	<0.001	0.020	n.d.	<0.001	0.001

* glucuronide and sulfate conjugates

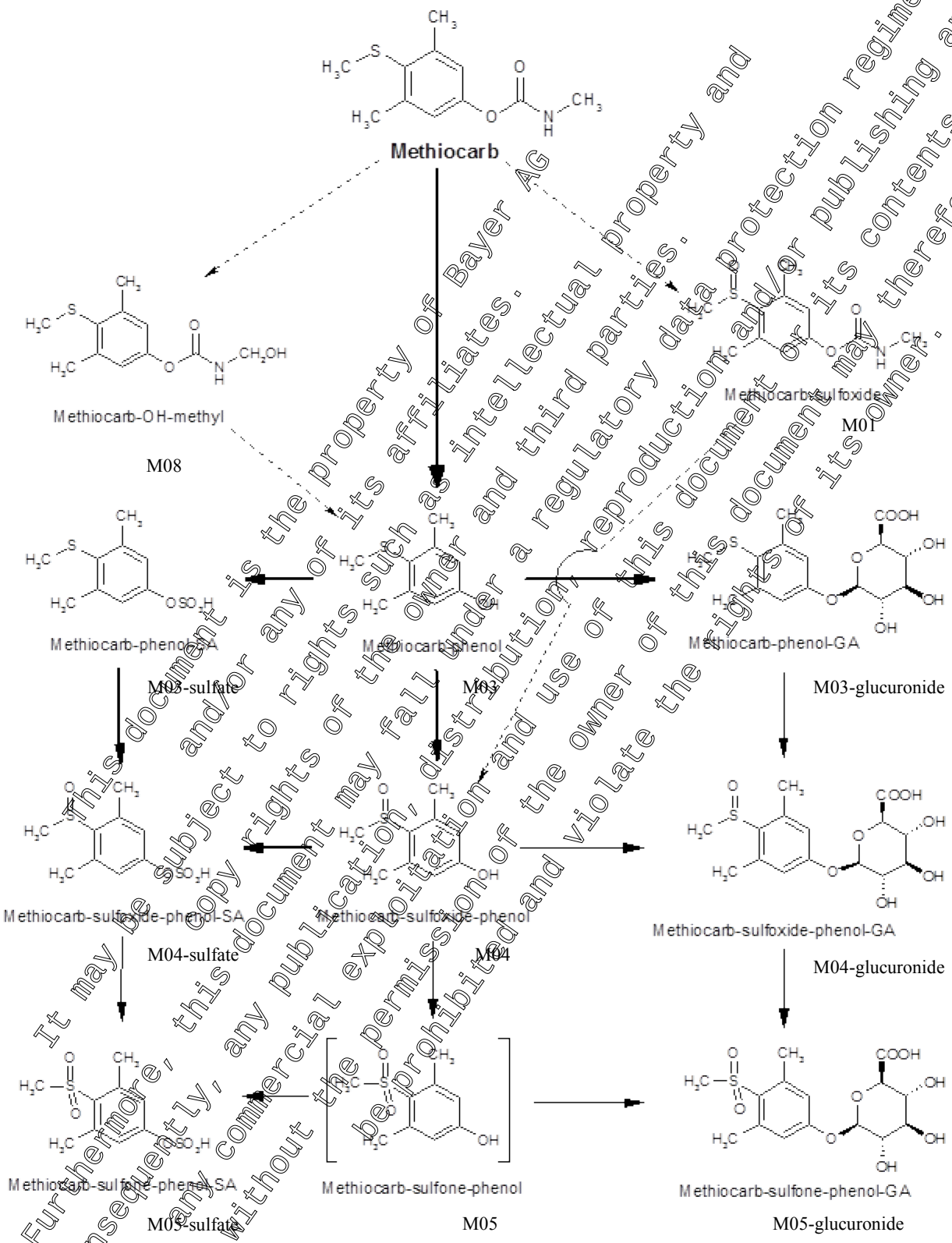
Table 5.1.1- 4: Renally excreted methiocarb and its metabolites following oral administration of ^{14}C-methiocarb at dose level of 0.91 mg/kg bw

Code	Report name (Methiocarb-)	Test No. A 0 - 0.25 h p. dose	Test No. 7 0 - 8 h p. dose
		[% of dose]	[% of dose]
M04-GA	sulfoxide-phenol-GA*	0.15	10.95
M05-GA	sulfone-phenol-GA	0.01	-
M04-SA	sulfoxide-phenol-SA	1.84	55.37
M04	sulfoxide-phenol	0.46	2.16
M05	sulfone-phenol	0.04	2.48
M05-SA	sulfone-phenol-SA	0.08	0.93
M03-GA	phenol-GA	0.25	8.94
M03	phenol	0.03	0.63
Parent subst.	methiocarb	0.02	-
Total		**2.88**	**81.76**

* two isomers
GA: glucuronic acid conjugate
SA: sulfonic acid conjugate

Figure 5.1- 2: Proposed metabolic pathway of methiocarb in the rat

(The metabolic pathway is updated, see Figure 5.1- 11)



Comparative *in vitro* metabolism using human and rat blood

The following *in vitro* study provides data on the degradation rate and metabolic route of methiocarb in fresh blood of human and rat. Due to the fast metabolic conversion of methiocarb in blood of both species this study can apply for the requirement "Comparative *in vitro* metabolism" mentioned as supporting study in Section 5.1.1 of Commission Regulation (EU) No 283/2013 of 1-March-2013 specifying the data requirements for active substances in accordance with Regulation (EC) No. 1107/2009. The proposed metabolic matrices "microsomes or intact cell systems" could well be replaced by fresh blood in this approach.

Report:	KCA 5.1.1/06 [redacted].; 2009; M-347682-01-1
Title:	In vitro: Blood stability tests with [phenyl-1-14C]methiocarb
Report No.:	MEF-09/287
Document No.:	M-347682-01-1
Guideline(s):	No guideline avialable
Guideline deviation(s):	not applicable
GLP/GEP:	**yes**

Executive Summary

In a comparative *in vitro* study [phenyl-1-^{14}C]methiocarb was incubated in blood samples of human and rat at 37°C for different time periods from 2 minutes to 2 hours.

^{14}C-Methiocarb was dissolved in water at pH 6 (approx. 0.020 mg/L) and added to 1 mL-samples of fresh blood from a male human donor and male rats. The concentration of the test substance in the blood was approx. 1 µg/mL. At the end of the incubation period the same volume of acetonitrile was added to stop the enzyme activity in blood, haemolyse the blood cells and precipitate enzymes and blood cell debris. The supernatant was radioassayed showing a complete recovery of the applied radioactivity and analysed by radio-HPLC for the radioactive components.

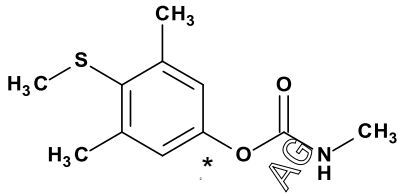
This study demonstrated that methiocarb is extremely instable in blood. It is hydrolysed very rapidly to the major metabolite methiocarb phenol (M03) indicating a high esterase activity of blood. (Hydrolysis in pure water is significantly slower.) The pattern of metabolites is similar in blood taken from both species with methiocarb phenol as major and methiocarb sulfoxide phenol (M04) as a minor metabolite. In human blood, the sulfate conjugate of methiocarb phenol was additionally observed. However, this conjugate was also be detected in blood and urine in of living rats (see ADME study above).

The rate of hydrolysis was significantly faster in human blood than in rat blood as demonstrated by degradation half-lives of methiocarb: 2.4 min in human blood and 11.4 min in rat blood. The parent substance could no longer be detected in human blood after a time period of 20 minutes, whereas it was observed in rat blood even after the longest incubation interval (2 hours).

Therefore, it can be concluded that toxic effects observed in the rat after exposure to methiocarb should be less pronounced in human.

Material and Methods

Test substance

Structural formula	 * denotes the ^{14}C label
Chemical name	4-methylthio-3,5-xylyl methylcarbamate (ISO) Phenol, 3,5-dimethyl-4-(methylthio)-, methylcarbamate (CAS, 9CI)
Common name	Methiocarb, Mercaptodimethur (ISO)
CAS RN	2032-65-7
Empirical formula	C_{11} H_{15} N O_2 S
Company code	BAY 37344
Molar mass (non-labelled)	225.3 g/mol
Label	Phenyl-1-^{14}C (see structural formula)
Specific radioactivity	9.43 MBq/mg = 5.658 x 10^5 dpm/µg = 254.86 µCi/mg = 57.42 Ci/mol
Radiochemical purity	> 99% by HPLC and TLC
Chemical purity	> 99% by HPLC
Water solubility	0.027 g/L (20°C)
Log Po/w	3.18 (20°C, pH 7)
Hydrolytic stability	>1 year (pH 4), 34.6 d (pH 7), 6 h (pH 9) at 22 °C
Use	Non-systemic insecticide and molluscide with contact and stomach action to control slugs, snails and pest insects (including soil insects)

Test material

Blood from a male human donor and from male "Wistar" rats were collected in heparinized test tubes at the respective application day.

Duplicate samples of human and rat blood with 1 mL each were prepared for the individual incubation periods: 2, 5, 10, 15, 20, 30, 60, and 120 min.

Test method

Application of the test substance and incubation in blood

The radiolabelled test substance was dissolved in acetonitrile (0.48 mg/mL) and analysed of the purity and identity using chromatographic and spectroscopic methods. Approx. 68 µL of this solution was diluted with 1.6 mL water resulting in a concentration of approx. 0.020 mg/L and adjusted to pH 6.0.

An aliquot of 50 µL (approx. 1 µg) of this aqueous solution was added to 1 mL of human or rat blood using a pipette. The final mixture was incubated in a water bath at 37°C.

After reaching the selected incubation periods the enzymatic activity of the blood was stopped by addition of acetonitrile at a ratio 1/1 (v/v). The acetonitrile also haemolysed the blood cells and

precipitated the enzymes. The precipitation was supported by centrifugation. The supernatant was radioassayed and analysed by radio-HPLC.

Radioassaying
Radioassaying (measurement of the radioactivity) of liquid samples was carried out by liquid scintillation counting with automatic quench correction (LSC). The counting time was stopped after reaching a 2σ error of 0.7% or after maximum 20 min. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC. All LSC measurements were conducted repeatedly (≤ 5) and averaged. Based on the instrument background, the specific radioactivity of the test substance and the aliquot taken for radioassaying the LOQ was set to 0.002 mg eq/kg.

Radio-HPLC
Radio-HPLC was conducted on a Phenomenex C18 column (250 x 2 mm, 5 µm particle size) operated with a gradient mixture of 1% formic acid in water and 1% formic acid in acetonitrile at 40°C. The system was equipped with an autosampler, precolumn (such C18), an UV detector (254 nm) and a radiomonitor with a glass scintillator. The flow was adjusted to 0.2 mL/min. The column recovery of approx. 100% was proven by comparison of the radioactivity of an injected blood sample and the eluted radioactivity. A quantifiable radioactive peak was regarded as relevant giving a signal approx. 2.5 times higher as the background noise.

For identification of peaks radiolabelled and non-labelled reference standards were co-injected. The HPLC profiles of this study were additionally compared with the blood profiles of the former metabolism study in rats after oral administration of ^{14}C-methiocarb (see above).

Sample storage
All individual samples were kept frozen at ≤ -18°C at all times except during aliquotation for analysis.

Findings

Recovery of radioactivity
The total radioactive residues (TRR) detected in the supernatants of the blood samples ranged from 0.528 to 0.601 mg eq/kg incubate with human and rat blood at termination of the incubation. Based on the applied amount of 1 µg ^{14}C-methiocarb applied to 1 mL (ca. 1 g) blood and on a one-to-one dilution with acetonitrile these TRR values imply a complete recovery of the applied radioactivity.

Incubation of ^{14}C-methiocarb in human blood (Table 5.1.1- 5)
Methiocarb is extremely instable in human blood. The ester hydrolysis of carbamate moiety of methiocarb started immediately after application of the test substance forming methiocarb-phenol (M03). Already after 20 to 30 minutes, the parent substance methiocarb was completely hydrolysed. This reaction rate should be caused a high esterase activity of blood, since pure hydrolysis of methiocarb in sterile water is significantly slower as indicated by a hydrolysis half-life of 34.6 days at pH 7.

Two minor metabolites were identified as methiocarb-sulfoxide-phenol (M04) and the sulfate conjugate of methiocarb-phenol. This is shown by a sequence of HPLC profiles performed after the different incubation periods, see Figure 5.1- 3.

To show the velocity of methiocarb hydrolysis a ratio of the formed metabolite methiocarb-phenol and the degraded parent methiocarb has been calculated and is presented in the last column of Table 5.1.1- 5. This ratio increased to 356 already after 20 min of incubation indicating the highly efficient esterase activity in human blood.

Incubation of ^{14}C-methiocarb in blood of rats (Table 5.1.1- 6)
As in human blood, methiocarb and methiocarb-phenol (M03) were also the major radioactive residue components. One minor metabolite was identified as Methiocarb-sulfoxide-phenol (M04). The corresponding sequence of HPLC profiles for different incubation periods is shown in Figure 5.1.1-4.

Although the ester hydrolysis of methiocarb also started immediately in blood of rats its extent is significantly lower than in human blood. Therefore, the parent substance methiocarb could still be observed after 120 min of incubation at a significant portion (11 – 17% of the radioactive components in incubate). In turn, the ratio between the hydrolysis product methiocarb phenol (M03) and the parent substance accounted only for 3.4 after 20 min of incubation and did not exceed a maximum of 7.8.

Degradation half-lives of methiocarb in blood of human and rat
The half-lives (DT50) of the equivalent concentrations of methiocarb in human and rat blood were calculated for the time period 2 – 20 minutes by non-compartmental evaluation single-first-order degradation kinetic using the TOPFIT software (version 2.0). They amounted to

DT50 in human blood = 2.4 min
DT50 in rat blood = 11.4 min

Conclusion

In this comparative *in vitro* study methiocarb was incubated in blood samples of human and rat for different time intervals up to two hours at 37°C. This test demonstrated that methiocarb is extremely instable in blood and is hydrolysed very rapidly the major metabolite methiocarb phenol (M03) indicating a high esterase activity of blood.

The pattern of metabolites is similar in blood taken from both species with methiocarb phenol (M03) as major and methiocarb sulfoxide phenol (M04) as minor metabolite in human blood only, the sulfate conjugate of methiocarb phenol was additionally observed. However, this conjugate was also be detected in blood and urine in of living rats (see ADME study above).

The rate of hydrolysis was significantly faster in human blood than in rat blood as demonstrated by degradation half-lives of methiocarb: 2.4 min in human blood and 11.4 min in rat blood. The parent substance could no longer be detected in human blood after a time period of 20 minutes, whereas it was observed in rat blood even after the longest incubation interval (2 hours). It can therefore be concluded that toxic effects observed in the rat after exposure to methiocarb should be less pronounced in human.

From the detected metabolites a metabolic pathway can be constructed (Table 5.1.1- 6) that is part of the pathway derived from metabolism studies with living rats.

Table 5.1.1- 5: Time course of the incubation pattern of [phenyl-1-^{14}C]methiocarb in human blood (mean of two replicates)

Incubation period [min]	Methiocarb-sulfoxide-phenol (M04)	unknown metabolite 2	Methiocarb-phenol (M03)	Methiocarb	Methiocarb-phenol-SA	Ratio: Methiocarb-phenol to Methiocarb
	Percent of TRR in the incubation sample					
2	0.74	0.15	58.20	40.91	n.d.	1.4
5	1.12	n.d.	87.50	11.05	0.32	7.9
10	1.22	n.d.	94.95	2.68	1.16	35.4
15	1.27	n.d.	96.71	0.70	1.32	137.9
20	1.54	n.d.	96.69	0.27	1.49	355.5
30	1.56	n.d.	96.06	n.d.	2.38	---
60	1.46	n.d.	94.23	n.d.	4.31	---
120	1.80	n.d.	89.21	n.d.	8.99	---
	Equivalent concentration [mg eq/kg]					
2	0.004	0.001	0.324	0.228	n.d.	
5	0.006	n.d.	0.479	0.061	0.002	
10	0.007	n.d.	0.528	0.015	0.006	
15	0.007	n.d.	0.543	0.004	0.007	
20	0.008	n.d.	0.526	0.001	0.008	
30	0.008	n.d.	0.507	n.d.	0.013	
60	0.008	n.d.	0.504	n.d.	0.023	
120	0.010	n.d.	0.506	n.d.	0.051	

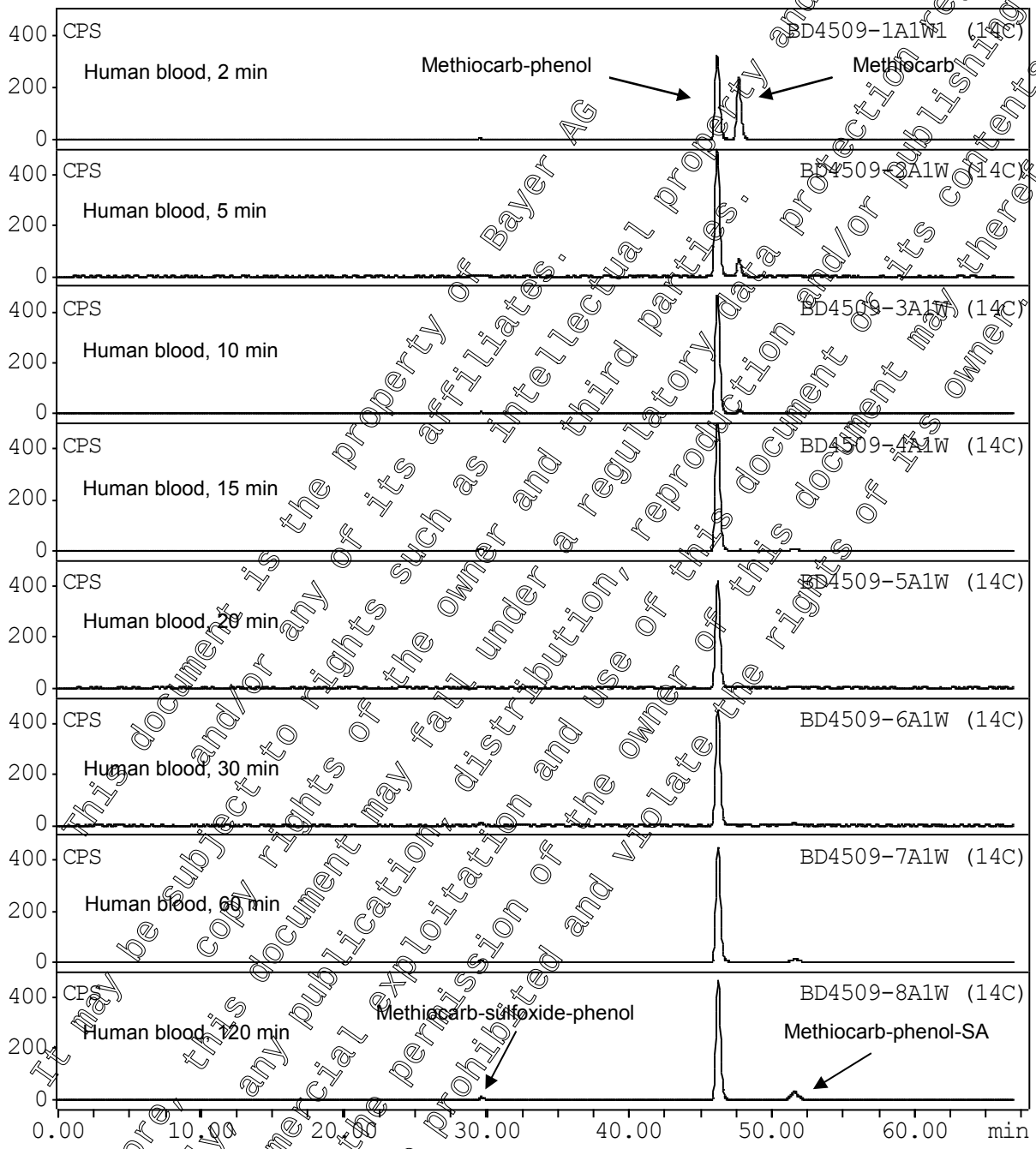
n. d.: not detectable

Table 5.1.1- 6: Time course of the incubation pattern of [phenyl-1-^{14}C]methiocarb in blood of rats (mean of two replicates)

Incubation period [min]	Methiocarb-sulfoxide-phenol (M04)	unknown metabolite 2	Methiocarb-phenol (M03)	Methiocarb	Ratio: Methiocarb-phenol to Methiocarb
	Percent of TRR in the incubation sample				
2	0.17	0.12	34.96	64.76	0.5
5	0.30	n.d.	45.70	54.00	0.8
10	0.44	n.d.	58.74	40.82	1.4
15	0.58	n.d.	69.35	30.07	2.3
20	0.39	n.d.	76.93	22.68	3.4
30	0.53	n.d.	80.45	19.09	4.2
60	0.80	n.d.	87.97	11.22	7.8
120	0.55	0.91	81.31	17.23	4.7
	Equivalent concentration [mg eq/kg]				
2	0.001	0.001	0.207	0.384	
5	0.002	n.d.	0.275	0.324	
10	0.003	n.d.	0.344	0.239	
15	0.003	n.d.	0.406	0.176	
20	0.002	n.d.	0.439	0.129	
30	0.003	n.d.	0.458	0.108	
60	0.005	n.d.	0.496	0.063	
120	0.003	0.005	0.452	0.096	

n. d.: not detectable

Figure 5.1- 3: Comparison of radio-HPLC profiles of [phenyl-1-^{14}C]methiocarb incubated in human blood samples for different incubation time periods (2 - 120 min)



SA: sulfate conjugate

Figure 5.1- 4: Comparison of radio-HPLC profiles of [phenyl-1-^{14}C]methiocarb incubated in rat blood samples for different incubation time periods (2 - 120 min)

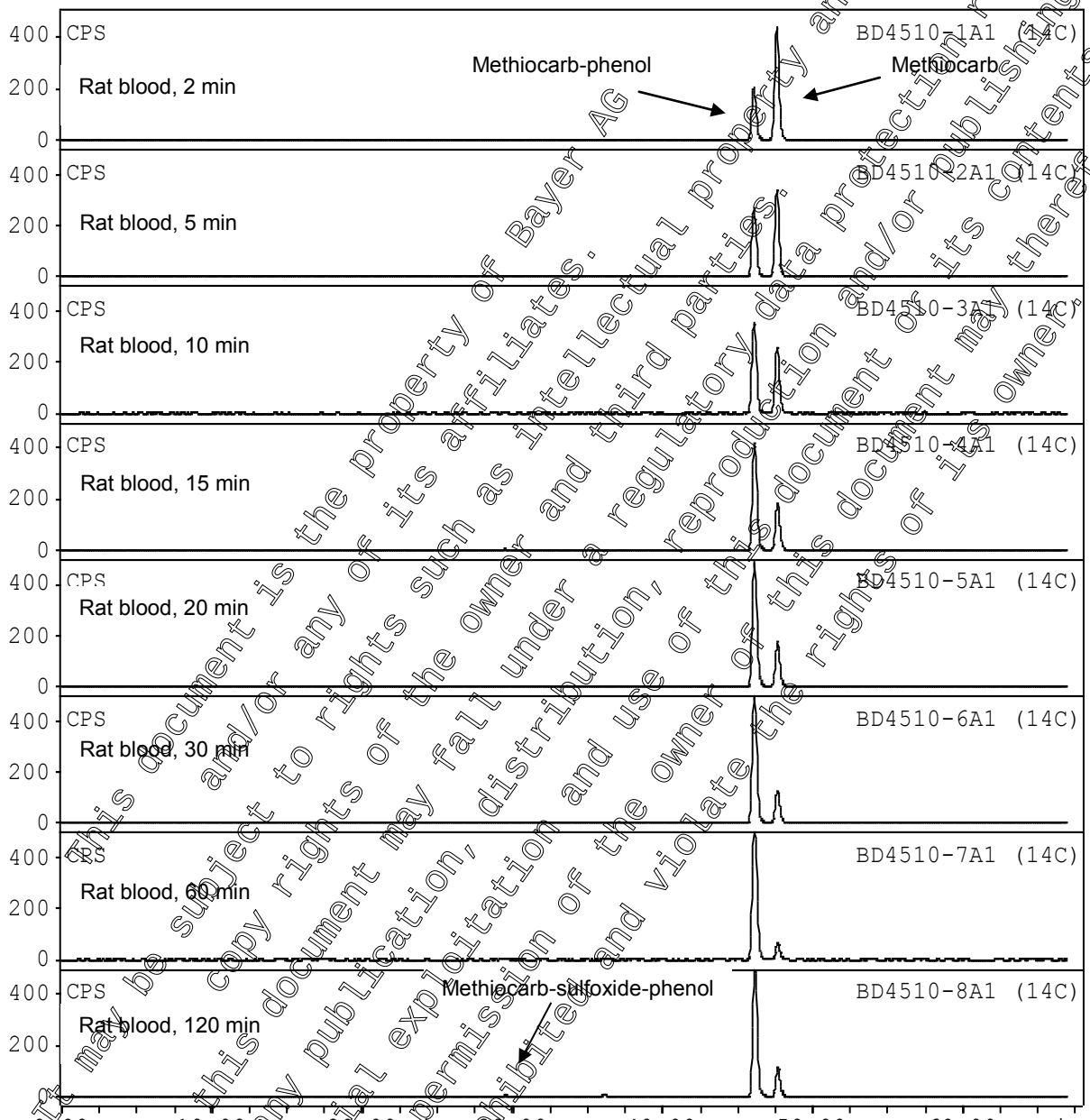
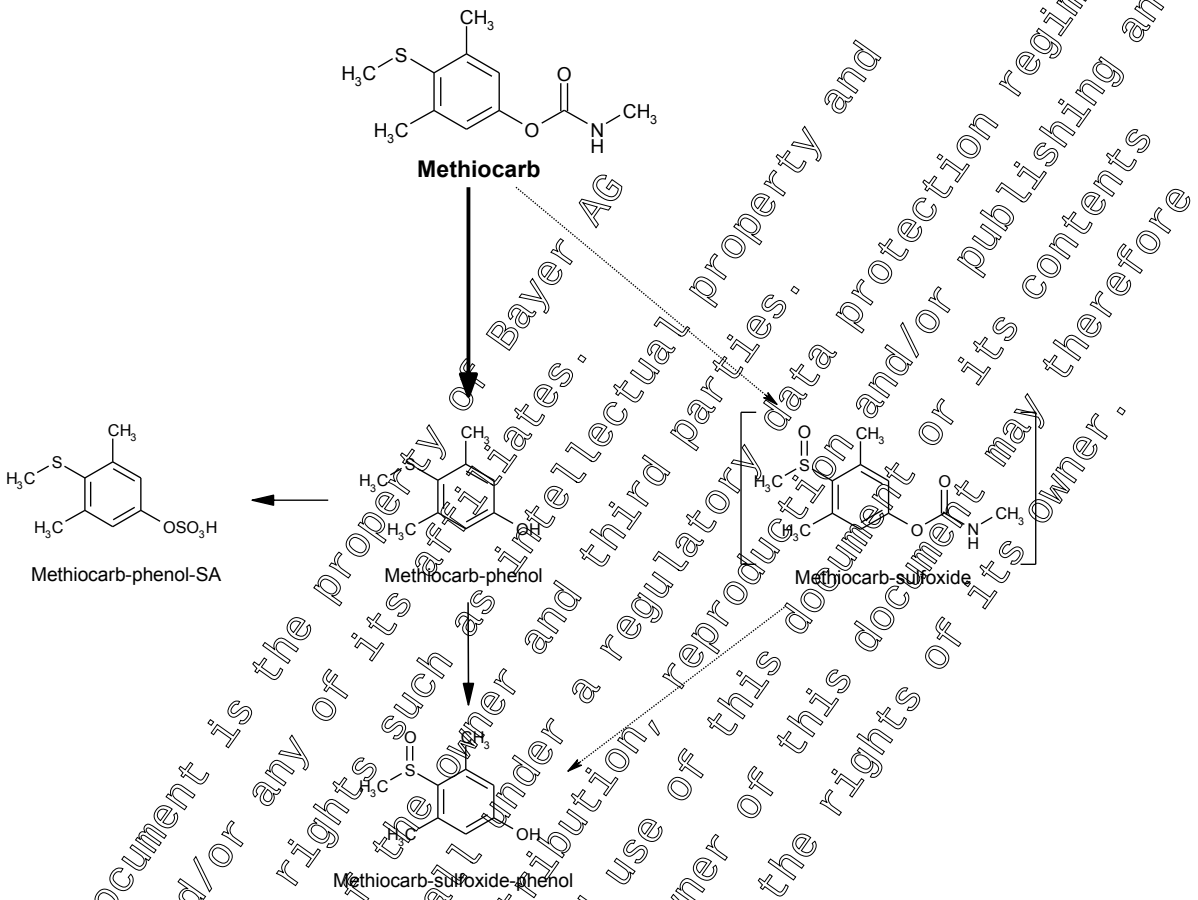


Figure 5.1- 5: Proposed biotransformation pathway of [phenyl-1-^{14}C]methiocarb incubated in human and rat blood (part of the metabolic pathway in the rat)



Determination of the HSA-Binding and Membran Affinity

Report: KCA 5.1.1/07 [redacted] 2009, M-347387-01-1
Title: Determination of the HSA-binding and membran affinity for 3 compounds
Report No.: M-347387-01-1
Document No.: M-347387-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: **no**

1. Materials and methods

A. Materials

1. Test material:

Test substance 1:	Methiocarb
Code:	AE F082618 00 1B99 0001
Test substance 2:	Methiocarb-sulfone
Code:	AE C417187-PU-1

Test substance 3:	Methiocarb-sulfoxide
Code:	AE F1371422 00 1B99 0001
Description:	solids
2. Solvent:	DMSO PBS
3. Test system:	
Species:	Human, rat
Strain:	Rat: Wistar
Age:	Not reported
Sex:	Male
Specimen:	Blood (collected in heparinized test tubes)

B. Study design and methods

1. Test substance preparation:

Stock solution: For all three test substances 10 mM stock solutions in DMSO were prepared.

Test solutions: The stock solutions were pre-diluted with PBS and DMSO to a 10-fold concentration compared to the final assay concentration. Final test concentrations were 2.5 µM in 1% DMSO.

2. Treatment:

Blood sample size:	1 mL
Test substance volume:	50 µL (approx. 1 µg of ^{14}C-methiocarb)
Incubation time:	2, 5, 10, 15, 20, 30, 60 and 120 min
Test conditions and blood sample preparation:	Tests were incubated in a water bath at about 37°C and terminated with acetonitrile at a ratio 1/1 (v/v) at the respective time points. Haemolysed blood cells (debris) and precipitated enzymes were removed by centrifugation. The weight and total radioactivity were determined in the blood/ACN-supernatants which were then analysed by chromatographic methods
Replicates:	2 per time point per species
TRANSIL®HSA assay:	TRANSIL® HSA consists of human serum albumin immobilized on inert and soft surfaces. These surfaces were designed to retain the protein properties and to have minimum interactions with drug molecules, thus preventing non-specific interactions between drug and immobilization layer. The affinity of drugs to HSA is determined by quantifying the free drug concentration in the buffer after binding to TRANSIL® and comparison with the start concentration (without TRANSIL®, i.e. reference) Dissociation constants (K_D-values) were obtained by titrating constant compound concentrations of 2.5 µM in phosphate buffered saline (pH 7.4) against increasing amounts of immobilized proteins. The suspension of TRANSIL® beads in buffer was mixed eight times to give a homogenous suspension and incubated for at least two minutes. Then the

beads were separated by low speed centrifugation (759 g, 10 minutes). Subsequently, the unbound fraction of the respective compound in the supernatant was determined by LC/MS.
Based on the results of the HSA-binding assay the dissociation constant K_D^{HSA} is determined according to the following equation assuming one binding-site

$$K_D^{HSA} = [X]_{unbound} \times \frac{1 - \frac{[X]_{unbound}}{[HSA]}}{\frac{[X]_{bound}}{[HSA]}}$$

$[X]_{unbound}$ = concentration of the unbound compound X in the aqueous phase
$[X]_{bound}$ = concentration of the bound compound X bound to HSA
[HAS] = concentration of HSA

TRANSIL membrane affinity assay (lipid/water partition coefficient):

In an experimental set-up that is comparable to the TRANSIL® HAS assay the membrane affinity (lipid/water partition coefficient) of the three compounds was determined by using TRANSIL® beads coated with a single non-covalently attached lipid bilayer. Again, constant compound concentrations in phosphate buffered saline were titrated against increasing amounts of TRANSIL® beads. After mixing and incubation the unbound fraction of the respective compound was determined in the aqueous phase.
The lipid water partition coefficient or membrane affinity is defined as:

$$\log MA = \log\left(\frac{C_{lipid}}{C_{Water}}\right)$$

C_{lipid} = concentration of the compound in the lipid phase
C_{water} = concentration of the compound in the aqueous phase

II. Results and discussion

A. HAS-binding and membrane affinity

The results of the two assays are summarized in the table below.

Table 5.8.2/05- 1: Summary of the HAS-binding and membrane affinity assays

Compound	HAS-binding		Membrane affinity	
	K_D^{HSA} [mol/L]	SD	log MA	SD
Methiocarb	4.93^{-06}	$\pm 6.3^{-07}$	2.7	±0.1
Methiocarb-sulfoxide	4.74^{-04}	$\pm 4.2^{-05}$	1.2	--
Methiocarb-sulfone	1.27^{-05}	$\pm 3.3^{-06}$	2.1	±0.1

K_D^{HSA} = dissociation constant; log MA = lipid water partition coefficient (or membrane affinity)

III. Conclusion

The dissociation constant of methiocarb is lower than for the two metabolites. Thus, compared to the two metabolites more methiocarb will be bound to protein (HAS), and the unbound fraction of methiocarb in plasma will be lower as for the metabolites.
Based on the results methiocarb has a higher membrane affinity than methiocarb-sulfone and methiocarb–sulfoxid.

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

The following study on *in-vivo* dermal absorption study of methiocarb with the rat the extent of dermal absorption, the distribution, the metabolism in blood and the excretion of the absorbed residues were investigated and compared to the previous study with oral administration in order to aid the risk assessment of occupational, non-dietary exposure (e.g. during spray application). Therefore, this study was conducted at a similar sacrifice regimen as in the previous study.

Report:	KCA 5.1.2/01 [redacted].; 2009; M-347596-01-1
Title:	[Phenyl-1-14C]methiocarb: Mechanistic study in male rats after dermal application
Report No.:	MEF-09/115
Document No.:	M-347596-01-1
Guideline(s):	OECD 427; US EPA OPPTS 870.7600; EU 91/414/EC amended by 94/79/EC
Guideline deviation(s):	Not specified in the report. Use of one concentration only: a 0.2 percent spray dilution to simulate exposure of an operator to the spray fog.
GLP/GEP:	**yes**

Executive Summary

Dermal absorption as well as toxicokinetics and metabolism of the insecticide and molluscide methiocarb in blood were investigated in male rats. A 0.2% spray dilution of an SC 500 formulation of [phenyl-1-^{14}C]methiocarb was applied to a 10 cm^2 area of the shaved dorsal skin of rats within a spacer ring affixed to the skin. The dose level was 10 µg as/cm^2 corresponding to 0.366 mg as/kg bw. The ring was covered by an air-permeable filter paper and in case of longer exposure periods additional by a non-occlusive tube-gauze dressing.

Seven animal groups with each 4 rats were exposed dermally to the radioactive spray dilution for different exposure periods from 15 min to 8 hours. Urine and faeces were collected separately. At the end of the exposure period the animals were sacrificed and exsanguinated. The treated skin was washed with mild soap solution. The outer cornified layer (*stratum corneum*) of the treated skin was removed by repeated tape stripping. Blood after sacrifice, treated and untreated skin, GIT including the faeces and the residual carcass were sampled. All samples were radioassayed. Acetonitrile was added to the blood immediately after collection to stop ongoing enzyme activity, haemolyse the blood cells and precipitate the proteins. Precipitation was completed by centrifugation. The supernatant was analysed for radiolabelled residue components by radio-HPLC, LC-MS/MS and an off-line combination of LC-MS and ^{1}H-NMR. The respective samples of the individual animals were pooled within each animal group to compensate for individual variances.

The radioactive mass balance determined only for the animal group with the longest exposure (8 hours) was excellent (recovery 102.7% of the applied dermal dose).

Following an 8-hour dermal exposure of an aqueous spray dilution of a SC 500 formulation of radiolabelled methiocarb to rats the **systemic absorption** (sum of radioactivity in urine, faeces, blood, non-treated skin, GIT plus faeces, carcass and cage wash) accounted for **28.7% of the applied dose**. An additional portion of 7.32% of the dose remained in the treated skin after removal of the *stratum corneum* by tape stripping. If this portion is added to the systemic absorption a **potential maximum absorption of approx. 35 - 36% of the dermal dose** could be expected.

The absorbed residue was efficiently excreted with urine (17.0% of the dose renally excreted already after a 4-hour exposure). Faecal excretion reached approx. 5% of the dermal dose within the 8-hour exposure period.

The total radioactive residues (TRR) in pooled blood samples showed a very quick peak level (t_{max} = 15 min after beginning of dermal exposure, C_{max} = 0.032 mg eq/kg or dose-normalized concentration CN_{max} = 0.096) followed by variations between 0.010 and 0.020 mg eq/kg (CN: 0.022 – 0.068). [Ed.: CN is calculated as ratio between absolute residue concentration in a sample and the dose level.]

The unchanged parent compound was detected in all analysed blood samples; however at rather low levels. The maximum level was detected at the first sampling point, after a 15-min dermal exposure amounting to 12.7 µg/kg blood followed by lower varying levels of (1.5 – 5.6 µg/kg).

At least 10 metabolites (and conjugates) were detected in blood after a 4-hour dermal exposure. Beyond a 0.5-hour exposure period the carbamate moiety was cleaved off from all metabolites in blood indicating extensive esterase activities in blood. Following cleavage the free phenol was conjugated subsequently with sulfuric acid and glucuronic acid.

The predominant metabolite in blood was identified as methiocarb-sulfoxide-phenol (M04) and its conjugates, which peaked to a level of 12.8 µg eq/kg after 1-hour exposure (CN: 0.043). The metabolite methiocarb-phenol (M03) and its conjugates peaked after a 15-min exposure reaching a level of 7.51 µg eq/kg (CN: 0.023). Methiocarb-sulfone-phenol (M05) and its conjugates as well as methiocarb sulfoxide (M01) were only detected in negligible amounts (maximum: 0.5 µg eq/kg; CN: ≤ 0.001). Methiocarb-sulfone (M02) was not observed in blood after dermal exposure.

The HPLC profiles of the urine samples collected at 2 to 8 hours dermal exposure were very similar. They were also comparable with urine profile of the former metabolism study with oral administration (see above).

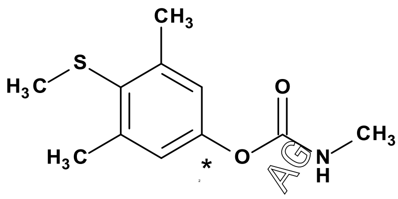
The same four types of metabolic reactions as after oral dosing were also observed after dermal application:

- Ester hydrolysis of the carbamate group (major metabolic step)
- Hydroxylation of the methyl group of carbamate group (minor reaction)
- Oxidation of the thioether group to form the sulfoxide and the sulfone
- Conjugation of the phenolic hydroxy group with sulfuric acid or glucuronic acid

The proposed metabolic pathway is the same as following oral administration. It is shown in Figure 5.1- 2.

Material and Methods

Test Material

Structural formula	 * denotes the ^{14}C label
Chemical name	4-methylthio-3,5-xylyl methylcarbamate (ISO) Phenol, 3,5-dimethyl-4-(methylthio)-, methylcarbamate (CAS, 9CI)
Common name	Methiocarb, Mercaptodimethur (ISO)
CAS RN	2032-65-7
Empirical formula	C_{11} H_{15} N O_2 S
Company code	BAY 37344
Molar mass (non-labelled)	225.3 g/mol
Label	Phenyl-1-^{14}C (see structural formula)
Specific radioactivity	9.43 MBq/mg = 5.658 x 10^5 dpm/µg = 254.86 µCi/mg = 57.42 Ci/mol
Radiochemical purity	> 99% by HPLC and TLC
Chemical purity	> 99% by HPLC
Water solubility	0.027 g/L (20°C, pH 7)
Log Po/w	3.18 (20°C)
Use	Non-systemic insecticide and molluscide with contact and stomach action to control slugs, snails and pest insects (including soil insects)

Test Animals

Test animals	Rat (*Rattus norvegicus domesticus*)
Breed	Wistar, Hsd/Cpd: WU
Number, sex	28 male animals (4 per test)
Age	Approx. 7 weeks at delivery
Body weight	232 - 271 g
Identification	Cage cards, additionally by water-insoluble spots at tail
Accommodation period	Approx. 8 days prior to administration
Housing during test	Individually in Makrolon® metabolism cages allowing separate collection of urine and faeces
Feed and water	Rat/mice maintenance long life diet, *ad libitum*, tap water *ad libitum* Starvation: 16 hours prior to administration
Housing conditions	Temperature: 23 – 24°C Relative humidity: 45 – 58% Photoperiod: alternating 12- to 12-hours light / dark cycles Air change: 10 – 15 times per hour

Study regimen

The animals were separated in seven groups of each four individual rats. They were sacrificed at different intervals after administration as shown in the following table

Test No.	Time of sacrifice [hrs p. admin.]	Samples collected
1	0.25	Blood
2	0.5	Blood
3	1	Blood
4	2	Blood and urine
5	3	Blood and urine
6	4	Blood and urine
7	8	Blood and urine, rinsing of filter paper, spacer ring, tube gauze, cotton swabs (washing of treated skin at exposure end); tape strips (*stratum corneum*), skin at application site; cage wash, non-treated skin, carcass plus blood cells debris, GIT plus faeces

Preparation of the application mixture

The radiolabelled test substance was dissolved in acetonitrile. Aliquots of this solution were taken for radioassaying by LSC and purity and identity checks by radio-HPLC and LC-MS/MS. The major volume containing 20.38 mg test substance was concentrated to near dryness. 26.08 mg of SC 500 blank formulation and two small steel balls (for homogenization) were added and the mixture homogenized using a Vortex mixer and an ultrasonic bath. The resulting radioactive SC 500 formulation was suspended stepwise in water using an ultrasonic bath and a magnetic stirrer. This suspension was filled up to a volume of 16 mL resulting in a 0.2% aqueous field spray dilution. Aliquots were taken for radioassaying and for a purity and identity check of the radiolabeled test substance.

Dermal application procedure

One day prior to application the animals were slightly anaesthetised with ether and an area of approx. 15 cm^2 was shaved on their dorsal skin using an electric hair clipper. The shaved area was wiped carefully with acetone-soaked cotton swabs to remove fat and skin oils and then checked for abrasions. Only rats with intact skin were used for application.

At the day of application, the animals were weighed. While the animals were kept under slight anaesthesia, a rubber spacer ring (36 mm inner diameter, 10 cm^2) was glued onto the shaved skin using cyanoacrylate contact glue. 100 µL of the aqueous spray dilution containing 71 – 113 µg ^{14}C-methiocarb was applied on the skin within the ring using a pipette tip and evenly spread over the skin area with a glass rod. The rod was rinsed with acetonitrile to determine the non-applied portion of application solution.

The mean applied dose level was 0.1 mg/10cm^2 = 10 µg/cm^2. Based on the body weight this dose corresponded to a mean of 0.366 mg/kg bw.

The spacer ring was covered by a protective air-permeable filter paper that was pasted to the ring. The filter paper of the last two animal groups was protected by a tube-gauze dressing that was fixed with an adhesive tape under non-occlusive conditions. The rats were then kept individually in metabolism cages allowing separate collection of urine and faeces.

Collection and processing of excreta
Urine and faeces were collected separately from the animals specified in the study regimen.

Sacrifice and washing of the treated skin
The animals were sacrifized in Pentobarbital-Na anaesthesia by transection of the cervical vessels and exsanguination at the end of the scheduled in-life period.

Of group-7 animals, the protective covers (adhesive tape, protective tube-gauze dressing, filter paper) were removed from the application site. The treated area was washed three times with a mild soap solution using cotton swabs. The cornified skin layer (*Stratum corneum*) of the treated area was removed by tape stripping (10 successive strips). All samples were radioassayed.

Sample processing
The tube-gauze dressing, filter paper and spacer ring were removed and rinsed with acetonitrile. The acetonitrile solutions, washing solutions of the treated area, tape strips of the horny layer, urine and cage wash (aqueous soap solution), other biological samples (blood, treated and untreated skin, GIT and carcass) and all material that was in contact with the skin during exposure were radioassayed.

Treated and non-treated skin was first solubilized in an alkaline solvent. Before radioassaying the basic solution was neutralized with hydrochloric acid. The GIT was dissected form the body. Together with the faeces the mixture was freeze-dried and homogenated. The residual carcass was minced in half-frozen state, homogenized and freeze-dried. Aliquots of the freeze-dried samples were radioassayed after combustion.

The oozed blood obtained at sacrifice by exsanguination was collected in heparinized test tubes and immediately diluted with acetonitrile in a ratio of approx. 1/1 (v/v) in order to stop any possible enzyme activity, to haemolyse blood cells and to precipitate crude protein and cell debris. The supernatant was separated by centrifugation. Supernatant was directly radioassayed. The solids and carcass and GIT samples were radioassayed after homogenization and combustion.

Radioassaying and rendering of the results
Radioassaying (measurement of the radioactivity) of liquid samples was carried out by liquid scintillation counting with automatic quench correction (LSC). The counting time was stopped after reaching a 2σ error of 0.7% or after maximum 20 min. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC. All LSC measurements were conducted repeatedly (up to 5) and averaged.

The results of radioassaying were given as percentage of sample radioactivity per administered radioactivity, as absolute concentration and as a dose-normalized concentration (CN). A CN-value of 1 is equivalent to the so-called equidistribution. CN can be estimated by division of the absolute concentration in the tissue sample given in mg eq/kg by the dose given in mg/kg bw. CN values easily allow a comparison of organ concentrations obtained from experiments with different doses.

Radio-HPLC of blood (supernatant after precipitation) and urine
Radio-HPLC was conducted on a Phenomenex C18 column (250 x 2 mm, 5 µm particle size) operated with a gradient mixture of 1% formic acid in water and 1% formic acid in acetonitrile at 40°C. The system was equipped with an autosampler, a precolumn (also C18), an UV detector (254 nm) and a radiomonitor with a glass scintillator. The flow was adjusted to 0.2 mL/min. The column recovery of approx. 100% was proven by comparison of the radioactivity of an injected blood sample and the eluted radioactivity. A quantifiable radioactive peak was regarded as relevant if its signal exceeded the background noise by a factor of approx. 2.5 times

For identification of peaks radiolabelled and non-labelled reference standards were co-injected.

LC-MS of blood (supernatant after precipitation) and urine

LC-MS was conducted by a combination of a LTQ Orbitrap mass spectrometer and a HPLC system with a C18 or Phenyl-hexyl columns (250 x 2 mm, 5 µm particle size) that was operated with gradient mixtures of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The HPLC effluent was split into a line connected with the mass spectrometer and a line connected with a UV detector and a radiomonitor. The flow was adjusted of 0.2 mL/min. MS ionisation was performed by electro-spray ionisation (ESI).

LC-MS for isolation of peaks further analysed by NMR

LC-MS for isolation of an NMR sample was conducted by a combination of a Esquire HCT mass spectrometer and a HPLC system with a Nucleodur C18 column (250 x 2 mm, 5 µm particle size) that was operated with a gradient mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The effluent of the column was split into two lines, one entering the mass spectrometer, the other one was led to a SPE cartridge (solid phase extraction with a resin polymer as absorbing phase). The absorption in the SPE unit was controlled by its quasi molecular ion detected in the mass spectrometer. After trapping the HPLC peak the SPE cartridge was dried and transferred to an NMR probe head using deuterated solvents.

^{1}H-NMR spectroscopy was performed using a 600 MHz spectrometer. Deuterated methanol was used as solvent.

Identification of parent substance and metabolites in blood and urine

For identification of the residue components of methiocarb in blood and urine the following strategy was applied.

- Co-chromatography of composite blood samples with authentic reference standards of methiocarb-sulfoxide, methiocarb-sulfoxide-phenol, methiocarb-sulfone, methiocarb-phenol and the parent substance methiocarb
- Comparison of the HPLC profiles of different samples (e.g. blood vs. urine),
- Comparison of the HPLC retention times of individual components in the samples with a characteristic urine profile of the former methiocarb rat ADME study (already evaluated, see above) and the methiocarb mechanistic study in male rats after oral application (see above). In these studies, the metabolites were identified by chromatographic and spectroscopic methods.
- The radioactive residues in the composite urine sample of test 6 (0 – 4 h) were identified by direct LC-MS/MS analysis. Based on the interpretation of these mass spectra the single individual components were identified as methiocarb-sulfoxide-phenol-GA, methiocarb-sulfoxide-phenol, Methiocarb-sulfoxide-phenol-SA, methiocarb-phenol-GA, methiocarb-sulfone-phenol-SA, methiocarb-phenol, and parent methiocarb (GA: glucuronic acid conjugate, SA: sulfuric acid conjugate)

Definition of characteristic figures of dermal absorption

The *systemic absorption* was defined as sum of radioactivity in urine and cage wash, residual non-treated skin, blood, GIT plus faeces and carcass plus blood cell debris.

The *relative percentage systemic absorption* was defined as ratio of the systemic absorption and the dermal dose multiplied by 100.

The *relative percentage systemic absorption related to the recovered radioactivity* was defined as ratio of the systemic absorption and the dermal dose multiplied by 100 and by a normalization factor. The normalization factor is defined as ratio 100/% recovery. This is based on the assumption that the operator is exposed in "real life" situations also to very different amounts of the pesticide in relation to a possible contaminated skin area.

The *potential absorption* was defined as sum of radioactivity in the treated skin (after washing) and the tape strips representing the cornified skin layer (*stratum corneum)*. Addition of this figure to the systemic absorption provides a potential worst-case value for the overall absorption.

The *relative percentage potential absorption* was defined as ratio of the potential absorption and the dermal dose multiplied by 100.

The *fraction not absorbed* was identified as sum of radioactivity in the skin wash, spacer ring, covering filter paper and the rinse of the tube-gauze.

Sample storage
Freeze dried samples were stored at room temperature or at approx. +4 °C in a refrigerator. All liquid samples were kept frozen at ≤ -18 °C at all times except during aliquotation for analysis.

Findings

Recovery of radioactivity
The mean radioactivity balance of in test group 7 (4 animals) with 8 hours dermal exposure accounted for 102.27% of the dermal dose based on radioassaying of the applied amount and all radioactive samples (see Table 5.1.2- 1).

Extent of dermal absorption (Table 5.1.2- 1)
Following an 8-hour dermal exposure of an aqueous spray dilution of a SC 500 formulation of radiolabelled methiocarb to rats approx. two thirds of the dose could be washed off with a mild soap solution. In turn, the **systemic absorption** (sum of radioactivity in urine, faeces, blood, non-treated skin, GIT plus faeces, carcass and cage wash) accounted for **28.7% of the applied dose**. An additional portion of 7.32% of the dose remained in the treated skin after removal of the outer *stratum corneum* by tape stripping. If this portion is added to the systemic absorption a potential **maximum absorption of approx. 35 - 36% of the dermal dose** could be expected.

Toxicokinetics of radioactivity in blood following dermal absorption (Table 5.1.2- 2)
The toxicokinetics of the total radioactive residues (TRR) in blood following dermal absorption was determined after different exposure intervals. Mean values (% of dose, TRR and dose-normalized concentrations CN) were calculated of the 4 individuals in an animal group to compensate for differences within the single animals.

TRR in blood showed a very quick peak level (t_{max} = 15 min after beginning of dermal exposure, C_{max} = 0.032 mg eq/kg, equivalent to CN_{max} = 0.096) followed by blood level variations between 0.010 and 0.020 mg eq/kg (equivalent to CN: 0.022 and 0.068) until study end, 8 hours after beginning of exposure.

Excretion with urine and faeces (Table 5.1.2- 2 and Table 5.1.2- 1)
Renal excretion of the dermally absorbed residues was quick and extensive. Already 4% of the dermal dose was excreted with the urine within 2 hours of exposure. Renal excretion increased to 17% of the dermal dose within 4 hours of exposure. Faecal excretion of the absorbed residues achieved 5% of the dermal dose within 8 hours of exposure and is expected to increase further.

Parent substance and metabolites in blood following dermal absorption (Table 5.1.2- 3)
To simulate the reality, blood and not plasma was chosen as analytical matrix, since methiocarb is rapidly cleaved by esterases in blood. Ongoing cleavage after sampling was stopped by immediate addition of acetonitrile that inactivated the enzyme activity and caused haemolysis of blood cells and precipitation of the proteins. Precipitation was completed by centrifugation. The concentration of parent methiocarb and its metabolites was determined in the supernatant taking into account the dilution by acetonitrile.

The unchanged parent compound was detected in all analysed blood samples, however at rather low levels. The maximum level was detected at the first sampling point, after a 15-min dermal exposure amounting to 12.7 µg/kg blood (CN: 0.039) followed by lower varying levels of (1.5 – 5.6 µg/kg).

At least 10 metabolites (and conjugates) were detected in blood after an 4-hour dermal exposure. Beyond a 0.5-hour exposure period the carbamate moiety was cleaved off from all metabolites in blood. These were conjugated with glucuronic acid and sulfuric acid indicating extensive esterase and glucuronosyl-transferase and sulfotransferase activities in blood (as already observed after oral application, see above).

The predominant metabolite in blood was identified as methiocarb-sulfoxide-phenol (M04) and its sulfate and glucuronic acid conjugates, which peaked together to a level of 12.8 µg eq/kg after 1-hour exposure (CN: 0.043) followed by a varying levels between 6.1 and 8.3 µg eq/kg (CN: 0.013 – 0.019).

The metabolite methiocarb-phenol (M03) and its conjugates peaked after a 15-min exposure reaching a level of 7.51 µg eq/kg (CN: 0.023). Thereafter, their level varied between 2.27 and 3.54 µg eq/kg (CN: 0.005 – 0.009).

Methiocarb-sulfone-phenol (M05) and its conjugates as well as methiocarb sulfoxide (M01) were only detected in negligible amounts (maximum: 0.5 µg eq/kg; CN: ≤ 0.001). Methiocarb-sulfone (M02) was not observed in any blood sample.

Renally excreted metabolites

The HPLC profiles of the urine samples collected at 2 to 8 hours dermal exposure were very similar. They were also comparable with urine profile of the former metabolism study with oral administration (see above).

The sulfate conjugate of methiocarb-sulfoxide-phenol was the predominant metabolite. Unchanged methiocarb was also detected, however on a rather low level: 0.16 - 0.19% of the dose after 4 and 8 hours of exposure, respectively. Methiocarb-sulfoxide and Methiocarb-sulfone were not found.

Conclusion

After *in vivo* dermal application of a spray dilution of ^{14}C-methiocarb (SC 500 formulation) to rats at a dose level of 10 µg/cm^2 (0.366 mg as/kg bw) the following conclusion was drawn.

- Following 8-hour dermal exposure the systemic absorption in the rat amounted to 28.7% of the dermal dose. Approximately 2/3 of the dermal dose could be washed off with a mild soap solution at the end of the exposure period.
- An additional portion of 7.4% of the dose remained in the treated skin following washing. If this portion is assumed to penetrate into the body a potential maximum dermal absorption of approx. 35 - 36% of the dermal dose could be expected.
- In the blood, the unchanged parent compound was detected at any time points (15 min – 8 hours), however at only very low concentrations (maximum of 12.7 µg/kg after 15-min exposure)
- The absorbed radioactive residues were efficiently excreted with urine (17% of the dermal dose after 4 hours of exposure)

- Dermally absorbed ^{14}C-methiocarb was metabolised extensively. Beside the parent substance at least 10 metabolites were identified in rat blood and urine. The same four types of metabolic reactions as after oral dosing were also observed after dermal application:
  - Ester hydrolysis of the carbamate group (major metabolic step)
  - Hydroxylation of the methyl group of carbamate group (minor reaction)
  - Oxidation of the thioether group to form the sulfoxide and the sulfone
  - Conjugation of the phenolic hydroxy group with sulfuric acid or glucuronic acid
- Methiocarb was almost completely metabolised by hydrolysis of the carbamate structure and subsequent conjugation of the formed phenol group. Conjugation with sulfate was preferred when compared to conjugation with glucuronic acid.
- The main metabolite in the blood was identified as methiocarb-sulfoxide-phenol and its glucuronide and sulfate conjugates. It peaked after 1-hour dermal exposure to 12.8 µg eq/kg. Methiocarb-phenol and its conjugates peaked already after a 15-min exposure indicating very efficient esterase enzymes in the blood (or skin).

The proposed metabolic pathway is the same as following oral administration. It is shown in Figure 5.1- 2

Table 5.1.2- 1: Dermal absorption of a 0.2% aqueous spray dilution of [phenyl-1-^{14}C]methiocarb formulated as SC 500 formulation by rats (mean of 4 animals)

Dose level	**10 µg/cm² 0.366 mg/kg bw**	
Target dose: Actual dose:	100 µg/rat 93.4 µg/rat	
Exposure period Sacrifice	8 hours at the end of the exposure period	
	% of dose applied	**% of dose recovered**
Washing of treated skin at the end of exposure (Cotton swab rinsing)	64.70	63.39
Tube-gauze	0.02	0.02
Filter paper	0.69	0.68
Spacer ring	0.78	0.77
Not absorbed *	**66.19**	**64.86**
Tape strips (stratum corneum)	0.05	0.05
Skin, application site	7.32	7.22
Potential absorption **	**7.37**	**7.27**
Cage wash	0.41	0.41
Non-treated skin, total	0.67	0.66
Carcass+blood cells debris	5.61	5.44
GIT+faeces	4.76	4.63
Blood	0.15	0.14
Urine	17.12	16.59
Systemic absorption ***	**28.71**	**27.87**
Balance	102.27	
Norm.-factor		0.99 ******

* sum of radioactivity of tube-gauze, filter-paper, spacer ring and cotton swabs rinsing

** sum of radioactivity of skin at application site (after washing) plus tape strips

*** sum of radioactivity of urine, residual skin, blood, GIT+faeces, carcass+blood cells debris, and cage wash

**** mean of (100 / individual recovery of the 4 rats in this group)

Table 5.1.2- 2: Time course of radioactivity in blood and urine of rats following dermal application an aqueous dilution of a ^{14}C-methiocarb-SC 500 formulation at dose level of 10 µg as/cm^2 (0.366 mg as/kg bw)

Animal groups		TRR in Blood			Urine
Test no.	Exposure time [hours]	% of dose administered	TRR [µg eq/kg]	CN*	% of dose administered (cumulative)
1	0.25	0.173	31.592	0.096	---
2	0.5	0.092	16.676	0.045	---
3	1	0.125	20.378	0.068	---
4	2	0.082	12.752	0.041	3.82
5	3	0.036	10.083	0.022	4.10
6	4	0.071	15.226	0.035	17.09
7	8	0.146	15.350	0.041	17.12

* CN: Dose-normalized concentration (approx. TRR / dose level)

Table 5.1.2- 3: Methiocarb and its metabolites in blood of rats after dermal application of an aqueous dilution of ^{14}C-methiocarb-SC 500 formulation at dose level 10 µg as/cm² (0.366 mg as/kg bw)

Exposure Time [hours]	Methiocarb-sulfoxide-phenol (M04) + conjugates*	Methiocarb-sulfone-phenol (M05) + SA-conjugate	Methiocarb-phenol (M03) + conjugates*	Methiocarb-sulfoxide (M01)	Methiocarb-OH-methyl	Methiocarb (parent substance)
	[% of administered radioactive dose]					
0.25	0.052	n.d.	0.041	0.002	0.008	0.070
0.5	0.042	n.d.	0.014	0.002	0.003	0.031
1	0.079	n.d.	0.014	n.d.	0.003	0.029
2	0.053	0.001	0.015	n.d.	0.002	0.010
3	0.022	0.001	0.008	n.d.	n.d.	0.005
4	0.039	0.003	0.017	n.d.	n.d.	0.013
8	0.066	0.004	0.032	n.d.	0.003	0.040
	[µg eq/kg blood]					
0.25	9.424	n.d.	7.510	0.455	1.488	12.715
0.5	7.626	n.d.	2.604	0.310	0.571	5.565
1	12.838	n.d.	2.268	n.d.	0.468	4.805
2	8.329	0.220	2.332	n.d.	0.280	1.591
3	6.141	0.179	2.284	n.d.	n.d.	1.479
4	8.296	0.561	3.541	n.d.	n.d.	2.828
8	6.994	0.412	3.405	n.d.	0.326	4.213
	Dose normalised concentration CN					
0.25	0.029	n.d.	0.023	0.001	0.005	0.039
0.5	0.021	n.d.	0.007	0.001	0.002	0.015
1	0.043	n.d.	0.008	n.d.	0.002	0.016
2	0.027	0.001	0.007	n.d.	0.001	0.005
3	0.013	0.001	0.005	n.d.	n.d.	0.003
4	0.019	0.001	0.008	n.d.	n.d.	0.007
8	0.019	0.001	0.009	n.d.	0.001	0.011

* glucuronide and sulfate conjugates

Physiologically based pharmacokinetic modelling in the rat after oral and dermal exposure of methiocarb: lower toxicity after dermal exposure

In the following study a modelling approach of the pharmacokinetics of the methiocarb metabolism in the rat following oral and dermal exposure is summarized. Based on these results a route-specific difference of the pharmacodynamics with regard to the acetyl choline esterase (AChE) inhibition of methiocarb and its metabolite methiocarb sulfoxide is explained. Due to different metabolic activities of the gut wall and the skin it was concluded that dermal exposure of methiocarb results in a lower toxicity (AChE inhibition) than oral exposure of the same amount of this substance.

Report:	KCA 5.1.2/02[redacted],;[redacted];[redacted]; 2008; M-308644-01-1
Title:	Physiologically based pharmacokinetic and pharmacodynamic modelling of orally and topically administered methiocarb in the rat
Report No.:	MEF-08/364
Document No.:	M-308644-01-1
Guideline(s):	No guideline available
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Executive Summary

A PBPK model was adjusted to the biokinetics of methiocarb and its metabolite methiocarb-sulfoxide in the rat using compound-specific properties and toxicokinetic data. The time-course of their plasma levels was simulated following oral and dermal application of methiocarb to the rat. Both compounds, parent and metabolite, have an intact methyl carbamate function and are known as potent AChE inhibitors. Other methiocarb metabolites in the rat are cleavage products no longer containing the carbamate function and therefore do not impair the AChE enzyme. Therefore, they were not considered in this modelling. This PBPK modelling revealed a strong indication of exposure route-dependent differences in metabolism of methiocarb: The plasma levels of the AChE inhibiting carbamates that are unbound to plasma proteins are significantly lower following dermal exposure than after oral administration. As a consequence, dermal exposure of methiocarb causes lower toxic effects than oral exposure.

Following oral administration the calculated plasma levels lay significantly above the measured data when only based on the physico-chemical properties of methiocarb. A reduction of the permeability through the gut wall resulted in a lower and proper C_{max}, but in a too long T_{max}. An alternatively assumed higher degradation rate in blood or liver also resulted in a proper C_{max}, but in a too rapid decrease of the plasma levels of methiocarb. The only solution for a better approximation was the introduction of a significant metabolisation step of methiocarb in the gut wall (in the enterocyte compartment). This is very reasonable as the gut wall contains high amounts of CYP 450 enzymes that are able to oxidize methiocarb to methiocarb sulfoxide.

Based on this calculated formation rate of methiocarb sulfoxide (in the gut wall) and its physico-chemical properties the calculated plasma levels of the sulfoxide were also too high. Therefore, an additional degradation step of the sulfoxide in the gut wall was introduced (hydrolysis of the carbamate group) and combined with a lower hydrolysis rate in the blood. This resulted in a good agreement of simulated and measured data.

Summarizing these results demonstrated an initial rapid metabolism of methiocarb in the gut wall following oral administration. This leads to an initial concentration of the toxic metabolite methiocarb-sulfoxide in plasma comparable to that of the parent substance. Since hepatic metabolism seems to be less relevant for the initial metabolisation step, methiocarb entering the blood circulation is first cleaved by blood esterases before a metabolic oxidation step can take place in the liver. Consequently a modified metabolic pathway is proposed for methiocarb in the rat as shown in Figure 5.1- 11:.

These PBPK results were extended to the inhibition of acetylcholine esterase (AChE) by these substances in the red blood cell compartment. Methyl carbamates like methiocarb and methiocarb sulfoxide are substrates of this enzyme. The inhibition is caused by occupation of AChE that makes the occupied enzyme unavailable for further substrates and therefore inactive. Based on data for the *in-vivo* time-course of erythrocyte AChE inhibition by methiocarb in rats (from literature data) the inhibition was calculated separately for methiocarb and methiocarb-sulfoxide and concurrently in combination for an oral dose of 25 mg/kg bw.

This modelling showed that the major part of the observed inhibition effect can be attributed to methiocarb sulfoxide. This is due to its lower plasma binding (f_u(methiocarb) = 2 %; f_u(methiocarb-sulfoxide) = 39 %) and the respective 20-fold higher availability of the sulfoxide, since the intrinsic inhibition constants K_i are very similar (K_i = 0.34 μM^{-1} min^{-1} for methiocarb and K_i = 0.216 μM^{-1} min^{-1} for methiocarb sulfoxide).

After dermal exposure of methiocarb the sulfoxide metabolite was not observed in the blood circulation because the skin has no oxidative capacity and could only lead to an ester cleavage due to the abundance of esterases. Therefore, the plasma level was only calculated for methiocarb based on its physico-chemical properties and a maximum 40% dermal absorption within 8 hours of dermal exposure. A significant overestimation of the plasma levels was also calculated in this case. Therefore, a reduced absorption was assumed by introduction of a metabolic degradation step in the skin. This was of course no oxidative step as in case of the gut wall, but a hydrolytic step that cleaved the carbamate function. Following reduction of the absorbed fraction of intact methiocarb to 0.3% and adjusting the absorption rate (velocity) a quite good approximation to the measured plasma levels was achieved.

Using the pharmacodynamics AChE inhibition model, as developed before, together with the calculated unbound concentration of methiocarb in red blood cells after dermal exposure the *in-vivo* AChE inhibition in rats was estimated.

This resulted in a maximum inhibition of about 6 % for a dermal dose of 35 mg/kg bw in comparison to 75 % inhibition that was reached for the same dose applied orally. An inhibition of 50 %, which is assumed to lead to first toxic signs is reached only for dermal doses of 500 mg/kg bw and above according to this estimate.

Therefore, it can be concluded that the same amount of methiocarb causes significantly lower toxic effects (AChE inhibition) after dermal exposure (for an 8-hour period) than administered as a single oral dose.

Material and methods

Experimental data used

Toxicokinetic studies on rats were conducted following oral (1 mg/kg bw per gavage) and dermal application (undiluted 50 mg and spray dilution 250 µg per 10 cm^2 of shaved skin; exposure periods 15 min – 8 hrs) of ^{14}C-methiocarb. The animals were sacrificed at different time points after (beginning of) dosing (0.25, 0.5, 1, 2, 4, and 8 hrs) and the whole blood was collected. The plasma of the blood samples was separated by addition of acetonitrile and centrifugation. It was analysed for methiocarb and its metabolites by radio-HPLC.

In addition, the stability of methiocarb in rat blood was investigated by incubation of 10 µg ^{14}C-methiocarb in blood for the same incubation intervals as the sacrifice times above. The incubation was stopped by addition of acetonitrile, centrifugation and radio-HPLC analysis of the supernatant plasma.

In-vitro and *in-vivo* acetylcholine esterase (AChE) inhibition data were taken from the literature. The *in-vitro* inhibition of bovine AChE by parent methiocarb and its active metabolite methiocarb-sulfoxide is described in (Buronfosse, 1995)[1] with the inhibition constants K_i = 0.34 $\mu M^{-1} min^{-1}$ for

[1] Buronfosse, 1995: T. Buronfosse, P. Moroni, E. Benoit and J.L. Riviere, "Stereoselective Sulfoxidation of the Pesticide Methiocarb by Flavin-Containing Monooxygenase and Cytochrome P450-Dependent Monooxigenases of Rat Liver Microsomes. Anticholinesterase Activity of the Two Sulfoxide Enantiomers.", Journal of Biochemical Toxicology, 10, 179-189 (1995)

methiocarb and K_i = 0.216 $\mu M^{-1}min^{-1}$ for methiocarb-sulfoxide. The time-dependent inhibition of AChE in red blood cells of rats after oral administration of 25 mg methiocarb/kg bw was taken from (Padilla, 2007)[2].

Structure of the PBPK model

A generic physiologically based pharmacokinetic (PBPK) model was adjusted to a substance specific model using the physicochemical properties of modelled substances. The model includes the following tissues as explicit compartments: venous blood, arterial blood, bone, brain, fat, heart, kidney, large intestine, liver, lung, muscle, pancreas, portal vein, skin, small intestine, spleen, stomach, gonads. Each compartment except the blood compartments and the gall bladder are subdivided into four sub-compartments (blood plasma, red blood cells, interstitial space and cellular space). All these sub-compartments are separated from each other by a permeation barrier through which substance can be exchanged by passive diffusion. The permeation rate for the exchange between cellular space and interstitial space, given by the product of permeability x surface area, is calculated from compound properties (lipophilicity and molar mass). Depending on the ratio between this rate and the blood-flow rate in the respective tissue the distribution into this tissue is either blood flow limited or permeation limited. Correspondingly, also the permeation into the red blood cells is treated. The permeation rate for the exchange between plasma and interstitial space is set to a very high value above the blood flow rate, because low molar mass compounds are usually quickly exchanged between those compartments. The separation of plasma and interstitium is therefore only used to allow the consideration of different pH values in both spaces that imply changes of the physicochemical properties of charged molecules (weak acids and bases) which then influence the distribution behaviour.

An exception is given for the brain. There the distribution limiting barrier is that between vascular space and interstitium, since this is the actual location of the blood brain barrier that determines the exchange of substances between blood and the brain in reality.

Active ADME processes like metabolisation or active transport across the permeation barriers can be described in the model either by first order processes using fixed rate constants or as Michaelis-Menten like processes with a concentration dependent rate.

Intestinal absorption is modelled similarly as described for the advanced compartmental absorption and transport model (Agoram, 2001)[3]. The whole gastrointestinal tract is composed of a stomach compartment, several small intestine and two large intestine compartments. These compartments represent the lumen of the GI-tract. Substance entering the stomach is released into the small intestine following a first order kinetic in case of a fasted state and following a linear function in the fed state. The passage through the small intestine is described using fixed flow rates between adjacent compartments. Substances contained within the intestinal lumen are assumed to be solved until their solubility in intestinal fluid is reached. If the calculated concentration in the compartment exceeds the solubility the exceeding amount is assumed to be present in a crystalline form. Solved substance is able to be absorbed from the lumen into the portal vein either directly in the large intestine or via enterocytes in the small intestine. The introduction of the enterocyte compartments in the small intestine allows for the consideration of metabolic processes in the gut wall. Additionally carrier mediated active transport may be considered in the permeation process out of the intestinal lumen. The rates of passive uptake from the gut lumen are determined by assuming a constant compound specific permeability in each compartment multiplied by a surface area that is site specific and thus varies from compartment to compartment.

[2] [redacted] 2007: S. Padilla, R.S. Marshall a, D.L. Hunter a, A. Lowit, "Time course of cholinesterase inhibition in adult rats treated acutely with carbaryl, carbofuran, formetanate, methomyl, methiocarb, oxamyl or propoxur", Toxicology and Applied Pharmacology 219, 202–209 (2007)

[3] [redacted], "Predicting the impact of physiological and biochemical processes on oral drug bioavailability". Advanced Drug Delivery Reviews 50, 41–67 (2001)

Volumes and surface areas of the intestinal compartments as well as the stomach emptying times were taken from different literature sources. The surface areas used include all surface enhancement factors given by the intestinal folds, villi and micro-villi of the enterocytes. The intestinal flow rates for the rat were determined by fitting model results to experimental data of the intestinal transit.

Modelling strategy

Only the acute toxic residue components of methiocarb detected in blood of rats were considered for the biokinetic modelling. These are the parent substance methiocarb and its metabolite methiocarb-sulfoxide (M01) both having an intact methylcarbamate structure. These carbamate compounds cause a significant inhibition of the acetylcholine esterase (AChE). A further metabolite with an intact carbamate structure, methiocarb-sulfone (M02), was not detected in the rat blood. All other metabolites of methiocarb in the rat did not contain the intact carbamate structure and, therefore, have been neglected.

Compound specific parameters

Tissue/plasma partition coefficients and passive permeation rates are estimated from physicochemical properties of the compound to be simulated. All other substance specific model parameters (e.g. clearance, metabolic rates, rates of active transport) have to be input directly. Their values must either be known from dedicated experiments or can be estimated by manually adjusting the simulation for a good fit to observed data in the model calibration step.

The tissue/plasma partition coefficients are calculated from measures of the substances' lipophilicity (octanol/water distribution coefficient (D) and Membrane Affinity (MA) = phospholipid/water partition coefficient) and plasma binding (Schmitt, 2008)[4]. Roughly for each tissue a partition coefficient related to water is calculated as a weighted mean of the partition coefficients for each of its constituents (water, lipids, protein):

$$K_{tissuewater} = \frac{C_{tissue}}{C_{water}} = \frac{1}{f_u^{tissue}} = F_{water} + K_{n\_L} F_{n\_L} + K_{n\_PL} F_{n\_PL} + K_{a\_PL} F_{a\_PL} + K_P F_P$$

where K are the partition coefficients, F are volume fractions and C are concentrations. The subscripts W, n_L, n_PL, a_PL and P mean water, neutral lipid, neutral phospholipids, acidic phospholipids and protein respectively. f_u^{tissue} is the unbound fraction of the concentration in the tissue which is equal to the concentration in water.

For the rate constants of the substance exchange between vascular space and tissue as well as between plasma and erythrocytes a scaling rule based on lipophilicity and molar mass was developed. For the diffusion coefficient a power law for its dependence on molar mass is assumed. As partition coefficient the octanol/water distribution coefficient D is considered (see footnote 4).

The following **compound-specific parameters** were applied as input data for kinetic modelling

[4] [redacted] "General approach for the calculation of tissue to plasma partition coefficients", Toxicology in Vitro 22, 457–467 (2008)

Parameter	Methiocarb	Methiocarb-sulfoxide	Description /Remark
Molar mass (g/mol)	225	241	-
Log MA	3.2	0.9	Logarithm of membrane affinity (phospholipid membrane/water partition coefficient
Log Kow	3.08	1	Logarithm of octanol/water partition coefficient (determined by RP-HPLC at pH 2.3)
pKa	-	-	Logarithm of dissociation constant for human serum albumin
Log K_{d_HSA}	- 4.6	- 3.2	Unbound fraction in plasma; from these values are calculated: f_u = 2% for methiocarb, f_u = 39% for methiocarb-sulfoxide
Rate of metabolism in plasma (min^{-1})	0.04	n.d.	-

<u>Model calibration</u>

This PBPK-model can simulate the fate of only one substance only at a time. Therefore the calibration had to be done for methiocarb and methiocarb-sulfoxide separately. First the adjustments were made in order to achieve a good agreement between calculated methiocarb concentration and respective observed levels in the plasma. From a simulation with this model the calculated time course of methiocarb-sulfoxide generation by the respective metabolic process was used as an input table in the model then parameterised for methiocarb-sulfoxide. This model was then in a second step calibrated for an optimized agreement with observed methiocarb-sulfoxide concentrations.

Findings

<u>Methiocarb modelling following oral dosing</u>

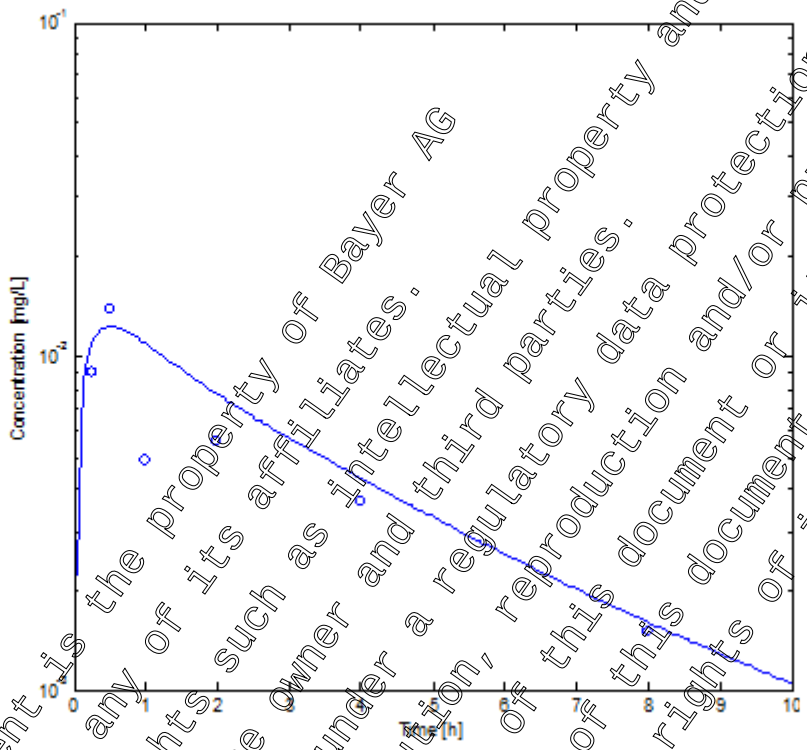
The PBPK-model parameterised for methiocarb was used to calculate plasma concentrations after oral administration of 1 mg/kg bw. The amount of methiocarb in the blood of the rat was dramatically overestimated by the model in this first step.

This may have two reasons. Either the calculated fraction absorbed is much too high or the rate of metabolisation is significantly higher than considered in the model. A reduction of the two model parameters that influence the intestinal absorption, i.e. solubility in the gastro-intestinal tract and intestinal permeability, led to reduced plasma concentration levels. But simultaneously this led to a retarded absorption that contradicts the rapid absorption that was observed (short Tmax). Therefore, a lower than estimated absorption can be excluded as reason for the discrepancies.

Thus alternatively, the degradation rate in blood was increased. By this it was possible to reduce Cmax to a level close to the observed one. However, the rate that has to be considered is implausibly high and results in a very rapid degradation that does not agree with the experimental results. Alternatively, it had been checked if a better result can be achieved by introducing a hepatic clearance into the model, but this led to a very similar result as after assumption of a higher degradation rate in the blood.

The only hypothesis that could explain the observed behaviour is a <u>rapid metabolisation in the gut wall</u> (oxidation of methiocarb to methiocarb-sulfoxide). Accordingly such a metabolic process with a concentration independent rate constant (first order process) was introduced into the enterocyte compartment of the intestine model. The methiocarb model for oral administration was calibrated by introducing one additional metabolic process in the gut wall and adjusting only the respective metabolic rate constant. The optimum clearance value used is CL_{int} = 500 mL/min which was considered in each enterocyte compartment of the small intestine. After an adjustment of this metabolic rate methiocarb levels in plasma, observed and modelled, are in a very good agreement (Figure 5.1- 6).

Figure 5.1- 6: Calculated (line) and observed (symbols) methiocarb plasma concentrations in rats after oral administration of 1 mg/kg bw. Calculation considered gut wall metabolism of methiocarb.



Remarkably, the degradation rate of methiocarb (via cleavage of the carbamate function) after entering the blood circulation is very well explainable with the degradation rate determined in blood *in-vitro*. No additional hepatic clearance is needed in the model. This implies that the metabolisation in blood, which is likely provided by esterases, is the dominant first metabolic step after methiocarb enters the body.

Since also the non-cleaved methiocarb-sulfoxide (M01) was observed in the plasma after oral absorption it can be assumed that this metabolite is mainly produced in the enterocytes. This is also likely because the gut wall contains considerably high amounts of (cytochrome P 450) CYP 450 enzymes which are able to catalyze the respective oxidation of the sulfur. Apart from monooxygenases the gut wall also contains esterases. Therefore, it may also be that some ester cleavage occurs in the gut wall as well. Since no further reliable information is available it is in first instance assumed for the present calculations that the metabolisation in the gut wall is solely due to oxidation to methiocarb-sulfoxide.

Methiocarb-sulfoxide modelling following oral dosing of methiocarb

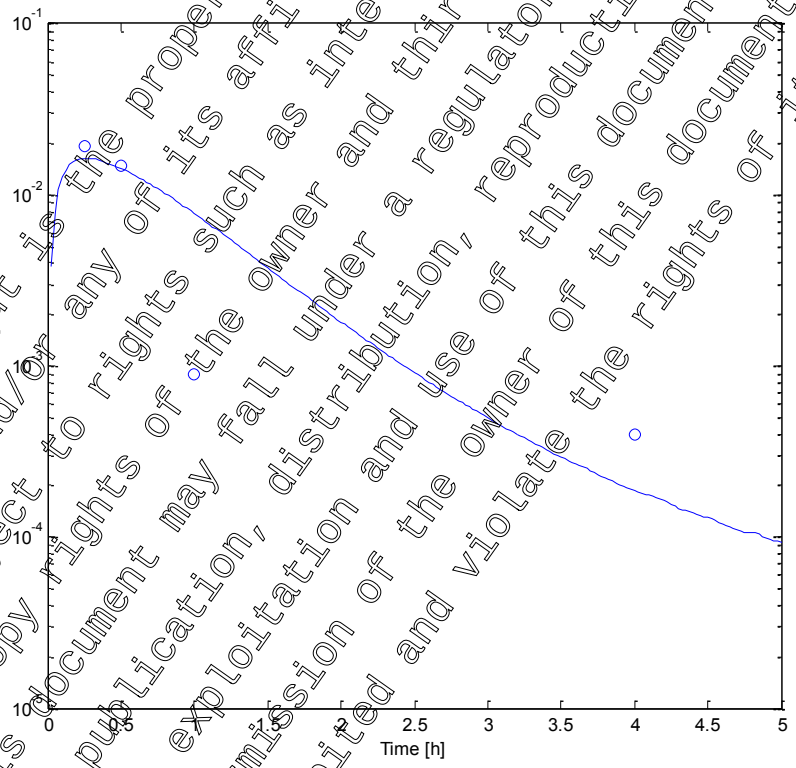
The methiocarb model as calibrated in the last step was used to calculate the amount of methiocarb-sulfoxide generated in the enterocytes. These data were output into a time-series table which was then used as a look-up table for substance input into the cellular space of the small intestine in the methiocarb-sulfoxide model. Using the parameterisation as described above and the same degradation rate in blood as for the parent compound, this calculation was done in the first instance.

However, similar to methiocarb, the calculated concentrations of methiocarb-sulfoxide were by far higher than those observed in the experiment. The slope of the concentration curve in the decreasing part is, however, comparable to that indicated by the observed data points. This implies that either the

fraction of sulfoxide and of the metabolites generated in the gut wall (assumed to be 100 %) is over estimated or that the sulfoxide is rapidly metabolised further in the enterocytes before entering the blood circulation. Also a combination of both might apply.

For the result of the methiocarb-sulfoxide model in terms of calculated plasma concentrations it has no impact which of these alternative hypotheses is considered. Therefore, simply a metabolic process, equivalent to the methiocarb model, was implemented in the enterocyte compartment. After adjustment of the respective rate constant a good agreement with the observed concentration was achieved. This could be further improved by reducing the metabolic rate in the blood by a factor of 2.5 leading to the approximation as shown in Figure 5.1- 7. Considering the low number of data points and their significant scatter the achieved quality of the model was deemed sufficient.

Figure 5.1- 7: Calculated (line) and observed (symbols) methiocarb-sulfoxide plasma concentrations in rats after oral administration of 1 mg/kg bw. In the calculation an additional metabolic degradation in the enterocytes is considered.



Pharmacodynamic model for the AChE inhibition in the rat after oral dosing of methiocarb

The PBPK models for methiocarb and its metabolite methiocarb-sulfoxide were extended by introducing an additional equation in the red blood cell compartment that describes the inhibition of acetylcholine esterase by these substances. This inhibition process facilitated by carbamates is well described in the literature. The carbamates are substrates of the enzyme and the inhibition is caused by a prolonged binding of one of the reaction products to the enzyme after ester cleavage that makes the occupied enzyme molecules unavailable for further substrates (e.g. acetyl choline) and therefore inactive. Activity is recovered after the inhibitor concentration decreases. The whole process is sketched in the following scheme:

$$E + CX \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ECX \xrightarrow{k_e} EC \xrightarrow{k_r} E + C$$

where E is the enzyme, CX the carbamate and EC the carbamylated enzyme.

Binding of the carbamate to the enzyme is a reversible process with on- and off-rates k_{on} and k_{off} which determine the dissociation constant $k_d = k_{off}/k_{on}$. k_e is the reaction rate of the esterase reaction and k_r is the release rate of the reaction product that leads to a recovery of the enzyme. For short times where the last release step can be neglected the time dependence of enzyme activity is determined by the dissociation constant k_d and the reaction rate k_e. The respective formulas are given by Buronfosse (1995, see footnote 1).

Unfortunately, information about all three kinetic constants (k_d, k_e and k_r) cannot completely be derived from the literature for methiocarb and methiocarb-sulfoxide. Solely the inhibition constants K_i for methiocarb (K_i = 0.34 $\mu M^{-1}min^{-1}$) and methiocarb-sulfoxide (K_i = 0.216 $\mu M^{-1}min^{-1}$, value for the racemic mixture) are reported by (Buronfosse, 1995; see publication in footnote 1) indicating a comparable intrinsic inhibitory potential for the two substances.

k_d and k_e could, however, not be determined separately by the authors. k_e values for various carbamates, but not for methiocarb, can be found in the publication of (Hastings, 1970). The values for dimethylcarbamates vary in the range of 0.14 – 44 min^{-1}. Due to this uncertainty one of these values, k_d or k_e, as well as that of k_r have to be defined by fitting the model results to *in-vivo* data. In the present case k_e was fitted and k_d is then calculated using the measured K_i values by the relation $k_d = k_e/K_i$.

Respective data for the *in-vivo* time course of erythrocyte ChE inhibition by methiocarb in rats were reported by (Padilla, 2007; see publication in footnote 2). The ChE-inhibition was calculated for methiocarb and methiocarb-sulfoxide for an oral dose of 25 mg a.i./kg bw.

The kinetic constants were fitted under the consideration that both k_r and k_e are equal for both substances. Therefore only two values (k_d for methiocarb and methiocarb-sulfoxide) had to be optimized. The k_e-value of 0.09 min^{-1} is somewhat lower than the values reported for other dimethylcarbamates but it is still in a reasonable range. Also the value for k_r lies well in the range of values (0.018 – 0.026 min^{-1}) given for other carbamates in (O'Brien, 1966)[5] and references therein. The optimized parameters in the ChE-inhibition model are compiled in the following table.

Kinetic parameter	Methiocarb	Methiocarb-sulfoxide
k_d [µM]	0.28	0.44
k_e [min^{-1}]	0.09	0.09
k_r [min^{-1}]	0.02	0.02

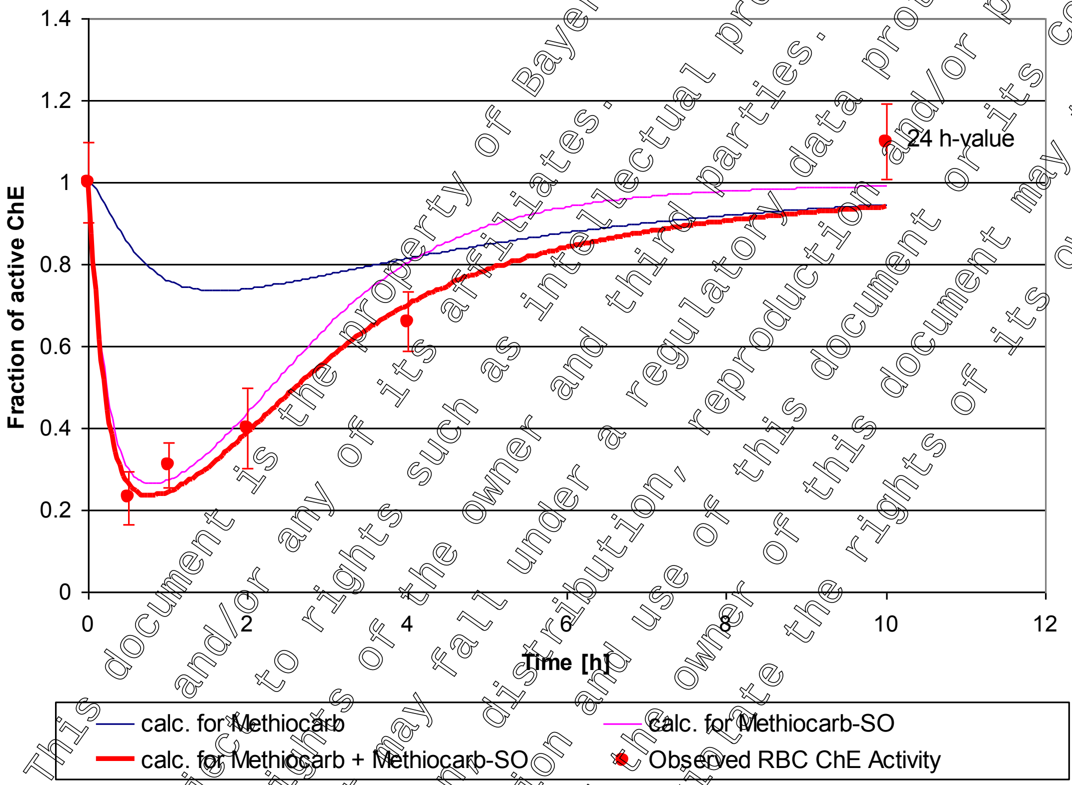
The inhibition curves calculated with these values are shown in Figure 5.1- 8 in comparison to the observed values. The calculated results are given for methiocarb and methiocarb-sulfoxide separately and for the case when both are present concurrently.

The figure demonstrates that the major part of the observed ChE inhibition effect can be attributed to the inhibition by the sulfoxide metabolite. This is due to its lower plasma binding (f_u(methiocarb) = 2%; f_u(methiocarb-sulfoxide) = 39 %, see Table with compound-specific parameters) and the respective higher availability, since the intrinsic activity measured by the inhibition constants K_i of the two compounds is nearly equal.

5 [redacted] "The Reaction of Carbamates with Cholinesterase", Molecular Pharmacology 2, 593-605 (1966)

Figure 5.1- 8: Time-dependence of erythrocyte ChE activity in rats inhibited by methiocarb and methiocarb-sulfoxide following oral administration of methiocarb at a dose level of 25 mg/kg bw

The thin lines show separately the model results for methiocarb and methiocarb-sulfoxide (methiocarb-SO). The bold line depicts the combined effect of both compounds when present simultaneously. The points represent measured ChE activity of red blood cells.



The release rate k_r is quite well determined when fitted to the experimental data, because all observed values belong to the recovery phase of the inhibition. Therefore, its optimised value is only little dependent on different boundary conditions. The value of k_e (0.09 min^{-1}), however, is less certain. Therefore a sensitivity analysis was conducted in order to find out the dependence of the results on this parameter. For this purpose the total inhibition was calculated for a range of different k_e values (k_e = 0.1, 0.15, 1, 10 min^{-1}). There is some influence on the maximum inhibition for values of ke between 0.1 min^{-1} and 1 min^{-1}, but the extend of this variation is roughly only in the range of the standard variation of the observed values. Therefore it can be concluded that the fitted ke value is uncertain, but it has no large influence on the model results and can thus be considered reliable enough for use in further applications of the model.

Methiocarb modelling following dermal dosing

For an initial simulation of the dermal absorption of methiocarb the model was parameterised using values for the total absorbed fraction and the absorption rate that were derived from the results of an

in-vivo dermal absorption study (Weber, 2001)[6]. The total absorption was about 40% of applied dose (based on radioactivity measurements) if the radiolabelled compound was applied in a diluted spray solution (Formulation SC 500 diluted in water 1+ 999; approx. 5 µg/cm^2; approx. 0.2 mg/kg bw; exposure period: 8 hours, excreta sampling: 160 hours). The absorption rate was only roughly estimated from an absorption curve determined from the cumulative excretion. About 50% of the totally absorbed amount was reached within 8 h. From that a rate was calculated to $k_{abs} = \ln(2)/AbsT_{50} = 0.001\ min^{-1}$.

Using these values for total absorption and absorption rate and considering the degradation rate for metabolism in blood a dramatic overestimation (100-fold) of the plasma concentrations were calculated compared to the measured levels of methiocarb after dermal exposure. Since no toxic metabolite (with the intact carbamate structure, Table 5.1.2- 3) was observed in the case of dermal application the calculation was performed solely for methiocarb.

Reasons for this discrepancy could be a fast metabolism in the skin reducing the concentration in blood, similar to the case of gut wall metabolism after oral absorption, or a generally much lower absorbed fraction. Both hypotheses have been tested. A reasonable result could only be achieved using a reduced absorbed fraction. Introducing a metabolic process with a high rate in the skin led to plasma concentration curve that decreased much too fast. However, considering the quality of the observed data with their large scatter, a certain decision for one of the hypotheses cannot be made. Moreover a mixture of both may be existent in reality. A reduction of the total absorbed fraction to 0.3 % led to the result shown in Figure 5.1- 9. Additionally the absorption rate had to be adjusted in order to achieve a maximum concentration in the time interval between 1 and 2 hrs as observed experimentally. The absorption rate used is $0.02\ min^{-1}$.

Independent of the explanation for the low methiocarb concentrations and due to the lack of any additional toxic metabolite in plasma after dermal absorption it can be concluded that the dermal toxicity of methiocarb must be much lower than after oral absorption.

Using the pharmacodynamic model, as developed in the preceding section, together with the calculated unbound concentration in red blood cells after dermal exposure an *in-vivo* ChE inhibition in rats was estimated.

This resulted in a maximum inhibition of about 6 % for a dermal dose of 25 mg/kg bw in comparison to 75 % inhibition that was reached for the same dose applied orally.

An inhibition of 50 %, which is assumed to lead to first toxic signs is reached only for doses of 500 mg/kg bw and above according to this estimate. Calculated inhibition curves for 25 and 500 mg/kg bw are depicted in Figure 5.1- 10. For these estimates it is assumed that the totally absorbed fraction is independent of the dose, what might lead to an overestimate as the relative absorption decreases with the dose level due to saturation effects.

[6] ([redacted]: "[Phenyl-1-^{14}C]Methiocarb 500 FS (MESUROL®: Dermal absorption in the rat." Report of Bayer AG, 2001, Comp. No. M-035400-01-1.

Figure 5.1- 9: Calculated (line) and observed (symbols) methiocarb plasma concentrations in rats after dermal administration of 1 mg/kg bw. For the calculation the total absorbed fraction was adjusted to 0.3 %

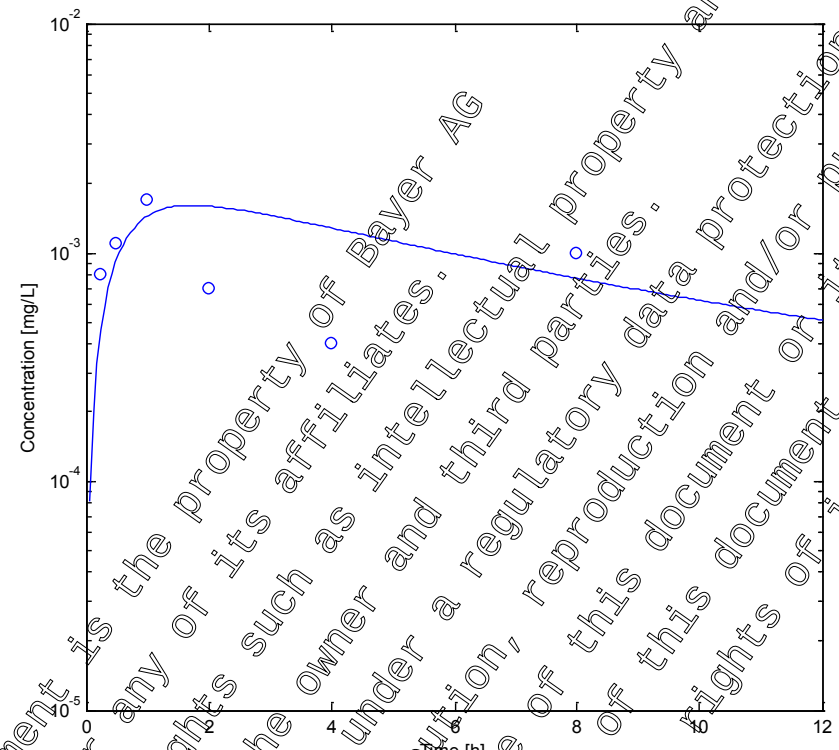
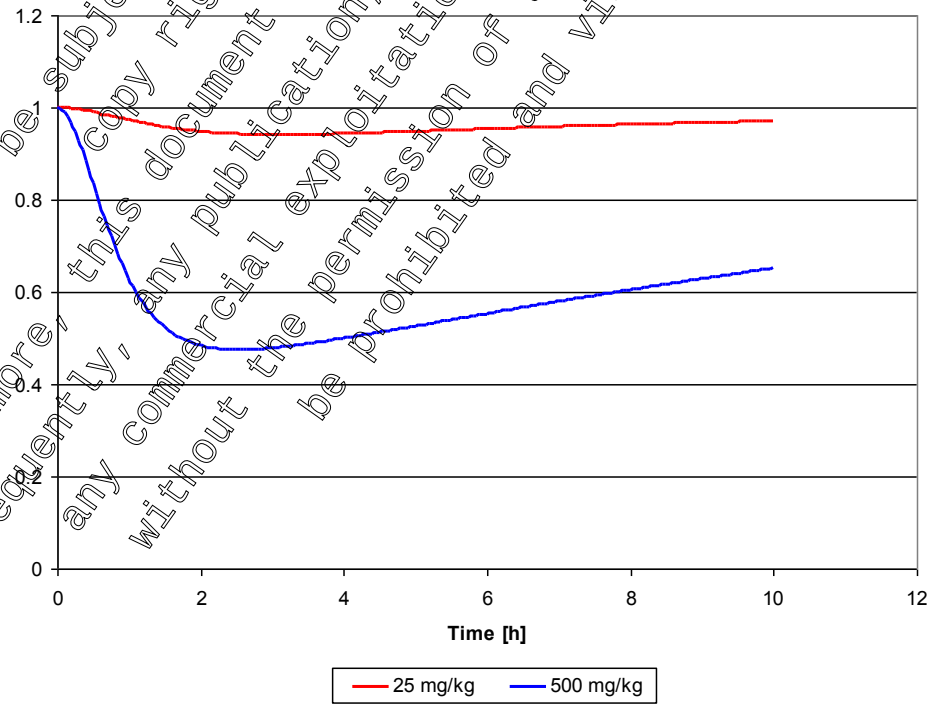


Figure 5.1-10: Calculated erythrocyte ChE inhibition of methiocarb following dermal exposure



Conclusion

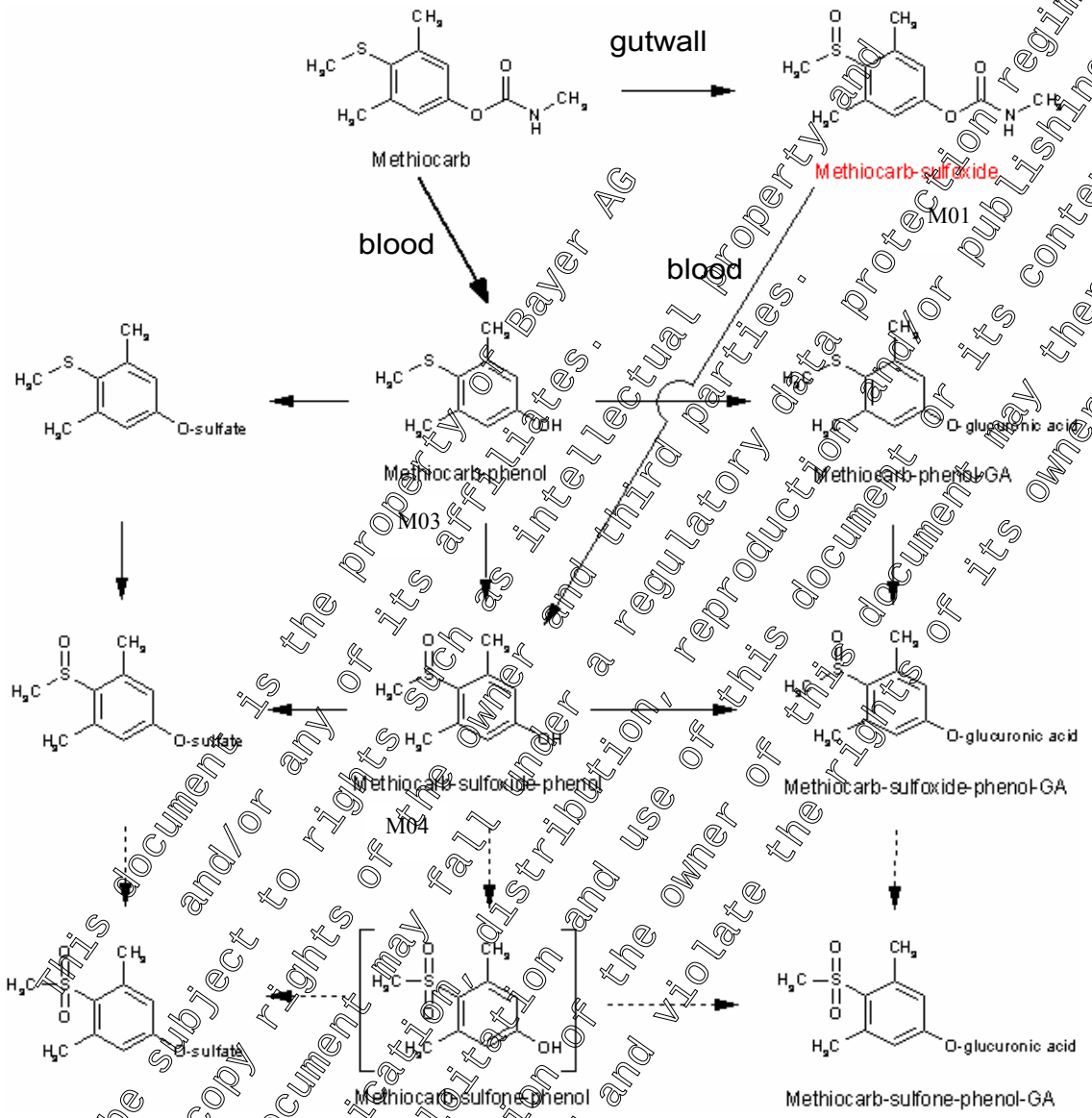
A generic PBPK model of the biokinetics of methiocarb and its metabolite methiocarb-sulfoxide in the rat was adjusted to using compound-specific properties and toxicokinetic data. The time-course of the plasma levels was simulated following oral and dermal application of methiocarb to the rat. Both compounds have an intact methyl carbamate function and are known as potent AChE inhibitors. Other methiocarb metabolites in the rat are cleavage products no longer containing the carbamate function and therefore do not impair the AChE. Therefore, they were not considered in this modelling. This PBPK modelling revealed a strong indication of exposure route-dependent differences in metabolism of this substance with a lower toxicity after dermal exposure.

The proposed rapid CYP450-induced metabolism in the gut wall following oral administration leads to plasma levels of the toxic metabolite methiocarb-sulfoxide comparable to those of the parent substance. In contrast, the sulfoxide was not observed in the blood circulation after dermal administration because the skin has no oxidative capacity and could only lead to an ester cleavage of the carbamate structure due to the abundance of esterases. Since hepatic metabolism seems to be less relevant for the initial metabolisation step methiocarb entering the circulation is first cleaved by blood esterases before an oxidation can take place in the liver. Consequently the modified metabolic pathway for methiocarb in rats shown in Figure 5.1- 11. is proposed.

The results of the pharmacodynamic model with regard to inhibition of erythrocyte AChE showed that the metabolite methiocarb-sulfoxide causes a higher toxic effect (AChE inhibition) following oral absorption than the parent substance itself. This is mainly due to the higher unbound fraction of this less lipophilic metabolite in plasma than that of the parent methiocarb. Thus, the metabolism in the gut wall is a toxifying step rather than a detoxification. Since the skin is missing this toxifying (oxidative) step dermal exposure of methiocarb did not result in methiocarb sulfoxide. The PBPK modelling also resulted in s significantly lower absorbed fraction of parent methiocarb (0.3% of the dose due to significant metabolic hydrolysis of the carbamate structure in the skin) than measured via radioactivity comprising parent and metabolites.

Therefore, methiocarb dermally exposed leads to a significantly lower substrate level for AChE inhibition than after oral administration. As a conclusion, it has to be stated that dermally applied methiocarb is significantly less toxic than orally applied methiocarb.

Figure 5.1- 11: Modified metabolic pathway of methiocarb in the rat following oral administration including the metabolic oxidation step in the gut wall



CA 5.2 Acute toxicity

Summary of acute toxicity

The acute oral LD_{50} of methiocarb in the rat from the submitted study was 33 mg/kg bw in males and 47 mg/kg bw in females.

However, in the previous review an overall acute oral LD_{50} of 19 mg/kg bw (range 13 – 35 mg/kg bw) was considered, based on several other available acute oral toxicity studies in rats, that were not submitted by Bayer. Toxicity after dermal exposure was low. The acute dermal LD_{50} was > 5000 mg/kg bw in the rat and > 2000 mg/kg bw in a limit dose study in the rabbit. The LC_{50} (4-hour exposure) values obtained after acute inhalation exposure to rats were 585 mg/m³ / 433 mg/m³ air in males / females. Methiocarb did not cause skin or eye irritation in rabbits, and was not sensitising to the skin of the guinea pig in tests by the Buehler method and by the Magnusson and Kligman method.

Furthermore, methiocarb was tested in an *in vitro* 3T3 NRU phototoxicity test. The test did not give any indication for a phototoxic potential of methiocarb.

Table 5.2- 1: Summary of acute toxicity studies*

Route / Study	Species / test system	Sex	Result [mg/kg bw or mg/m³ air] NSD#	LLD##	LD_{50}/LC_{50}	Reference
Oral	Rat (Sprague-Dawley)	M F	6.3 15.0	15.0 36.0	33 47	[redacted] 1979 M-026415-01-1
Oral[1)]	Rat (Wistar)	M F	10 10	50 [redacted]	Not determined	[redacted], 1973 M-009378-01-1
Dermal	Rabbit (NZW)	M F	2000 < 2000	2000 2000	> 2000 > 2000	[redacted] 1972 M-015282-01-1
Dermal	Rat (Wistar)	M F	< 100 < 100	> 5000 > 5000	> 5000 > 5000	[redacted] J., 1977 M-010260-01-1
Inhalation (aerosol, 4h)	Rat Sprague-Dawley)	M F	199 199	550 550	585 433	[redacted] 1987 M-009885-01-1
Skin irritation	Rabbit (NZW)	n.s.	Not irritating			[redacted] 1970 M-015323-01-1
Eye irritation	Rabbit (NZW)	n.s.	Not irritating			[redacted] 1970 M-015323-01-1
Skin sensitisation M&K method	Guinea pig	M	Not sensitising			[redacted] 1984 M-008929-01-1
Skin sensitisation Buehler method	Guinea pig (Hartley Dunkin)	M	Not sensitising			[redacted]., 1988 M-010126-01-1
In vitro 3T3 NRU phototoxicity test	BALB/c 3T3 cells		Not phototoxic			[redacted] M., 2015 M-536022-01-1

*: New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

1) Study was conducted to evaluate the AChE-inhibition

M: male F: female n.s.: not stated

#: NSD: no-symptoms dose ##: LLD: lowest lethal dose

Comparison of the study results / derived LD_{50} and LC_{50} values with CLP criteria according to REGULATION (EC) No 1272/2008[7]

According to the actual CLP criteria taking into account the derived oral LD_{50} of 19 mg/kg bw/day, and the LC_{50} of 433 mg/m^3 (for females) methiocarb is considered to be fatal after acute oral and inhalation exposure. No classification is triggered for acute dermal exposure, skin and eye irritation, and skin sensitisation.

Classification/labelling regarding acute toxicity, irritation and sensitisation for methiocarb:

- Regulation (EC) No 1272/2008 (CLP):	Acute Toxicity Category 2 H300 (Fatal if swallowed) H330 (Fatal if inhaled)

Comparison of the study results with CLP criteria for Specific Target Organ Toxicity – Single exposure (STOT-SE) according to REGULATION (EC) No 1272/2008[7]

According to the CLP criteria a classification for STOT-SE need to be considered if the substance causes non-lethal target organ toxicity after a single exposure (i.e. significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed and not covered by acute toxicity, skin corrosion / irritation, eye damage / irritation, respiratory or skin sensitization, genotoxicity, carcinogenicity and reproductive toxicity) should be taken into consideration.

Based on the results of the studies with acute exposure of methiocarb, there were no significant toxic effects observed at non-lethal dose levels. Cholinesterase inhibition is the toxic principle of acute toxicity of methiocarb and is therefore already addressed by the classification for acute toxicity. Thus, classification of methiocarb for STOT-SE category 1 or 2 is not warranted.
There is also no indication of transient effects like respiratory tract irritation (RTI) and narcotic effects (NE) after single exposure to methiocarb. Therefore, classification of methiocarb for STOT-SE category 3 is not warranted.

Classification/labelling regarding STOT-SE for methiocarb:
- Regulation (EC) No 1272/2008 (CLP): none

CA 5.2.1 Oral

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.2.2 Dermal

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.2.3 Inhalation

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

[7] REGULATION (EC) No 1272/2008 of the European parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006

CA 5.2.4 Skin irritation

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.2.5 Eye irritation

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.2.6 Skin sensitization

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.2.7 Phototoxicity

According to the new data requirements (COMMISSION REGULATION (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1 03.4.2013) (1), the conduct of a phototoxicity study is required under certain conditions. As the ultraviolet/visible molar extinction coefficient of methiocarb of 182 L x mol^{-1} x cm^{-1} at 290 nm (determined in the photo degradation study on methiocarb by [redacted] 1991, M-013352-01-2) exceeds the trigger of 10 L x mol^{-1} x cm^{-1} for the conduct of a phototoxicity study, a cytotoxicity assay *in vitro* with BALB/c 3T3 cells has been performed.

Report: KCA 5.2.7/01 [redacted] 1; 2015; M-536022-01-1
Title: Methiocarb, technical: Cytotoxicity assay in vitro with BALB/c 3T3 cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Report No.: 1690602
Document No.: M-536022-01-1
Guideline(s): OECD 432; Commission Regulation (EC) No. 440/2008, B 41; Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on Photosafety testing, EMEA/CPMP/SWP/398/01
Guideline deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test materials:

Name:	Methiocarb technical
Synonyms:	AEF082618, technical
Description:	White solid
Lot/Batch no:	NLL 9134-1-5
Purity:	98.2% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2016-06-23

2. Vehicle and/or positive control: vehicle: dimethyl sulfoxide (DMSO), 1% (v/v) in Earle´s Balanced Salt Solution (EBBS)
Solvent control: EBSS containing 1% (v/v) DMSO
Positive control: chlorpromazine (Sigma) dissolved in EBSS

3. Test system:

Culture medium: Dulbecco´s Minimal Essential Medium (DMEM) supplemented with 10% (v/v) Newborn calf Serum (NCS).

Cell cultures:

BALB/c 3T3 cell clone 31 (supplied by Dr. [redacted], [redacted], [redacted], Germany).
Large stocks (Master Cell Stock) of the BALB/c 3T3 31 cell line are stored in liquid nitrogen in the cell bank of [redacted] GmbH. A working cell stock is produced by multiplying from the master cell stock. Thawed stock cultures were propagated at 37 ± 1.5 °C in 75 cm^2 plastic flasks. Seeding was done with about 1 x 10^6 cells per flask in 15 mL DMEM, supplemented with 10 % NCS. Cells were sub-cultured twice weekly. The cell cultures were incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

B. Study design and methods

1. Treatment:

Dose:

Test item	+/–UV	Final concentrations in µg/mL
Methiocarb	+/–	0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125
Positive control	–	6.25, 12.5, 25, 37.5, 50, 75, 100, 200
	+	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0
Solvent control	+/–	EBSS containing 1 % (v/v) DMSO

The test item methiocarb was dissolved in DMSO. The final concentration of the solvent in EBSS was 1 % (v/v). Due to limited solubility the highest applied concentration of the test item was 125 µg/mL in accordance with the OECD Guideline.

Solar simulator:

Irradiation was performed with a Dr. Hönle Sol 500 solar simulator. The filter H1 was used to keep the UVB irradiation as low as possible. The produced wavelength of the solar simulator with the filter was > 320 nm. Due to the heterogeneous distribution of irradiation intensity the UVA intensity was measured at the complete area with a UV-meter. The homogeneous area was marked and the cultures were irradiated in this area. The solar simulator was switched on about 30 minutes prior to the start of experiment. The absorption spectrum of the test item was determined in the range from 270-800 nm. The test item showed absorption maxima at 284.9 nm.

Seeding of cultures:

2 x 10^4 cells per well were seeded in 100 µL culture medium in two 96-well plates

Replicates:

2 (one for exposure to irradiation, one for treatment in the dark)

Treatment & irradiation:

24 h after seeding the cultures were washed with EBSS. 100 µL of the dissolved test item were added/well and the plates were pre-incubated for 1 hour in the dark. Afterwards one plate was irradiated at 1.65 mW/cm^2 (~5 J/cm^2) for 50 min at 25 - 27 °C, the other plate was stored for 50 min at 26°C in the dark. The test item was removed and both plates

	were washed twice with EBSS. Fresh culture medium was added and the plates were incubated overnight at 37 ± 1.5 °C and 7.5 ± 0.5 % CO_2.
Cytotoxicity determination:	For measurement of Neutral Red uptake the medium was removed and 0.1 mL serum-free medium containing 50 µg Neutral Red / mL were added to each well. The plates were incubated for another 3 hours at 37°, before the medium was removed completely and the cells were washed with EBSS. For extraction of the dye 0.15 mL of a solution of 49% (v/v) deionized water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well. After approximately 10 minutes at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.
Number of measurements:	Methiocarb and positive control: 6 times per concentration Solvent control: 12 times

2. Evaluation

The mean absorption (OD_{540}) value per concentration was calculated. The ED_{50} values were determined by curve fitting by software, except for the non-irradiated plate, which was calculated as follows:

$$ED_{50} = Conc_{>50} - \frac{(Conc_{>50} - Conc_{<50}) \times (\%_{>50} - 50)}{(\%_{>50} - \%_{<50})}.$$

The Photo-irritancy factor (PIF), as well as the Mean Phototoxic effect (MPE) was calculated according to OECD guideline 432.

Evaluation criteria:	PIF < 2 or MPE < 0.1	=> no phototoxic potential
	PIF > 2 and < 5 or MPE > 0.1 and < 0.15	=> probable phototoxic potential
	PIF > 5 or MPE > 0.15	=>phototoxic potential

II. Results and discussion

In the range finding experiment (RFE) cytotoxic effects were observed with and without irradiation. Without irradiation only the highest tested concentration of 125 µg/ml showed a cytotoxic potential. With irradiation cytotoxicity occurred already starting with the concentration of 15.6 µg/mL. The ED_{50} value for the irradiated test item was 21.3 µg/mL. For the non-irradiated test item the ED_{50} value could not be calculated, since the viability values did not decrease below 50%. Because there was no ED_{50} value for the non-irradiated test item, a Photo-Irritancy-Factor (PIF) could not be calculated. The MPE value (Mean Phototoxic Effect) was determined as 0.170. According to the results of the RFE the test item seems to have a phototoxic potential. However, this is in contradiction to the fact that methiocarb does not absorb UVA in the wavelength range of > 320 nm that is used in the experiment.

In the main experiment (ME) no cytotoxic effects were observed after exposure of the cells to methiocarb, neither in the presence nor in the absence of irradiation with artificial sunlight. Therefore, ED_{50}-values and PIF could not be calculated. The resulting MPE-value was –0.021.

Based on this result, methiocarb does not possess any phototoxic potential.

Due to the different results obtained in the range-finding and main experiment a confirmatory experiment (CE) was conducted. In this experiment cytotoxic effects occurred after exposure of the cells to the highest test item concentration of 125 µg/mL in the presence as well as in the absence of irradiation with artificial sunlight. Since the viability values were not reduced below 50% with irradiation, the ED_{50}-value for the irradiated plate and following consequently also a PIF could not be calculated. The resulting MPE value was −0.025. This result confirmed the result of the main experiment that methiocarb is not phototoxic.

The mean of solvent control values of the irradiated versus the non-irradiated group met the acceptance criteria. The positive control chlorpromazine induced phototoxicity in the expected range in the presence of irradiation.

The result of the RFE differs clearly from the results of the ME and CE. Based on the clear negative results of the ME and CE, as well as the fact that methiocarb did not absorb UVA in the tested wavelength range, a phototoxic potential of methiocarb is ruled out. A possible explanation for the inconsistent result of the RFE compared with those of the ME and of the CE may be that the test item was contaminated after weighing and prior to application to the cells, and that the possible contaminating substance possesses a phototoxic potential.

The results are summarised in the tables below.

Table 5.2.7/01- 1: OD_{540} values in the Neutral Red assay of the range finding experiment (RFE)

OD_{540} with artificial sunlight				OD_{540} without artificial sunlight			
Concentration [µg/mL]	Mean	SD	% of solvent control	Concentration [µg/mL]	Mean	SD	% of solvent control
Treatment with methiocarb							
Solvent control	0.8040*	0.0557	100.00	Solvent control	0.8966*	0.0384	100.00
0.98	0.8243	0.0336	102.52	0.98	0.8780	0.0448	97.92
1.95	0.7323	0.0414	91.08	1.95	0.8735	0.0651	97.42
3.91	0.6587	0.0753	81.92	3.91	0.8876	0.0735	98.99
7.81	0.6068	0.0417	75.47	7.81	0.8601	0.0582	95.92
15.6	0.4919	0.0602	61.18	15.6	0.7937	0.0439	88.52
31.3	0.3506	0.0332	43.61	31.3	0.7200	0.0483	80.31
62.5	0.1962	0.0398	24.40	62.5	0.6613	0.0269	73.76
125	0.1672	0.0592	20.79	125	0.5397	0.0581	60.19
Treatment with positive control chlorpromazine							
Solvent control	0.7128*	0.0410	100.00	Solvent control	0.7736*	0.1156	100.00
0.125	0.5884	0.0282	82.55	6.25	0.7622	0.0933	98.53
0.250	0.1795	0.0439	25.19	12.50	0.5981	0.0925	77.32
0.500	0.0897	0.0105	12.59	25.00	0.1151	0.0139	14.87
0.750	0.1339	0.0277	18.78	37.50	0.0694	0.0086	8.97
1.000	0.0870	0.0048	12.21	50.55	0.0705	0.0073	9.12
1.500	0.0792	0.0038	11.11	75.00	0.0692	0.0053	8.95
2.000	0.0809	0.0049	11.35	100.00	0.0705	0.0072	9.11
4.000	0.0820	0.0044	11.50	200.00	0.0704	0.0086	9.10

*: mean $OD_{540\ nm}$ out of 12 wells

Table 5.2.7/01- 2: OD_{540} values in the Neutral Red assay of the main experiment (ME)

OD_{540} with artificial sunlight				OD_{540} without artificial sunlight			
Concentration [µg/mL]	Mean	SD	% of solvent control	Concentration [µg/mL]	Mean	SD	% of solvent control
Treatment with methiocarb							
Solvent control	1.116*	0.072	100.0	Solvent control	1.280*	0.109	100.0
0.98	1.197	0.097	107.2	0.98	1.263	0.122	98.7
1.95	1.187	0.091	106.3	1.95	1.273	0.096	99.5
3.91	1.166	0.077	104.5	3.91	1.206	0.107	94.2
7.81	1.110	0.058	99.4	7.81	1.209	0.047	94.5
15.6	1.088	0.064	97.5	15.6	1.232	0.118	96.3
31.3	1.055	0.062	94.5	31.3	1.233	0.133	96.4
62.5	0.957	0.088	85.7	62.5	1.089	0.067	85.1
125	0.816	0.140	73.1	125	1.065	0.088	83.2
Treatment with positive control chlorpromazine							
Solvent control	1.1391*	0.0861	100.00	Solvent control	1.1686*	0.1141	100.00
0.125	1.1911	0.0920	104.56	6.25	1.1846	0.0971	101.36
0.250	1.1490	0.1155	100.86	12.50	0.6616	0.1375	56.62
0.500	0.9489	0.1016	83.30	25.00	0.1042	0.0129	8.92
0.750	0.6980	0.1498	61.27	37.50	0.0682	0.0053	5.84
1.000	0.2497	0.0488	21.92	50.55	0.0652	0.0040	5.58
1.500	0.0967	0.0225	8.48	75.00	0.0610	0.0021	5.22
2.000	0.0935	0.0170	8.20	100.00	0.0590	0.0024	5.05
4.000	0.1075	0.0114	9.44	200.00	0.0640	0.0081	5.48

*: mean $OD_{540\ nm}$ out of 12 wells

Table 5.2.7/01- 3: OD_{540} values in the Neutral Red assay of the confirmatory experiment (CE)

Concentration [µg/mL]	OD_{540} with artificial sunlight Mean	SD	% of solvent control	Concentration [µg/mL]	OD_{540} with artificial sunlight Mean	SD	% of solvent control
Treatment with methiocarb							
Solvent control	1.1780*	0.0922	100.00	Solvent control	1.2526*	0.1163	100.00
0.98	1.2689	0.0489	107.72	0.98	1.2997	0.0561	103.76
1.95	1.2301	0.0533	104.42	1.95	1.2358	0.0857	98.66
3.91	1.2070	0.0396	102.46	3.91	1.2367	0.0749	98.73
7.81	1.1958	0.0638	101.51	7.81	1.1964	0.0922	95.51
15.6	1.1440	0.0849	97.12	15.6	1.1659	0.1062	93.08
31.3	1.1080	0.1128	94.06	31.3	1.1583	0.1256	92.47
62.5	1.0488	0.1054	89.03	62.5	1.0445	0.1036	83.39
125	0.5967	0.0875	50.65	125	0.5859	0.0533	46.77
Treatment with positive control chlorpromazine							
Solvent control	1.0668*	0.1001	100.00	Solvent control	1.2844*	0.0690	100.00
0.125	1.0681	0.0905	100.12	6.25	1.2736	0.0386	99.16
0.250	1.0640	0.0637	99.74	12.50	0.1383	0.0296	10.77
0.500	0.9921	0.0649	92.99	25.00	0.0798	0.0137	6.22
0.750	0.8898	0.0662	83.41	37.50	0.0818	0.0142	6.37
1.000	0.5537	0.0869	51.90	50.55	0.0813	0.0217	6.33
1.500	0.1545	0.0521	14.48	75.00	0.0848	0.0159	6.60
2.000	0.0898	0.0083	8.42	100.00	0.0822	0.0151	6.40
4.000	0.0948	0.0098	8.89	200.00	0.0804	0.0110	6.26

*: mean $OD_{540\,nm}$ out of 12 wells

Table 5.2.7/01- 4: Summary of the results of the Neutral Red assay

	Substance	ED_{50} (+UV) [µg/mL]	ED_{50} (–UV) [µg/mL]	PIF	MPE	% viability of solvent control of irradiated vs. non-irradiated plate
Range finding experiment	Methiocarb	21.3	nd	nd	0.170	89.7
	Positive control	0.18	18.10	98.32	0.768	92.1
Main experiment	Methiocarb	nd	nd	nd	–0.021	87.2
	Positive control	0.80	13.19	16.47	0.467	97.5
Confirmatory experiment	Methiocarb	nd	119.49	nd	–0.025	94.0
	Positive control	1.03	10.32	10.08	0.434	83.1

PIF: Photo-Irritancy-Factor MPE: Mean Phototoxic Effect
nd: could not be determined, since the viability of the cells was not reduced below 50% with and without irradiation

III. Conclusion

Based on the study results methiocarb does not possess any phototoxic potential.

CA 5.3 Short-term toxicity

Summary of short-term toxicity

Short-term dietary toxicity studies have been conducted in rats and dogs. Studies with dermal or inhalation exposure were conducted in rabbits and rats, respectively.

It has to be noted that most of the short-term toxicity studies with methiocarb were pre-GLP and not according to current guidelines. However, the 13-week rat study (M-088469-01-1) and the 3-month dog study (M-030181-01-1) are according to GLP and according to currently valid guidelines. These two studies cover all the required parameters for the assessment of short-term toxicity. They also cover the parameters that are required for the assessment of neurotoxicity according to OECD guideline 424, except that there was no automatic assessment of motor activity in the rat study, and no *in situ* fixation for histopathology, and no histopathological examination of some PNS tissues (dorsal root ganglia, dorsal and ventral root fibres proximal tibial nerve (at knee), tibial nerve calf muscle branches, skeletal muscle (calf muscle)). Therefore, the submitted data package is considered to be adequate for the evaluation of short-term toxicity, as well as the neurotoxic potential of methiocarb.
A summary of the study results are presented in the following.

The target effects are cholinesterase inhibition and reduction of body weight gain.
In rats, the NOAEL in a 16-week dietary study was 10 ppm (equivalent to 1 mg/kg bw/day) based on possible toxicologically significant reduction in cholinesterase in the submaxillary glands at 50 ppm. Inhibition of plasma cholinesterase activity was noted at the 50 ppm dose level but changes in erythrocyte and brain cholinesterase were not considered toxicologically significant. In a 90-day dietary toxicity study in rats, the NOAEL was 100 ppm (equivalent to 7.34 mg/kg bw/day in males and 10 mg/kg bw/day in females) based on reduction in body weight gain (14%) at 300 ppm. This finding was associated with sporadic findings in clinical chemistry and changes in erythrocyte parameters of uncertain toxicological significance at 900 ppm. Biologically significant cholinesterase inhibition was not observed at dose levels of ≤ 900 ppm (67.6 mg/kg bw/day in males or 90.7 mg/kg bw/day in females) in this study. This observation of low cholinesterase inhibition was noted to be inconsistent with findings in other dietary studies in rats. However, it has to be considered that rats were not fasted prior to blood sampling for cholinesterase assays.

In dietary toxicity studies in dogs, the NOAEL in a 3-month dietary study with investigation of neurological function was 50 ppm (1.33 mg/kg bw/day) based on increased incidence of vomiting, reduced body weight gain, erythrocyte cholinesterase inhibition and retinal cholinesterase inhibition at 250 ppm (5.9 mg/kg bw/day). Brain cholinesterase inhibition was not observed at the highest test dose and there was no evidence of functional impairment in a functional observation battery of tests.

In a two year feeding study beagle dogs the derived NOAEL was 60 ppm (2.2 mg/kg bw/day) based on impaired feed consumption, vomiting and clinical signs including mild weakness in the hind limbs, trembling and slightly reduced alertness suggestive of significant cholinesterase effects at 240 ppm (8.6 mg/kg bw/day).

In a sub-acute dermal toxicity study in the New Zealand White rabbit, the NOAEL was 150 mg/kg bw/day based on reduction in food consumption at the 375 mg/kg bw/day dose level. Reductions in food consumption were associated with reductions in plasma cholinesterase activity at dose levels > 150 mg/kg bw/day. In a further study, the only observed effects at the 500 mg/kg bw/day dose in New Zealand White rabbits were reduction in body weight, reduction in food consumption and mild behavioural changes. Reductions in plasma cholinesterase at the 500 mg/kg bw/day dose level were very slight and not of biological significance.

The NOEL in the sub-acute inhalation study in the rat was 6 mg/ m^3 based on significant reduction in plasma and brain cholinesterase activities in rats at dose levels of ≥ 23 mg/m^3.

The lowest relevant NOAEL for short term toxicity is 1.33 mg/kg bw/day based on cholinergic signs, reduction in body weight gain, and erythrocyte cholinesterase inhibition and retinal cholinesterase inhibition observed in the 90-day dog study.

Table 5.3- 1: Summary of short-term toxicity studies*

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
Rat 4-week, oral (gavage) 0-3-10 mg/kg bw/day	M F	3 3	10 10	↓ of plasma, erythrocyte and brain AChE activity; cholinergic symptoms, No accumulation of AChE inhibition	[redacted], 1973 M-009378-01-1
Rat 4-week,oral (gavage) 0-0.5-2.0 mg/kg bw/day	F	0.5	2	Tremors (during the first days only) ↓ >25% of plasma and erythrocyte AChE activity (during the first week only)	[redacted], 1981 M-009348-01-1
Dog, 29-day, oral (capsule) 0-0.05-0.5 mg/kg bw/day	M F	0.05 0.05	0.5 0.5	Cholinergic signs, slight ↓ of erythrocyte AChE activity (2 h post application)	[redacted], 1981 M-009577-01-1
Rat 16-week, oral (diet) 0-5-10-50 ppm	M F	1 1 (10 ppm)	(50 ppm)	↓ of AChE activity in submaxillary glands with significant ↓ of plasma AChE activity (both sexes)	[redacted], 1962 M-016133-01-1
Rat, 13-week + 4 weeks recovery, oral (diet) 0-100-300-900 ppm 0-7.34/10.1-22.72/31.1-67.59-90.74 mg/kg bw/day (m/f)	M F	7.34 10.1 (100 ppm)	22.72 31.1 300 ppm	≥ 300 ppm: stat. sign. ↓ glucose (m), ↓ urine volume, ↑ absolute adrenal weights (+ due to lower bw	[redacted], C., 2001 M-088469-01-1
Dog, 3-month, oral (diet) 0-10-50-250 ppm 0-0.32/0.25-1.32/1.33-6.46/5.91 mg/kg bw/day	M F	1.32 1.33 (50 ppm)	6.46 5.91 (250 ppm)	250 ppm: vomiting, trend of ↓ food consumption, marked ↓ bw gain, non-significant ↑ N-DEM (m), stat. sign. ↓ erythrocyte AChE activity	[redacted], 2000 M-030181-01-1
Dog, 2-year, oral (diet) 0-15 (0.4**-60-240 ppm 0-0.2-2.2-8.6 mg/kg bw/day	M F	2.2 2.2 (60 ppm)	8.6 8.6 (240 ppm)	Cholinergic effects, vomiting, ↓ food consumption associated with ↓ plasma AChE activity	[redacted], 1980 M-010201-01-1

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
Rabbit 21-day.dermal 0-60-150-375 mg/kg bw/day	M F	150	375	stat. sign. ↓ plasma AChE activity (m), ↓ food consumption No effects on erythrocyte and brain AChE activity	[redacted] 1988 M-009558-01-1
Rabbit 21-day, dermal 0-500 mg/kg bw/day	M F	< 500	500	↓ Food consumption, ↓ bw (f), stat. sign. ↓ plasma AChE activity (not biol. relevant) No effects on erythrocyte and brain AChE activity	[redacted]., 1989 M-009552-01-1
Rat 3-week, inhalation 0-6-23-96 mg/m^3 (6h/day, 5 days/week)	M F	6 mg/m^3	23 mg/m^3	stat. sign. ↓ plasma AChE activity (m), moderate ↓ plasma AChE activity (f), moderate ↓ brain AChE activity (m)	[redacted] M-009879-01-1

M: male F: female ↑: increase(d) ↓: decrease(d) bw: body weight
Stat: statistically sign.: significant(ly) AChE: acetylcholine esterase
N-DEM: N-demethylase (m): males (f): females
*: New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.
**: The lowest test dose was reduced from 15 to 5 ppm after 15 days due to the depression of plasma AChE activity at 15 ppm.

Comparison of the study results with CLP criteria for Specific Target Organ Toxicity – Repeated Exposure (STOT-RE) according to REGULATION (EC) No 1272/2008[7]

According to CLP criteria a classification for STOT-RE need to be considered if the substance causes non-lethal target organ toxicity after a repeated exposure (i.e. significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed and not covered by acute toxicity, skin corrosion / irritation, eye damage / irritation, respiratory or skin sensitization, genotoxicity, carcinogenicity and reproductive toxicity).

Based on the results of the studies with repeated exposure of methiocarb, there were no significant toxic effects observed at non-lethal dose levels. Thus, classification of methiocarb for STOT-RE category 1 or 2 is not warranted.

Classification/labelling regarding STOT-RE for methiocarb:
- Regulation (EC) No 1272/2008 (CLP): none

CA 5.3.1 Oral 28-day study

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.3.2 Oral 90-day study

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.3.3 Other routes

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.4 Genotoxicity testing

Summary of genotoxicity testing

Methiocarb was tested in *in vitro* and *in vivo* genotoxicity studies. The *in vitro* genotoxicity studies were except for one Ames test, a Pol A-test in *E. coli* and a sister chromatid exchange assay (SCE) according to GLP and OECD guidelines that were valid at the time of study conduct. However, the SCE was comparable to the OECD guideline # 479 (1986), which was valid at the time of study conduct.

Methiocarb was not mutagenic in studies *in vitro* including the bacteria/mammalian microsome assay (Ames test) for point mutations in histidine auxotrophic strains of *Salmonella typhimurium*; the HGPRT assay for forward mutations in cultured Chinese hamster ovary (CHO) cells; the Pol test in *Escherichia coli* for the potential to induce chromosomal damage; the unscheduled DNA synthesis assay rat primary hepatocytes; and the Sister chromatid exchange (SCE) assay in CHO cells.

In addition to the already existing *in vitro* studies a new bacterial reverse mutation assay (Ames test) was conducted (M-524333-01-1), since in the available studies only four of the five test strains recommended by the guideline were used. The results of the new test confirmed the results of the existing bacterial reverse mutation assays. No increase in the mutation frequency was observed in any of the tested strains.

Methiocarb was clastogenic at high concentrations in the *in vitro* chromosomal aberration assay in CHO cells that was according to GLP and guideline conform at the time of conduct. Although this test would not fulfill the current guideline requirements it was not repeated due to the positive result. The positive result was not confirmed in a micronucleus test *in vivo*. It has to be noted that at the time of study conduct of the available *in vivo* micronucleus test (MNT) (i.e. 1979), no OECD guideline was in place. But the *in vivo* MNT was in general accordance to the previous OECD guideline #474 of 1983. Compared to the OECD guideline # 474 (1997[8]), the assay has the deficiency is that the number of erythrocytes investigated was 1000 instead of the 2000 recommended. Therefore, the available *in vivo* MNT was reassessed, taken into account a factor of two for counting the micronucleated PCEs. Due to the fact that the number of animals per group was twice as much as recommended (i.e. 10/groups instead of 5/group) by the current OECD 474 (2014) the resulting total number of PCEs evaluated per test substance group (i.e. 20,000) is identical to the requirements of OECD 474 (2014).

The reassessment of the *in vivo* MNT is presented in section CA 5.4.2. Based on the new evaluation it confirmed that methiocarb does not have a clastogenic potential in the *in vivo* MNT.

Furthermore, there was no evidence of genotoxicity in other *in vitro* and *in vivo* studies sensitive to potential clastogenic compounds. Carcinogenicity was not observed in chronic studies in the rat and mouse.

In addition, methiocarb was not mutagenic in the *in vivo* mouse dominant lethal test for germ cell chromosomal and dominant gene mutations.

Overall, based on the evaluation of the results of all *in vitro* and *in vivo* genotoxicity studies methiocarb is considered to have no genotoxic / mutagenic potential.

[8] It has to be noted that the currently valid OECD guideline 474 (2014) 2014 recommends 4000 PCEs to be evaluated. However, the reason for increasing the PCEs to be evaluated from 2000 to 4000 is just to increase the statistical power. Since fort he re-evaluation no statistics were applied, a factor of two was considered to be sufficient.

Table 5.4- 1: Summary of genotoxicity studies*

Study	Test system	Concentration / Dose	Result	Reference
In vitro				
Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA1535, TA1537 +/–S9 mix	Up to 2500 µg/plate	negative	[redacted]., 1978 M-009542-01-1
Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA1535, TA1537 +/–S9 mix	Up to 12,500 µg/plate	negative	[redacted]., 1986 M-010145-01-1
Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA102 +/–S9 mix	Up to 5000 µg/plate	negative	[redacted] 2015 M-524333-01-1
Mammalian cell gene mutation test (HGPRT)	Chinese hamster ovary (CHO) cells	Up to 30 µg/mL (with S9) Up to 60 µg/mL (without S9)	negative	[redacted]., 1989 M-008916-01-1
Pol A-Test	*E. coli* +/–S9 mix	Up to 10,000 µg/mL	negative	[redacted] 1983 M-010151-01-1
Unscheduled DNA synthesis (UDS) assay	Primary rat hepatocytes	Up to 60 µg/mL (without S9)	negative	[redacted]., 1988 M-009902-01-1
Mammalian chromosome aberration test	Chinese hamster ovary (CHO) cells	Up to 50.8 µg/mL (+S9) up to 197 µg/mL (–S9)	positive (+/–S9)	[redacted]., 1990 M-010190-01-1
Sister chromatid exchange (SCE)	Chinese hamster ovary (CHO) cells	Up to 40 µg/mL	negative	[redacted]., 1986 M-010134-01-1
In vivo				
Micronucleus test**	NMRI mouse bone marrow cells	0-5-10-20 mg/kg bw	Negative	[redacted].; 1979; M-009544-01-1 supplemental HCD: M-536382-01-1
Dominant lethal test	NMRI mouse	6 mg/kg bw	negative	[redacted]., 1979 M-009533-01-1

*: New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.
** New evaluation in comparison with OECD guideline 474, taken into account a factor of 2 for evaluation of PCEs. A new study summary is also provided.
HCD = Historical control data

Comparison of the study results with CLP criteria according to REGULATION (EC) No 1272/2008[7]
According to REGULATION (EC) No 1272/2008 a substance is considered to be a germ cell mutagen and also a mutagen if it induces heritable mutations in the germ cells of humans, or causes positive test results in *in vivo* germ cell and / or somatic cell mutagenicity tests in mammals. In addition, results from *in vitro* mutagenicity tests in mammalian cells have to be taken into account.

Based on the overall result of the *in vitro* and *in vivo* mutagenicity tests with methiocarb conducted in bacteria and somatic cells it is concluded that methiocarb does not possess a gentotoxic / mutagenic potential.

In addition, the dominant lethal test in mice was also negative. Thus, methiocarb has no potential to cause mutations in germ cells.

Thus, according to the actual CLP criteria no classification is triggered for methiocarb regarding mutagenicity / genotoxicity.

Classification/labelling regarding mutagenicity for methiocarb:
- Regulation (EC) No 1272/2008 (CLP): none

CA 5.4.1 In vitro studies

In addition to the genotoxicity studies already contained in the Monograph and Baseline Dossier of methiocarb a new bacterial reverse mutation assay was conducted because in the available assays only four of the recommended five test strains were investigated.

Reference

Report:	KCA 5.4.1/08[redacted], 2015, M-524333-01-1
Title:	Methiocarb technical: Salmonella typhimurium reverse mutation assay
Report No.:	1690601
Document No.:	M-524333-01-1
Guideline(s):	OECD 471; Commission Regulation (EC) No 440/2008, B13/14; US-EPA 712-C-98-247, OPPTS 870.5100
Guideline deviation(s):	none
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:

Description:	Methiocarb technical
Synonym:	AE F082618, technical
Lot/Batch no:	NIL 9134-1-5
Purity:	98.2 % (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2015-07-08

2. Vehicle and/or positive control: DMSO

Sodium azide (Na-azide), 4-nitro-o-phenylene diamine (4-NOPD), methyl methane sulfonate (MMS), 2-aminoanthracene (2-AA)

3. Test system: *Salmonella typhimurium* strains TA1535, TA1537, TA100, TA98, TA102

Metabolic activation: S9 mix

B. Study design and methods

Dose:

Experiment I (pre-experiment), plate incorporation:
3-10-33-100-333-1000-2500-5000 µg/plate
Experiment II, pre-incubation):
10-33-100-333-1000-2500-5000 µg/plate
positive controls:
without metabolic activation:

	Na-azide:	10 µg/plate (TA 1535, TA 100)
	4-NOPD:	10 µg/plate (TA 1537, TA 98)
	MMS:	2.0 µL/plate (TA 102)
	with metabolic activation:	
	2-AA:	2.5 (TA 1535, TA 1537, TA 98, TA 100)
		10.0 µg/plate (TA 102)
Application volume:	0.1 mL	
Incubation time /temperature:	Pre-incubation: 60 minutes, 37°C	
	48 hours, 37°C	

II. Results and discussion

The potential of methiocarb to induce gene mutations was investigated according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) in two independent experiments both with and without liver microsomal activation (S9 mix).
The plates incubated with the test item showed normal background growth up to the highest concentration with and without metabolic activation in all strains used.
The test item precipitated in the overlay agar in the test tubes from 1000 to 5000 µg/plate. Precipitation of the test item in the overlay agar on the incubated agar plates was observed in experiment I from 2500 to 5000 µg/plate without S9 mix and from 1000 to 5000 with S9 mix and in experiment II from 1000 to 5000 µg/plate with and without S9 mix. The undissolved particles had no influence on the data recording.

In experiment I, toxic effects, evident as a reduction in the number of revertants were observed at 10 µg/plate in strain TA1535 without S9 mix and at 5000 µg/plate in strain TA1537 with S9 mix.
In experiment II, toxic effects were observed at 5000 µg/plate in strain TA1537 without S9 mix and at 333 mg/plate in strain TA100 with S9 mix. The minor reduction of revertants observed in strains TA 1535 and TA 100 is not considered as a toxic effect since it is based on biological fluctuations.
No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with methiocarb technical at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.
In experiment II, with metabolic activation, the number of colonies did not quite reach the lower limit of the historical control data in the negative control of strain TA 1535. Since this deviation is rather small, this effect is judged to be based upon statistical fluctuations and has no detrimental impact on the outcome of the study.

Table 5.4.1/08- 1: Summary of results of the pre-experiment

Metabolic activation	Test Group	Revertant colony counts (Mean ± SD)					
		(µg/plate)	TA1535	TA1537	TA98	TA100	TA102
Pre-experiment – Experiment I							
Without activation	DMSO		19 ± 3	11 ± 3	18 ± 4	168 ± 14	473 ± 51
	Untreated		10 ± 3	14 ± 2	28 ± 5	169 ± 13	520 ± 16
	MTC	3	13 ± 2	13 ± 2	24 ± 4	193 ± 9	484 ± 30
		10	8 ± 1	9 ± 1	21 ± 5	190 ± 15	505 ± 18
		33	14 ± 3	11 ± 3	20 ± 7	193 ± 19	564 ± 24
		100	10 ± 4	14 ± 7	32 ± 5	190 ± 19	493 ± 27
		333	12 ± 2	19 ± 6	30 ± 6	179 ± 19	436 ± 41
		1000	14 ± 5	10 ± 3	31 ± 6	176 ± 8	507 ± 19
		2500	11 ± 3 P M	6 ± 2 P M	21 ± 1 P M	182 ± 5 P M	483 ± 21 P M
		5000	11 ± 4 P M	8 ± 2 P M	21 ± 1 P M	168 ± 7 P M	488 ± 29 P M
	NaN_3	10	1055 ± 138			2000 ± 211	
	4-NOPD	10			356 ± 5		
	4-NOPD	50		87 ± 3			
	MMS	2 µL					5838 ± 973
With activation	DMSO		16 ± 4	18 ± 4	36 ± 6	172 ± 27	610 ± 12
	Untreated		9 ± 3	15 ± 2	42 ± 1	162 ± 21	615 ± 9
	MTC	3	12 ± 3	14 ± 7	31 ± 2	189 ± 5	644 ± 58
		10	13 ± 7	14 ± 6	35 ± 12	179 ± 9	679 ± 64
		33	10 ± 4	15 ± 2	31 ± 6	168 ± 3	623 ± 111
		100	13 ± 6	14 ± 8	34 ± 8	166 ± 12	678 ± 55
		333	12 ± 7	17 ± 9	32 ± 3	174 ± 3	490 ± 50
		1000	14 ± 3 P M	11 ± 1 P M	24 ± 3 P M	182 ± 6 P M	520 ± 30 P M
		2500	11 ± 4 M P	9 ± 3 M P	23 ± 2 M P	168 ± 2 M P	483 ± 26 M P
		5000	8 ± 3 P M	8 ± 1 P M	18 ± 5 P M	166 ± 10 P M	525 ± 11 M P
	2-AA	2.5	490 ± 7	176 ± 21	4896 ± 130	4284 ± 259	
	2-AA	10.0					1866 ± 115

NaN_3 = sodium azide; 2-AA = 2-aminoanthracene, MMS = methyl methane sulfonate, 4-NOPD = 4-nitro-o-phenylene-diamine; P = Precipitate, M = Manual count

Table 5.4.1/08- 2: Summary of the results of the main experiment

Metabolic activation	Test Group	Dose (µg/plate)	Revertant colony counts (Mean ± SD) TA1535	TA1537	TA98	TA100	TA102
Experiment II							
Without activation	DMSO		11 ± 1	11 ± 1	21 ± 8	114 ± 18	491 ± 15
	Untreated		17 ± 2	13 ± 1	27 ± 3	117 ± 12	487 ± 21
	MTC	10	13 ± 1	8 ± 2	28 ± 5	131 ± 9	457 ± 18
		33	10 ± 5	12 ± 2	25 ± 2	129 ± 13	475 ± 32
		100	11 ± 4	11 ± 2	30 ± 4	130 ± 14	494 ± 72
		333	10 ± 3	11 ± 3	22 ± 1	67 ± 6	464 ± 25
		1000	13 ± 1 P	9 ± 1 P	18 ± 3 P	122 ± 13 P	510 ± 32 P
		2500	10 ± 3 P M	5 ± 2 P M	13 ± 4 P M	136 ± 7 P M	334 ± 10 P M
		5000	12 ± 3 P M	5 ± 2 P M	13 ± 4 P M	134 ± 7 P M	273 ± 13 P M
	NaN_3	10	1067 ± 32			1883 ± 145	
	4-NOPD	10			509 ± 88		
	4-NOPD	50		85 ± 6			
	MMS	2.0 µL					5083 ± 899
With activation	DMSO		10 ± 4	18 ± 3	30 ± 4	110 ± 3	568 ± 54
	Untreated		7 ± 2	21 ± 5	39 ± 6	122 ± 7	619 ± 7
	MTC	10	10 ± 4	14 ± 7	31 ± 6	116 ± 16	519 ± 48
		33	14 ± 1	23 ± 3	45 ± 3	113 ± 23	530 ± 34
		100	12 ± 3	16 ± 1	32 ± 4	107 ± 32	529 ± 72
		333	8 ± 0	23 ± 2	37 ± 1	47 ± 6	571 ± 39
		1000	6 ± 2 P M	15 ± 4 P M	19 ± 2 P M	50 ± 1 P M	473 ± 19 P M
		2500	8 ± 2 P M	10 ± 2 P M	17 ± 1 P M	56 ± 5 P M	518 ± 12
		5000	7 ± 1 P M	8 ± 4 P M	14 ± 3 P M	57 ± 2 P M	478 ± 21 P M
	2-AA	2.5	386 ± 15	181 ± 10	3870 ± 389	3067 ± 173	
	2-AA	10.0					1853 ± 34

NaN_3 = sodium azide; 2-AA = 2-aminoanthracene, MMS = methyl methane sulfonate, 4-NOPD = 4-nitro-o-phenylene-diamine; P = Precipitate, M = Manual count

III. Conclusion

Based on the study results and under the experimental conditions described Methiocarb technical did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used in this *Salmonella typhimurium* reverse mutation assay.

CA 5.4.2 In vivo studies in somatic cells

An *in vivo* micronucleus test (MNT) in NMRI mice is available that was already evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb. This *in vivo* MNT was in general accordance with the main requirements of OECD guideline 474 of 1983. However, the *in vivo* MNT had some deficiencies regarding sampling regime (i.e. only 1000 polychromatic erythrocytes (PCEs) counted rather than 2000[8], as required by modern standards). Therefore, the study was re-assessed again taking into account an additional conservative assumptions (i.e. increasing of the individual mutant frequencies by a factor of 2). To facilitate the re-evaluation a summary of the study report is provided in the following together with the new evaluation. According to this evaluation, methiocarb is not genotoxic/clastogenic in the *in vivo* MNT.

Report:	KCA 5.4.2/01 [redacted]; 1979; M-009544-01-1
Title:	H 321 (active ingredient of Mesurol) - Micronucleus test for mutagenic effect on mice
Report No.:	8426
Document No.:	M-009544-01-1
Guideline(s):	At the time the study was performed no guideline was compulsory.
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

A. Materials

1. Test material:

Description:	Methiocarb technical
Synonym:	H321, Mesurol
Lot/Batch no:	06111978 (composite batch)
Purity:	98.5% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: not reported

2. Vehicle and/or positive control: 0.5% Cremophor EL emulsion
Adriblastin®, containing doxorubicin, dissolved in physiological saline

3. Test animals:

Species:	Mouse
Strain:	NMRI
Age:	Approximately 8-12 weeks
Sex:	Male and female
Weight at dosing:	22-37 g
Source:	[redacted], Germany
Acclimatisation period:	Not reported
Diet:	Altromin ® pelleted diet, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Maximum 3 of one sex/cage in Type I makrolon cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	Two applications at an interval of 24 h

	Methiocarb: 0-5-10-20 mg/kg bw positive control: 5 mg/kg bw
Application route:	Control and methiocarb: oral gavage positive control: i.p.
Application volume:	Control and methiocarb: 10 mL/kg bw positive control: 5 mL/kg bw
Group size:	5/sex/dose
Observations:	Mortality, clinical signs
Sacrifice:	6 h after the second application
Sampling:	Femoral marrow was prepared after sacrifice
Tissue preparation for assay:	Bone marrow was prepared according to the method of Schmid (DFG, 1975).
Evaluation:	1000 polychromatic erythrocytes (PCE) per animal were counted and the frequency of the cells containing micronuclei was determined. In order to evaluate the ratio of polychromatic to normochromatic erythrocytes (NCE), which can provide information about the general activity of the test compound on the bone marrow, the number of normochromatic erythrocytes per 1000 polychromatic erythrocytes was determined.
Statistics:	The data were statistically evaluated in accordance with the WILCOXON non-parametric ranking method. A difference is regarded as statistically significant if the probability of error of this difference is less than 5% ($p < 0.05$).

II. Results and discussion

A. Mortality
At 20 mg/kg bw 5 of 8 animals died.
Thus, the dose of 2 x 20 mg/kg bw/day clearly exceeded the maximum tolerable dose (MTD).

B. Clinical signs
Mice treated with up to and including 2 x 10 mg/kg bw methiocarb exhibited no clinical signs. Their physical appearance and motor activity was normal and did not differ from the animals of the negative control group.
Mice treated with 2 x 20 mg/kg bw methiocarb exhibited severe clinical signs.

C. Assessment of micronuclei
The *in vivo* MNT had some deficiencies regarding the sampling regime when compared to the current standards (e.g. OECD guideline 474 (2014)). In this MNT only 1000 polychromatic erythrocytes (PCEs) were counted per animal instead of 2000, as recommended in the guideline version of 1997 or of 4000 as recommended in the current version of 2014. The reason for increasing the required counting of 2000 PCEs to 4000 PCEs was implemented to increase the statistical power of the test only.
In order to avoid the conduct of a new *in vivo* MNT on methiocarb for animal welfare reasons, the counted micronuclei of the methiocarb treated animals were multiplied with a factor of 2 to simulate a result after counting of 2000 PCEs. The control values were not multiplied with a factor of 2 to create a worst case. The idea was to see if also under this worst case the test still would reveal a negative result. In addition, in this *in vivo* MNT the test groups consisted of ten animals (5/sex) instead of the 5 animals that are required by the current OECD guideline #474 (2014). Thus, the reassessment based on the evaluation of 20,000 PCEs per test substance group, which is the same number of PCEs that is required by OECD guideline #474 (2014).

The simulation of counting 2000 PCEs and not 4000 PCEs was considered to be sufficiently conservative in this case.

The results together with available historical control data are summarised in the Table 5.4.2/01- 1 below. Individual animal values are provided in Table 5.4.2/01- 2 and Table 5.4.2/01- 3.

Table 5.4.2/01- 1: Summary of results

Dose group (mg/kg bw)	HCD◊	Control 0	Methiocarb 2 x 5	 2 x 10	 2 x 20##	Positive control# 2 x 5
PCE evaluated	35000	1000	1000	1000	1000	1000
NCE per 1000 PCE mean value	**473.1-1439.1**	**795.4**	**1050.3**	**680.9**	**883.7**	**1028.2**
minimum	213	579	620	504	726	351
maximum	3886	1080	1865	923	1080	1853
PCE / NCE mean value	**n.r**	**1.30**	**1.05**	**1.53**	**1.16**	**1.17**
minimum	n.r	0.93	0.54	1.08	0.93	0.54
maximum	n.r	1.73	1.61	1.98	1.38	2.85
MNNCE per 1000 cells	**0.52-2.9**	**2.123**	**1.57**	**1.49**	**0.39**	**1.74**
minimum	0	0	0	0	0	0
maximum	7.38	5.72	2.58	4.68	1.18	6.23
MNPCE per 1000 cells	**0.6-2.6**	**1.6**	**1.1**	**2.2**	**1.67**	**20.3**
minimum	0	0	0	1	1	3
Maximum	7	3	5	4	3	58
MNPCEs per 1000 cells x factor 2 ♦	**n.a.**	**n.a.**	**2.2**	**4.4**	**3.33**	**40.6**
minimum	n.a.	n.a.	0	2	2	6
maximum	n.a.	n.a.	10	8	6	116

PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes; MNNCE = micronucleated NCE; MNPCE = micronucleated PCE

\# Adriblastin, i.p.

\## Only 4 mice/sex assigned to this group; 5 animals (3 males and 2 females) died! Therefore, the values presented for this dose group are only from 3 animals! At this dose the MTD is clearly exceeded.

♦ Since only 1000 cells instead of 2000 as recommended by OECD guideline 474 were evaluated, the determined PCEs/1000 cells were multiplied with a factor of 2

◊ HCD = Historical control data. The historical control data were obtained from in *vivo* MNTs conducted in the same laboratory (i.e. Bayer Toxicology [redacted], Germany) between 1977 and 1982 using the same strain of mice (i.e. NMRI). For details please refer to M-536382-01-1

n.a. = not applicable
n.r. = not reported

As can be seen from the results the ratio of PCEs/NCEs is comparable between all groups. Thus no cytotoxic effect on the bone marrow is expected. The mean frequency of MNPCEs/1000 PCEs is also comparable in all groups.
Application of a factor of two revealed that the MNPCEs/1000 cells are higher than the control value. However, comparison of the values for MNPCEs/1000 PCEs, taking into account a factor of two, with the historical control values (see Table 5.4.2/01- 1) demonstrate that individual values of methiocarb treated animals are in the range of historical control except for one female of the low-dose group, and one male and one female of the high-dose group. In these animals MNPCEs/1000 NCEs taking into account a factor of two were 10 and 8, respectively, and therefore slightly outside the historical control range of 0 to 7. For the major part of the animals the MNPCE/1000 NCEs were clearly within the historical control range even if the factor two is applied. Therefore, it can be assumed that sampling 2000 PCEs would not result in a different outcome of the study. Furthermore, 10 mice (8 for the highest dose group), instead of 5 as currently recommended by OECD 474 were used for each group. Thus, in total 1000 PCEs per group were scored. This is the total number that will be evaluated if 2000 PCEs for 5 animals per group (acc. to OECD 474 (1997)[8]) will be scored. It has to be noted that

according to the current OECD 474 (2014) scoring of 4000 PCEs per animal is required. However this is only done to facilitate a proper statistical analysis.

Furthermore, in this study sampling was done only once 6 h after the second application (i.e. 30 h after first treatment). The current OECD 474 (2014) requires for two applications one sampling between 18 and 24 h after the last application. Thus, sampling time point is not according to guideline for two applications. The time point however fulfills the requirements of OECD 474 (2014) for one application (i.e. between 24 and 48 h after application). More than one sampling after one application is only required for the highest dose group.
Considering that the bone marrow cell cycle in mice is about 12 h, the 6 h sampling after the second application cannot cover effects that might be induced by the second application. However, with a sampling time 30 h after the first application it can cover effects that might be induced by the first application.

Overall, although the *in vivo* MNT was not conducted according to the currently valid guideline, the applied test protocol, as well as the reassessment with an application of a factor of two to the MNPCEs/1000 PCE ratios is considered to be sufficient for the evaluation of a clastogenic potential of methiocarb.

Table 5.4.2/01- 2: Individual results for positive and negative control groups

Dose Animal No.	sex	0 mg/kg bw (negative control)					positive control 2 x 5 mg/kg bw Adriblastin					
		PCEs examined	NCEs per 1000 PCEs	PCE / NCE	MNNCE per 1000 cells	MNPCE per 1000 cells	PCEs examined	NCEs per 1000 PCEs	PCE / NCE	MNNCE per 1000 cells	MNPCE per 1000 cells	PCEs per 1000 cells x 2 ♦
1	m	1000	709	1.41	2.82	2	1000	1013	0.99	2.96	6	12
2	m	1000	772	1.30	2.59	2	1000	663	1.51	0	33	66
3	m	1000	809	1.24	2.47	0	1000	869	1.15	0	13	26
4	m	1000	845	1.18	2.37	1	1000	1074	0.93	0.93	6	12
5	m	1000	873	1.15	1.15	1	1000	1117	0.90	0	9	18
6	f	1000	579	1.73	0	0	1000	351	2.85	2.85	11	22
7	f	1000	944	1.06	3.18	0	1000	802	1.25	6.23	58	116
8	f	1000	699	1.43	5.72	3	1000	1149	0.87	1.74	3	6
9	f	1000	644	1.55	0	3	1000	1391	0.72	2.16	34	68
10	f	1000	1080	0.93	0.93	3	1000	1853	0.54	0.54	30	60
mean		**1000**	**795.4**	**1.30**	**2.12**	**1.6**	**1000**	**1028.2**	**1.17**	**1.741**	**20.3**	**40.6**
Min			579	0.93	0	0		351	0.54	0	3	6
Max			1080	1.73	5.72	3		1853	2.85	6.23	58	116

♦ Since only 1000 cells instead of 2000 as recommended by OECD guideline 474 were evaluated, the determined PCEs/1000 cells were multiplied with a factor of 2

Table 5.4.2/01- 3: Individual results for methiocarb groups

Dose	2 x 5 mg/kg bw methiocarb						2 x 10 mg/kg bw methiocarb						2 x 20 mg/kg bw methiocarb##					
sex	PCEs exa-mined	NCEs per 1000 PCEs	PCE / NCE	MNNCE per 1000 cells	MNPCE per 1000 cells	MNPCEs per 1000 cells x 2♦	PCEs exa-mined	NCEs per 1000 PCEs	PCE / NCE	MNNCE per 1000 cells	MNPCE per 1000 cells	MNPCEs per 1000 cells x 2♦	PCEs exa-mined	NCEs per 1000 PCEs	PCE / NCE	MNNCE per 1000 cells	MNPCE per 1000 cells	MNPCEs per 1000 cells x 2♦
m	1000	776	1.29	2.58	1	2	1000	504	1.98	0	1	2	1000	845	1.18	1.18	3	6
m	1000	812	1.23	2.46	2	4	1000	738	1.36	4.07	3	6	✞					
m	1000	832	1.20	2.4	0	0	1000	855	1.17	4.68	4	8	✞					
m	1000	620	1.61	1.61	1	2	1000	651	1.54	3.07	1	2	✞					
m	1000	928	1.08	1.08	0	0	1000	561	1.78	0	1	2						
f	1000	1349	0.74	0.74	0	0	1000	590	1.69	0	3	6	✞					
f	1000	921	1.09	2.17	5	10	1000	923	1.08	0	3	6	1000	726	1.38	0	1	2
f	1000	1865	0.54	1.61	1	2	1000	863	1.16	1.16	1	2	1000	1080	0.93	0	1	2
f	1000	1405	0.71	0	0	0	1000	534	1.87	1.87	4	8	✞					
f	1000	995	1.01	1.01	1	2	1000	590	1.69	0	1	2	--					
mean	**1000**	**1050.3**	**1.05**	**1.57**	**1.1**	**2.2**	**1000**	**680.9**	**1.53**	**1.485**	**2.2**	**4.4**	**1000**	**883.7**	**1.16**	**0.39**	**1.67**	**3.33**
minimum		620	0.54	0	0	0		504	1.08	0	1	2		726	0.93	0	1	2
maximum		1865	1.61	2.58	5	10		923	1.98	4.68	4	8		1080	1.38	1.18	3	6
Historical control data ◊																		
minimum		213			0	0		213			0	0		213			0	0
maximum		3886			7	7		3886			7.38	7		3886			7.38	7

PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes; MNNCE = micronucleated NCE; MNPCE = micronucleated PCE

Only 4 mice/sex assigned to this group; 5 animals (3 males and 2 females) died! At this dose the MTD is exceeded.

✞ Animal died

♦ Since only 1000 cells instead of 2000 as recommended by OECD guideline 474 were evaluated, the determined PCEs/1000 cells were multiplied with a factor of 2

MNPCEs/1000 cells that are outside of the historical control range (i.e. 0-7) are shaded in grey

◊ The historical control data were obtained from in vivo MNTs conducted in the same laboratory (i.e. Bayer Toxicology [redacted], Germany) between 1977 and 1982 using the same strain of mice (i.e. NMRI). For details please refer to M-536382-01-1

III. Conclusion

Based on the study results and the re-evaluation taking into account a factor of two it is concluded that methiocarb is not genotoxic in this *in vivo* MNT.

CA 5.4.3 In vivo studies in germ cells

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.5 Long-term toxicity and carcinogenicity

Summary of long-term toxicity and carcinogenicity
Long-term studies have been conducted in the rat and mouse.

In rats the main effect was a significant reduction of body weights at the high dose of 600 ppm (3.3 / 4.98 mg/kg bw/day (males / females)). The NOAEL was 200 ppm (9.3 / 13.89 mg/kg bw/day (males / females). Plasma, erythrocyte and brain cholinesterase activities were not significantly depressed at the tested dose levels.

In mice the main effect was cholinesterase inhibition, as well as changes in alanine aminotransferase activity. The NOAEL in the 24-month feeding study in mice was 67 ppm (19.8 mg/kg bw/day) based on changes in alanine aminotransferase and transient but biologically relevant reductions of plasma and brain cholinesterase activities at 200 ppm (57 mg/kg bw/day).

The combined chronic toxicity / carcinogenicity studies in rats and mice were conducted before a validated guideline was in place. However, both studies were in general accordance with the previous OECD guideline 453 (1981), and are also in general accordance with the currently valid OECD guideline 453 (2009).
The rat study (M-009809-02-1) covers most of the required parameters for the assessment of chronic toxicity / carcinogenicity according to OECD 453 (2009). Missing were ophthalmology, some clinical chemistry (Ca, K, Na, alb) and urinalysis parameters (specific gravity), some organ weights (brain, epididymides, uterus) and some tissues for histopathology (caecum, coagulating gland, gall bladder, Harderian gland, lacrimal gland, mammary gland, peripheral nerve rectum, skin, spinal cord, thymus, vagina). However, there were no treatment-related gross-pathological findings in those tissues observed. In addition, in the sub-chronic toxicity study (M-088469-01-1) there were no treatment-related effects observed on the weight of the brain, epididymides and uterus. This study contains also histopathological data on the required tissues that were not examined in the 2-year study. There were no treatment-related effects observed. Furthermore, the rat study fulfills the acceptability criteria for a negative carcinogenicity study[9] regarding survival rates, ie. ≥ 25% (for rats). Overall, the 2-year rat study is considered acceptable for the assessment of chronic toxicity and carcinogenicity.

As for the rat study, the mouse study (M-008825-02-1) covers also most parameters required by OECD 453 (2009). Missing were ophthalmology, some haematological parameters (prothrombin time, APTT), most of the clinical chemistry (the only examined parameters were urea and ALAT after 12 and 24 month) and all urinalysis parameters, some organ weights (brain, epididymides, uterus) and some tissues for histopathology (coagulating gland, Harderian gland, lacrimal gland, mammary gland,

[9] Based on the OECD Guidance Document 116 on the conduct and design of chronic toxicity and carcinogenicity studies, supporting test guideline 451, 452 and 453, 2nd edition, ENV/JM/MONO(2011)47, OECD Environment, Health and Safety Publications, 2012-04-13

skin, spinal cord, thymus, vagina). Although not all recommended organ weights were determined, the gross pathological examinations, as well as additional information presented in the addendum on clinical examinations, and histopathology are considered suitable for the assessment of carcinogenicity.
In addition, the survival rate in the low- and mid-dose males and control females at termination after 24 month was below the acceptability criterion of ≥ 25%[9]. The survival rate in control, low-, mid- and high-dose groups was 34.7%, 14.3%, 14.3%, 27.7% in males and 15.7%, 25.5%, 30.0%, 32.7% for females. Although survival in the high-dose group was above 25%, obviously the study duration of 24 month was too long for CFW1 mice. However, the survival rates after 18 month met the acceptability criterion of >50% survival[9], except for low-dose males. Survival in control, low-, mid- and high-dose groups was 73.5%, 46.9%, 59.2%, 66% for males, and 68.6%, 52.9%, 64%, 69.2% in females. In addition, the acceptability criterion that not more than 10% of any group should be lost due to autolysis, cannibalism, or management problems[9] was met at termination after 24 month.
Regarding haematological investigations all mandatory parameters for chronic studies were investigated after 12 and 24 month. Missing were only the parameters prothrombin time and activated partial thromboplastin time, which are also not mandatory for carcinogenicity studies (OECD 453 (2001)). However, there were no effects on these parameters observed in short- and long-term studies in rats and dogs. It is therefore assumed that these parameters are also not affected in mice due to the treatment with methiocarb.
The important investigations for methiocarb, i.e. the assessment of acetylcholine esterase (AChE) inhibition, was conducted after 1 month, 12 month and at termination for plasma AChE, and at termination for brain AChE. The derived NOEL for plasma AChE inhibition was 67 ppm (14.7 / 19.8 mg/kg bw/day (males/females)). No effects were observed for the brain AChE.
Overall, the study is considered to be suitable for the assessment of a carcinogenic potential of methiocarb in mice.

In both studies in rats and mice there were no treatment-related increases in incidences of benign and malignant tumours.

Overall methiocarb was not carcinogenic in mice and rats.

Table 5.5- 1: Summary of long-term studies

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
Rat 2-year, oral (diet) 0-67-200-600 ppm 0-3.27/4.98-9.3/13.9-29/42 mg/kg bw/day	M F	9.3 13.9 (200 ppm)	29 42 (600 ppm)	Slight stat. sign. ↓ bw, (−6.2%/−7.6% (m/f))	[redacted] 1981 M-009809-02-1
Mouse 2-year, oral (diet) 0-67-200-600 ppm 0-14.67/19.8-42.8/57-131.9/173.3 mg/kg bw/day	M F	14.7 19.8 (67 ppm)	57 (200 ppm)	↑ ALT indicating liver toxicity Slight stat. sign. ↓ of plasma AChE (only after 1 month)	[redacted] 1983 M-008825-02-1

New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.
↑: increase(d) ↓: decrease(d)

Comparison of the study results CLP criteria according to REGULATION (EC) No 1272/2008[7]
According to REGULATION (EC) No 1272/2008 a substance is considered to have a carcinogenic potential if it induce tumours, increase tumour incidence and/or malignancy or shortens the time to tumour occurrence in humans or well conducted animal studies. In addition, benign tumours that are considered to have the potential to progress to malignant tumours are generally considered along with malignant tumours.

In the combined chronic toxicity / carcinogenicity studies conducted with methiocarb in rats and mice there were no treatment-related inductions of tumours and no increased incidences of benign and malignant tumours. Therefore, a carcinogenic potential for methiocarb can be excluded.

Therefore, according to the actual CLP criteria no classification is triggered for methiocarb regarding carcinogenicity.

Classification/labelling regarding carcinogenicity:
- Regulation (EC) No 1272/2008 (CLP): none

Previous evaluation	In DAR (2004) for original approval

Study	H 321 (Mercaptodimethur, the active ingredient of Mesurol) - Chronic toxicity on rats (2-year feeding experiment)
Reference	[redacted]. (1981)
Date performed	1981-07-02
Test facility	Bayer AG, [redacted], Germany
Report reference	10039

Guideline(s)	Satisfy the essential criteria of OECD guideline # 453
Deviations from the guideline	Some of the required parameters for the assessment of chronic toxicity / carcinogenicity according to OECD 453 (2009) are missing
GLP	No
Test material	Methiocarb (H321)
Study acceptable	Yes

In a study (Kroetlinger, F.; Loeser, E.; Vogel, O. 1981), groups of 60 male and 60 female SPF Wistar rats (40-45 days old, initial mean bw 92 g and 81 g for males and females) were administered in the diet methiocarb (98 % purity, Batch 75/66) at concentrations of 0 (control), 67, 200 or 600 ppm (0, 3.27, 9.3 or 29 mg/kg bw/day for males and 0, 4.98, 13.9 or 42 mg/kg bw/day for females) for a period of 24 months. Feed and water were provided ad libitum. Clinical laboratory tests were conducted on 10 rats/sex/dose group at 3, 6, 12 and 24 months. Cholinesterase activity (10 rats/sex/dose) in plasma and RBC was determined on days 1 and 2 and on weeks 1, 2, 4, 8, 13, 26, 52, 78, and 105 after initiation of the study and in brain at termination using a modified colorimetric method of Ellman *et al*. (1961). Blood samples for cholinesterase measurements were taken from non-fasted animals. All surviving animals were killed at the end of the treatment period and tissues and organs were grossly examined followed by histopathology. A full re-analysis of histopathology on the processed tissues was performed by Life Science Research in 1988-89 and presented in the addendum. Stability of the test compound and its stability in feed were stated to have been determined.

The main study was pre GLP and not quality assured. The addendum to the main report was quality-assured. Some of the parameters required in the OECD Guideline N 453 (2009) for the assessment of chronic toxicity / carcinogenicity were not evaluated; however, there were no treatment-related gross-pathological findings in the tissues observed. Furthermore, the rat study fulfils the acceptability criteria for a negative carcinogenicity study[10] regarding survival rates, i.e. > 25% (for rats). Overall, the 2-year rat study is considered to satisfy the essential criteria of OECD guideline 453 and acceptable for the assessment of chronic toxicity and carcinogenicity.

Cumulative mortalities among the study groups (including sacrifice of 14 moribund animals) by the completion of the study were: 8 (13 %), 8 (13 %), 12 (20 %) and 6 (10%) in the males and 19 (32 %), 16 (26 %), 17 (28 %) and 14 (23 %) in females at control, 67, 200 and 600 ppm respectively but deaths did not show any relation to treatment. No changes in animal behaviour or overt cholinergic symptoms were observed at all test doses. Food consumption was minimally affected by the treatment, approximately 5 % reduction during the second year of treatment. The mean compound intake was approximately 3, 9.3 and 28.5 mg/kg bw/day at dose levels of 67, 200 and 600 ppm respectively. Body weight gain was slightly but significantly lower compared to controls throughout the study in the top dose group. Terminal body weights were only slightly but significantly reduced at the 600 ppm dose level in males (6.2 %) and females (7.6 %).

Table: Body weight development

	Males				Females			
Dose level [ppm]	**0**	**67**	**200**	**600**	**0**	**67**	**200**	**600**
Dose level [mg/kg bw/day]	**0**	**3.27**	**9.28**	**28.85**	**0**	**4.98**	**13.89**	**42.07**
Bodyweight								
BW week 0 [g]	91	91	91	91	80	81	82	82*

[10] Based on the OECD Guidance Document 116 on the conduct and design of chronic toxicity and carcinogenicity studies, supporting test guideline 451, 452 and 453, 2nd edition, ENV/JM/MONO(2011)47, OECD Environment, Health and Safety Publications, 2012-04-13

	Males				Females			
Dose level [ppm]	**0**	**67**	**200**	**600**	**0**	**67**	**200**	**600**
Dose level [mg/kg bw/day]	**0**	**3.27**	**9.28**	**28.85**	**0**	**4.98**	**13.89**	**42.07**
Bodyweight								
BW week 1 [g]	127	122*	122*	111**	101	99	100	95**
% change from control	--	−4	−4	−13	--	−2	−1	−6
BW week 2 [g]	160	158	156	142**	116	113	113	109**
% change from control		−1.2	−2.5	−11	--	−3	−3	−6
BW week 4 [g]	216	211*	208*	194**	138	137	137	130**
% change from control	--	−2.3	−3.7	−10	--	−0.7	−0.7	−6
BW week 8 [g]	277	273	265**	249**	164	163	163	154**
% change from control	--	−1.4	−4	−10	--	−0.6	−0.6	−6
BW week 12 [g]	314	309	303**	290**	181	180	180	172**
% change from control	--	−1.6	−3.5	−8	--	−0.6	−0.6	−5
BW week 18 [g]	343	339	331**	316**	194	194	193	186*
% change from control	--	−1.2	−3.5	−8	--	0	−0.5	−4
BW week 24 [g]	363	358	354	339**	203	203	204	194*
% change from control	--	−1.4	−2.5	−7	--	0	+0.5	−4.4
BW week 37 [g]	389	383	388	365**	213	210	213	202**
% change from control	--	−1.5	−0.3	−6	--	−1	0	−5
BW week 53 [g]	394	394	411*	384	212	211	223**	213
% change from control	--	0	+4	−2.5	--	−0.5	+5	+0.5
BW week 61 [g]	412	409	409	396*	232	230	234	223
% change from control	--	−0.7	−0.7	−4	--	−1	+1	−4
BW week 73 [g]	420	418	419	400**	240	237	241	227**
% change from control	--	−0.5	−0.2	−5	--	−1.2	+0.4	−5.4
BW week 93 [g]	413	408	411	390**	250	250	249	231**
% change from control	--	−1.2	−0.5	−5.5	--	0	−0.4	−8
BW week 105 [g]	405	399	404	384*	252	252	250	234**
% change from control	--	−1.5	−0.2	−5	--	0	−1	−7
Bodyweight gain								
BWG week 0-24	272	267	263	248	123	122	122	112
% change from control	--	−2	−3	−9	--	−1	−1	–
BWG week 0-53	303	303	320	293	132	130	141	131
% change from control	--	0	+6	−3	--	−2	+7	−1
BWG week 0-105	314	308	313	293	172	171	168	152
% change from control	--	−2	−0.3	−7	--	−1	−2	−12

* Statistical significant different from control $p < 0.05$

** Statistical significant different from control $p < 0.01$

There were no significant treatment-related changes in haematology, clinical chemistry or urine analysis. Haematology parameters showed in the first year sporadic incidences of reduced erythrocyte, haemoglobin, haematocrit and increased MCHC. The observation of slightly reduced red blood cell parameters in females only at 6 months was noted to be inconsistent with the remaining observations during this study or in other studies in rats and is considered incidental.

Erythrocyte cholinesterase activity assays did not show any biologically significant change (> 20% change compared to controls) at all doses in males and females. Plasma cholinesterase activity assays showed transient > 20% reductions at 600 ppm in males between week 8-13 and in females during

week 2 to 26. At week 105 > 20% reduction in plasma AChE was noted in males at 200 ppm and in males and females at 600 ppm. Brain cholinesterase inhibition measured at termination (week 105) did not reveal any significant differences between treated animals and controls. Overall, although decreases > 20% were seen in plasma AChE, considering that these were not confirmed in brain and erythrocytes, no toxicologically significant changes in AChE were observed in this study up to the top dose.

Table B.6.5.1. Cholinesterase activities in rat plasma, erythrocytes and brain

Cholinesterase activity in plasma (U/mL)											
	Males										
Dose in ppm	D 1	D 2	W 1	W 2	W 4	W 8	W 13	W 26	W 52	W 78	W 105
0	0.40	0.36	0.53	0.47	0.46	0.41	0.45	0.47	0.50	0.66	0.89
67	0.42	0.39	0.53	0.47	0.51	0.45	0.44	0.49	0.47	0.79*	0.73
200	0.42	0.36	0.59	0.54	0.51	0.41	0.40	0.41	0.54	0.77	0.71
600	0.34*	0.36	0.52	0.47	0.42	0.30**	0.32**	0.38*	0.50	0.56**	0.64*
	Females										
0	0.48	0.44	0.78	0.87	1.10	1.21	1.26	1.62	1.35	1.78	1.64
67	0.47	0.46	0.75	0.92	1.07	1.18	1.29	1.67	1.48	1.81	1.92
200	0.47	0.44	0.67	0.87	0.95	1.11	1.30	1.66	1.91*	1.65	1.48
600	0.4*	0.42	0.63*	0.68**	0.65**	0.86*	0.99	1.21	1.80	1.60	1.23

Cholinesterase activity in erythrocytes (U/mL)											
	Males										
Dose in ppm	D 1	D 2	W 1	W 2	W 4	W 8	W 13	W 26	W 52	W 78	W 105
0	2.54	2.54	2.29	2.66	2.79	2.60	3.00	2.40	2.89	3.16	3.24
67	2.54	2.55	2.30	2.69	2.79	2.64	3.03	2.55	3.04	3.11	3.08*
200	2.63	2.31*	2.28	2.72	2.75	2.46*	2.92	2.51	3.09	2.93*	2.97**
600	2.59	2.48	2.21	2.59	2.72	2.43*	2.80*	2.46	3.13	2.87**	3.0*
	Females										
0	2.37	2.43	2.57	2.56	2.79	2.55	2.97	2.44	3.18	3.22	2.89
67	2.39	2.38	2.63	2.61	2.78	2.63	2.85	2.47	2.99	3.10*	2.95
200	2.43	2.28	2.49	2.57	2.57**	2.65	2.92	2.55	3.03	2.81*	2.87
600	2.49	2.28*	2.53	2.50	2.58	2.70*	2.99	2.59*	2.99	2.87**	2.86

D = day, W = week
* Difference from control is significant for p < 0.05
**Difference from control is significant for p<0.01

Cholinesterase activity in brain(U/mL)	
Dose in ppm	W 105
	Males
0	0.40
67	0.42
200	0.42
600	0.34*
	Females
0	0.48
67	0.47
200	0.47
600	0.4*

W = week
* Difference from control is significant for $p < 0.05$

No gross treatment-related abnormalities were observed at post-mortem of animals that died or were killed at termination. Organ weight changes were related to body weight and significant reduction in the absolute and relative spleen weights at 600 ppm was noted. Histopathology did not reveal any significant treatment-related findings but significant levels of parasitic infection in the bowel were noted. There were no significant treatment-related findings in the incidence of benign or malignant tumours.

Table. Organ weight - Males

Dose level [ppm]	0	67	Δ%	200	Δ%	600	Δ%
Terminal BW [g]	406	396	–2	403	–1	381*	–6
Liver (abs.) [mg]	14343	13681	–5	13790	–4	13278*	–7
Liver (rel.) [mg/100 g]	3526	3443	–2	3417	–3	3491	–1
Liver (rel.) [% BW]	3.53	3.45	–2.3	3.42	–3.1	3.49	–1.1
Thyroid (abs.) [mg]	23	30	+30	23	0	20**	–13
Thyroid (rel.) [mg/100 g]	6	8	+33	6	0	5	–17
Thyroid (rel.) [% BW]	0.006	0.008	+33	0.006	0	0.01	+67
Kidney (abs) [mg]	2618	2578	–2	2552	–3	2516	–4
Kidney (rel) [mg/100 g]	648	655	+1	638	–2	666	+3
Kidney (rel) [% BW]	0.64	0.65	+1.6	0.63	–1.6	0.66	+3.1
Spleen (abs) [mg]	782	735	–6	736	–6	659**	–16
Spleen (rel) [mg/100 g]	193	185	–4	183	–5	173**	–10
Spleen (rel) [% BW]	0.19	0.19	0	0.18	–5.3	0.17	–10.5
Testes (abs) [mg]	3614	3639	1	3542	–2	3592	–1
Testes (rel) [mg/100 g]	894	923	3	880	–2	953*	+7
Testes (rel) [% BW]	0.89	0.92	+3.4	0.88	–1.1	0.94	+5.6
Adrenal (abs) [mg]	49	75	+53	46	–6	45*	–8
Adrenal (rel) [mg/100 g]	12	20	67	12	0	12	0
Adrenal (rel) [% BW]	0.01	0.02	+100	0.01	0	0.01	0
Heart (abs) [mg]	1203	1140	–5	1199	–0.3	1138	–5.4
Heart (rel) [mg/100 g]	297	290	–2.4	300	+1	302	+2
Heart (rel) [% BW]	0.3	0.29	–3.3	0.3	0	0.3	0
Lungs (abs) [mg]	1655	1548	–6.5	1971	+19	1559*	–6
Lungs (rel) [mg/100 g]	412	396	–4	504	+22	412	0
Lungs (rel) [% BW]	0.41	0.39	–5	0.49	+20	0.41	0

NOTE: rel BW in % BW were calculated from absolute organ weights. In the study report rel organ weights were only reported in mg/100g BW; NOT in %

Δ% = % change from control

* Statistical significant different from control $p < 0.05$

** Statistical significant different from control $p < 0.01$

Table. Organ weight - Females

Dose level [ppm]	0	67	Δ%	200	Δ%	600	Δ%
Terminal BW [g]	252	252	0	250	–1	233**	–8
Liver (abs.) [mg]	9022	8927	–1	9117	+1	8372	–7
Liver (rel.) [mg/100 g]	3509	3576	+2	3658	+4	3588	+2
Liver (rel.) [% BW]	3.58	3.54	–1.1	3.65	+2	3.59	+0.3
Thyroid (abs.) [mg]	18	17	–6	18	0	33**	+83
Thyroid (rel.) [mg/100 g]	7	7	0	7	0	14	+100
Thyroid (rel.) [% BW]	0.007	0.007	0	0.0067	–4.3	0.014	+100
Kidney (abs) [mg]	1771	1691	–5	1711	–3	1690	–5
Kidney (rel) [mg/100 g]	704	680	–3	692	–2	729*	+4
Kidney (rel) [% BW]	0.7	0.67	–4.3	0.68	–2.9	0.73	+4.3
Spleen (abs) [mg]	590	597	+1	566	–4	483*	–18
Spleen (rel) [mg/100 g]	238	241	+1	228	–4	207	–13
Spleen (rel) [% BW]	0.23	0.24	+4.3	0.23	0	0.21	–8.7
Ovaries (abs) [mg]	133	119	–11	150	+13	447	+236
Ovaries (rel) [mg/100 g]	54	47	–13	60	+11	183	+236
Ovaries (rel) [% BW]	0.053	0.047	–11	0.06	+13	0.19	+259
Adrenal (abs) [mg]	68	68	0	70	+3	64	–6
Adrenal (rel) [mg/100 g]	27	28	+4	28	+4	27	0
Adrenal (rel) [% BW]	0.03	0.03	0	0.03	0	0.03	0
Heart (abs.) [mg]	912	908	–0.4	950	+4	904	–0.9
Heart (rel) [mg/100 g]	365	364	–0.3	385	+6	389	+7
Heart (rel) [% BW]	0.36	0.36	0	0.38	+6	0.39	+8
Lungs (abs) [mg]	1196	1130	–4.3	1174	–1.8	1146	–4
Lungs (rel) [mg/100 g]	480	456	–5	474	–1.2	495	+3
Lungs (rel) [% BW]	0.47	0.45	–4	0.47	0	0.49	+4

NOTE: rel BW in % BW were calculated from absolute organ weights. In the study report rel organ weights were only reported in mg/100g BW; NOT in %
Δ% = % change from control
* statistical significant different from control $p < 0.05$
** statistical significant different from control $p < 0.01$

Table. Incidence of selected non-neoplastic findings

	Males				Females			
Dose level [ppm]	**0**	**67**	**200**	**600**	**0**	**67**	**200**	**600**
No of animals	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**
Adrenals								
No examined	59	60	60	60	60	60	60	60
groups of vacuolated enlarged cells	10	23*	13	18	18	12	2**	19
inflammatory cell infiltration	1	0	2	0	2	1	3	3
degeneration	0	0	0	1	0	0	0	0
Eye								
No examined	59	60	60	60	60	60	60	60
Retinal detachment	0	0	0	2	1	1	1	1
Eosin red material	1	1	0	5	1	1	0	3
Cateract	0	3	6*	4	0	4	6*	2
Heart								
No examined	60	60	60	60	60	60	60	60

	Males				Females			
Dose level [ppm]	**0**	**67**	**200**	**600**	**0**	**67**	**200**	**600**
No of animals	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**
Fibrosis	6	9	2	5	2	1	5	2
Necrosis	6	0*	0*	5	0	0	1	1
Inflammatory cell infiltration	22	8**	5***	14	10	5	8	12
Kidney								
No examined	60	60	60	60	60	60	60	60
senile nephropathy	56	51	45*	51	30	14**	30	39
inflammatory cell infiltration	1	1	1	0	5	2	6	2
plethora, hyperaemia	3	0	4	1	0	0	0	2
epithelial proliferation	0	0	1	0	0	0	8**	0
calcification	0	0	4	0	1	10**	7	5
fatty degeneration	0	0	0	0	0	1	1	0
hyaline deposits in epithel. tubules	0	1	3	1	0	0	0	0
cystic structures	0	1	2	0	0	1	0	0
fibrosis	0	1	1	1	0	2	0	0
necrosis	0	1	1	0	0	0	1	0
pigment	0	0	0	0	0	6*	0	0
proliferation	0	0	0	0	0	0	1	0
dilatation	0	0	0	1	0	0	0	0
Liver								
No examined	60	60	60	60	60	60	59	60
bile duct proliferation	34	27	10**	17**	7	13	4	10
single vacuolated cells	5	0	0	5	0	0	1	2
fibrosis	1	3	0	0	0	2	0	0
clear cell foci	38	33	24*	30	9	8	7	10
inflammatory cell infiltration	14	7	6	8	11	11	7	14
fatty degeneration	5	6	3	5	8	8	7	4
Plethora, hyperaemia	5	5	5	3	3	0	7	5
basophilic cells - groups	1	0	0	2	3	3	3	5
proliferation of lymphoreticular tissue	0	0	0	0	2	1	0	3
nodular hyperplasia	0	1	0	0	1	0	0	0
necrosis	0	1	0	0	0	11***	1	0
Lung								
No examined	60	60	60	60	60	60	60	60
Inflammatory cell infiltration	30	22	26	24	14	17	18	14
Xanthoma cell	9	11	13	11	9	7	11	8
Ovaries								
No examined					55	58	60	60
Epithelial proliferation					3	2	3	1
Pancreas								
No examined	58	60	58	60	58	58	58	58
Inflammatory cell infiltration	3	3	6	5	4	1	3	5
Nodular hyperplasia	2	2	2	0	1	0	1	2
Pituitary								
No examined	58	58	58	60	49	56	57	55
Nodular hyperplasia	6	8	17*	11	0	1	3	2
Prostate								
No examined	60	60	60	59				
Inflammatory cell infiltration	1	9*	2	2				

	Males				Females			
Dose level [ppm]	**0**	**67**	**200**	**600**	**0**	**67**	**200**	**600**
No of animals	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**
Salivary gland								
No examined	59	59	60	60	59	57	60	59
Inflammatory cell infiltration	0	0	1	1	0	0	1	3
Spleen								
No examined	60	60	60	60	59	59	58	60
Proliferation of lymphoreticular tissue	0	0	3	0	2	3	11**	4
Testes								
No examined	60	60	60	60				
Polyarteritis nodosa	11	8	12	8				
inflammatory cell infiltration	8	7	10	3				
calcification	1	0	3	2				
proliferation of Leydig cells	5	1	5	6				
tubular atrophy	10	18	30**	7				
Atrophy	2	6	0	3				
Thyroid								
No examined	60	60	60	60	59	59	60	60
Nodular hyperplasia	8	1*	0**	7	2	0	0	3
Urinary bladder								
No examined	60	57	59	59	56	58	59	58
inflammatory cell infiltration	1	0	0	2	0	0	0	1
Uterus								
No examined					60	60	60	60
inflammatory cell infiltration					13	13	12	7
Endometial polyp					10	9	15	10

* statistical significant different from control $p < 0.05$
** statistical significant different from control $p < 0.01$
*** statistical significant different from control $p < 0.001$

Table. Incidence of selected neoplastic findings

		Males					Females			
Dose level [ppm]	**HCD range**	**0**	**67**	**200**	**600**	**HCD range**	**0**	**67**	**200**	**600**
No of animals		**60**	**60**	**60**	**60**		**60**	**60**	**60**	**60**
Adrenals										
No. examined	1925	59	60	60	60	1936	60	60	60	60
Phaeochromocytoma (b)	0-15	4	6	5	5	0-4	1	1	2	0
Adenoma (b)	0-7	0	0	0	0	0-5	1	0	1	1
Carcinoma (m)	0	0	0	0	0	0-1	0	2	0	0
Cholangioneurinoma (b)	0-1	0	0	0	1	0-1	0	0	0	0
Bone										
No. examined	1945	60	59	60	60	1909	60	60	60	60
Osteosarcoma (m)	0-1	0	0	1	0	--	0	0	0	0
Brain										
No. examined	1863	50	51	51	49	1862	49	50	50	50
Granular cell tumor (b)	0-1	1	1	0	0	0-1	0	0	0	0
Astrocytoma (b)	--	0	1	1	0	0-1	0	1	0	0
Epididymides										
No. examined	1946	60	60	60	60					
Mesothelioma (b)	0-1	0	0	0	1					
Hemopoietic system										
No. examined	1943	59	60	60	60	1936	60	60	60	60

	Males					Females				
Dose level [ppm]	**HCD range**	**0**	**67**	**200**	**600**	**HCD range**	**0**	**67**	**200**	**600**
No of animals		**60**	**60**	**60**	**60**		**60**	**60**	**60**	**60**
Myeloid leukosis (m)	--	1	0	0	0	--	0	1	0	0
Malignant lymphoma (m)	0-2	0	0	0	0	0-3	0	1	1	0
Liver										
No. examined	1952	60	60	60	60	1950	60	60	59	60
Adenoma (b)	0-1	0	0	0	0	0-1	0	1	0	0
Cholangioma (m)	0-1	0	1	0	0	0-2	0	0	0	0
Lungs										
No. examined	1954	60	60	60	60	1949	60	60	60	60
Squamous cell carcinoma (m)	0-1	1	0	0	0	--	0	0	0	0
Lymph node										
No. examined	695	57	56	53	57	--	42	43	44	46
Malignant lymphoma (m)	0-4	0	0	1	0	--	0	0	0	0
Mammary gland										
No. examined						457	3	2	4	1
Fibro adenoma (b)						0-6	3	1	4	0
Adenoma (b)						0-4	0	1	0	0
Adenocarcinoma (m)						0-6	3	2	4	0
Ovaries										
No. examined						1880	55	58	60	60
Granulosa theca cell tumour (b)						0-6	4	0	0	6
Carcinoma (m)						--	0	0	0	1
Granulosa theca cell tumour (m)						0-1	0	0	0	1
Pancreas										
No. examined	1913	58	60	58	60	1899	58	58	58	58
Islet cell tumour (b)	0-2	0	3	1	0	0-2	1	0	0	0
Pituitary										
No. examined	1858	58	58	58	60	1842	49	56	57	55
Adenoma (b)	4-22	9	8	5	8	7-24	4	3	12	6
Carcinoma (m)	0-1	0	2	1	0	0-2	1	0	2	0
Salivary glands										
No. examined	1913	59	59	60	60	1906	59	57	60	59
Adenoma (b)	--	0	0	1	0	0-1	0	0	0	0
Skin										
No. examined	363	0	2	4	0	360	1	1	0	0
Fibroma (b)	0-2	0	2	0	0	0-2	0	0	0	0
Fibro(myo)sarcoma (m)	0-2			2		0-1	0	0	0	0
Squamous cell carcinoma (m)	0-1	0	0	1	0	0-1	0	0	0	0
Spleen										
No. examined	1945	60	60	60	60	695	59	59	59	60
Haemangio-endothelioma (m)	0-1	1	1	0	0	0	0	0	0	0
Testes										
No. examined	1945	60	60	60	60					
Leydig cell tumor (b)	1-8	5	5	1	3					
Mesothelioma (b)	0-1	0	0	0	1					
Thyroid										
No. examined	1908	59	60	60	60	1892	60	60	60	60
Follicular adenoma (b)	0-2	1	0	1	0	0-3	0	2	0	0
Adenoma (C-cell) (b)	0-8	1	4	1	1	0-7	0	2	2	0
Medullary carcinoma (m)	0-8	5	9	8	4	0-5	0	3	7**	2
Follicular carcinoma (m)	0-2	0	2	0	0	0-1	0	1	0	0

	Males					Females				
Dose level [ppm]	**HCD range**	**0**	**67**	**200**	**600**	**HCD range**	**0**	**67**	**200**	**600**
No of animals		**60**	**60**	**60**	**60**		**60**	**60**	**60**	**60**
Urinary bladder										
No. examined	1915	60	57	59	59	1900	56	58	59	58
Adenocarcinoma (m)	--	0	0	1	0	--	0	0	0	0
Fibrosarcoma (m)	0-1	0	0	0	0	0-1	1	0	0	0
Uterus										
No. examined						1930	60	60	60	60
Adenoma (b)						0-5	1	1	0	1
Haemangioma (b)						--	1	0	0	0
Adenocarcinoma (m)						0-14	8	14	8	7
Leiomyosarcoma (m)						0-2	1	0	0	1
Squamous cell carcinoma (m)						0-1	1	0	1	1
Anaplastic carcinoma (m)						--	0	1	0	0
Fibrosarcoma (m)						0-5	0	0	3	1
Haematopoietic system										
No. examined	1943	1	0	0	1	1936	0	2	1	0
Myeloid leicosis (m)	--	1	0	0	0	--	0	1	0	0
Malignant lymphoma (m)	0-4	0	0	0	0	0-2	0	1	1	0
Leukaemic leucosis (m)	--	0	0	0	1	--	0	0	0	1

-- no Historical Control Data (HCD) available
* statistical significant different from control $p < 0.05$
** statistical significant different from control $p < 0.01$
(b) = benign, (m) 0 malignant
HCD from studies conducted from 05/1975 – 11/1984 with Wistar rats of the strain TNO/W74.

Methiocarb does not show any carcinogenic potential in the rat. However the study did not reach the maximum tolerated dose as no significant inhibition (> 20%) in acetylcholinesterase activity in erythrocytes or brain nor significant systemic toxicity was achieved. Nevertheless, taking into consideration that no specific organ toxicity was observed in the sub-chronic studies, it is unlikely that methiocarb has carcinogenic potential. The NOAEL for systemic toxicity was established at 200 ppm (9.3 mg/kg bw/day) based on reduction in body weight gain at the higher dose level. The NOAEL for carcinogenicity was 600 ppm (29/42 mg/kg bw/day), the top dose tested.

(Krötlinger *et al* 1981)

CA 5.6 Reproductive toxicity

Summary of reproductive and developmental toxicity studies

In a multi-generation study in rats from 1970, the NOAEL was 300 ppm (15 mg/kg bw/day) based on the absence of significant maternal, foetal or reproductive toxicity. No malformations were observed in parents or pups at dose levels of ≤ 300 ppm.

In a range-finding, one generation reproductive toxicity study in Wistar rats conducted in 1999 (M-035507-01-1, the NOAEL for parental toxicity was 100 ppm (equivalent to 6.1/8.1 mg/kg bw/day (m/f) in the premating period) based on retarded body weight gain in parent animals at ≥ 300 ppm. The NOAEL for reproduction was 100 ppm (6.1/8.1 mg/kg bw/day (m/f)) based on retarded body weight gain in pups at 300 ppm (18.5/22.4 mg/kg bw/day (m/f)). At the high dose of 900 ppm (76.5 mg/kg bw/day), in the presence of maternal toxicity (biologically relevant RBC AChE inhibition and severe body weight effects), there were some marginal / slight findings on reproduction parameters. Prenatal loss (1.63 pups/litter) was slightly increased in comparison to the controls; however the value was only marginally above or at the upper limit of historical control data (HCD)[11] of up to 1.6 pups/litter, respectively. This finding was associated with a lower mean litter size of 8.86 pups/litter, which was below the lower limit of the historical control data of 9.86 pups/litter. Pup viability on lactation day (LD) 4 of 86.92% was also slightly below the lower limit of the historical control data of 87.4%. The lactation index was marginally reduced at the high dose with 71.43% vs 78.7% in concurrent controls. However, the lactation index of the control rats was already outside the lower limit of historical control data of 85.1%, as were the lactation index of the low-dose group. There was no dose-relation obvious, since the lactation index in the mid-dose was within the HCD range. Thus, no effect on lactation index can be derived from these findings.

In the 1^{st} two-generation study conducted in 2000/01 (M-036790-01-1) dose levels were 0, 50, 150 and 500 ppm methiocarb (corresponding to 0-4.3/5.5-12.5/15.4-41/52.1 mg/kg bw/d (m/f) in F0, and 0-4.2/6-13.5/18.6-43.5/61.3 mg/kg bw/day (m/f) in F1 rats)). Signs of toxicity in parental F0 animals consisted of a significantly and biologically relevantly reduced AChE activity and significantly reduced body weights and / or body weight gains at 500 ppm. Body weights and / or body weight gain was also significantly reduced at ≥150 ppm in F1 parents during lactation of F2 and F2b. In this study prenatal loss was always in the range of the respective historical control data and showed no statistically significant increases. However, mean litter size of 9.78 and 9.68 pups/litter at 150 and 500 ppm (corresponding to 15.4 and 52.1 mg/kg bw/day) was statistically significantly reduced in comparison to controls, but only in the 1^{st} generation and only marginally in comparison to the respective historical control data (lower limit: 9.86 pups/litter). The fact, that in addition the mean value in the concurrent control group of 12.05 pups/litter was close to the upper limit of the historical control data of 12.75 pups/litter was contributing to the statistical significance. No effects on mean litter size were noted in F2 and F2b pups, all values were in the range of the respective historical control data. Therefore, the toxicological relevance of this finding is questionable. The viability index on lactation day 4 showed no effect in F1 and F2 pups, while it was reduced in F2b pups in all dose groups including controls (86.05 – 82.25 – 82.95 – 76.75% at 0 – 50 – 150 – 150 ppm (0 – 6.0 – 18.6 – 61-3 mg/kg bw/day) in comparison to the historical controls (87.4 – 100 %). There was also no effect on the lactation index in F1 pups. In the 2^{nd} generation the lactation index was overall low in F2 and F2b pups, with values below the lower limit of the historical controls (51.7 – 100%) in F2 pups at 50 and 500 ppm (with 46.23 and 42.7%) as well as in all doses including controls in F2b pups (43.87 – 37.3 – 34.04 – 28.68 % at 0 – 50 – 150 – 150 ppm (0 – 6.0 – 18.6 – 61.3 mg/kg bw/day)). Furthermore, pups in these groups displayed more frequently severe clinical signs indicative of a poor general condition. They were thin and had a food pasted mouth/nose and empty stomach (no

[11] The historical control data obtained from generation studies conducted in the same laboratory (i.e. Bayer Toxicology [redacted], Germany) between 1996 and 2004 (i.e. +/− 3 years of study conduct of methiocarb generation studies) using Wistar rats. For details pleaser refer to M-540041-01-1

milkspot). With also the control groups showing low viability (F2b) and lactation indices in the F2 and F2b pups, it is possible that other influences like an (subclinical) infection or a general, unspecific reduction in nursing capability in rats of this sub strain[12], however, expressed only in the second generation and not observed in the generation coming directly from the breeder, have contributed to pup deaths. The later possibility is considered as highly probable, because reduced lactation indices were noticed as well in F2 rats of a another two-generation study (including controls) running in parallel to the present study.
A clear treatment related effect cannot be derived from these data.
The parental NOAEL was 50 ppm (equivalent to 4.3 mg/kg bw/day/5.5 mg/kg bw/day (m/f)) based on reduced body weights in parental males and females (during lactation) at 150 ppm (12.5 mg/kg bw/day / 15.4 mg/kg bw/day (m/f)) and the NOAEL for reproduction was 50 ppm (4.3 mg/kg bw/day) based on a marginally reduced number of pups per litter in the 1st generation and a reduced lactation index at ≥ 150 ppm in the 2nd generation.

Due to the low lactation index observed in the 1st two-generation study in F2 / F2b control pups, an additional two-generation study (M-064945-01-1) was conducted. Dose levels were again 0, 50, 150 and 500 ppm methiocarb (corresponding in this case to higher doses of 0-4.6/6.9-14.8/21.3-55.1/83.9 mg/kg bw/d (m/f) in F0, and 0-5.6/7.6-16.3/22.3-69/89 mg/kg bw/d (m/f) in F1 animals)).
Effects on reproduction and offspring occurred exclusively at parentally toxic doses, indicated by significantly reduced body weights (up to −14%), and biologically relevant reduction of plasma AChE (up to −23% in F0 in week 5, −39% in F1-dams in week 7 after weaning) at 500 ppm.
In this study there was a borderline, but statistically significant increase in prenatal loss at 500 ppm (89 mg/kg bw/day) in the 2nd generation only. The mean value of 2.09 pups/litter was marginally above the upper limit of the historical control data (2.0 pups/litter) and statistical significance was at least supported by the fact that the mean value in the concurrent controls of 0.76 pups/litter was even below the lower limit of the historical control values of 1.0 pups/litter This was associated with a slight, statistically significant effect on litter size in the same dose and generation. Again, the mean value of 9.35 pups/litter was only slightly below the lower limit of historical controls of 9.5 pups/litter and the relatively high value of 11.88 pups/litter in the concurrent controls, which is above the higher limit of the historical controls (i.e. 11.28 pups/litter), contributed to statistical significance. There was no effect on pup viability on lactation day 4, all values were in the range of the historical control data. The lactation index, however, was statistically significantly reduced at 500 ppm in the 1st generation (53.1% vs. 76.2% in concurrent controls; 85.1 - 100% in historical controls). Pups in this group presented more frequently severe clinical signs indicating poor condition: they were thin, had a food pasted mouth/nose and empty stomach (no milkspot). Additionally, they had statistically significantly reduced body weights (up to −25%) and, as a consequence, slightly delayed preputial separation and vaginal opening.
The derived parental NOAEL was 50 ppm (6.9 mg/kg bw/day) based on reduced plasma AChE activity. The offspring NOAEL was 150 ppm based on reduced body weight, clinical signs, reduced plasma and RBC AChE, increased prenatal loss and litter size (both in F2 only), as well as a reduced lactation index (in F1 only) at 500 ppm (83,9 mg/kg bw/day).

Since in the 2nd two-generation study (M-064945-01-1) AChE activities measured in F1 animals were not conclusive to determine a NOAEL, a specific sub-chronic study (M-088195-01-1) with dietary levels of 0, 50, 150 and 500 ppm methiocarb (corresponding to 0-5.6/7.6-16.3/22.3-69/89 mg/kg bw/d (m/f)) was conducted with the objective to determine reliable AChE activities. F0 rats were treated during mating, pregnancy and lactation until weaning of F1 pups. F1 weanlings were then treated for further 12 weeks. Plasma and erythrocyte AChE activities were measured in F1 rats after 8 and 11 weeks of treatment, and brain AChE was determined at necropsy. Plasma AChE activity was biologically relevantly reduced in F1 rats (up to −27% and −47% at 150 and 500 ppm, respectively). RBC AChE activity was statistically significantly and biologically relevantly reduced only at the high

[12] As stated in the study report.

dose (up to −29%). The derived NOAEL of 50 ppm based on biologically relevant reduced plasma AChE activity in F1 rats confirmed the NOAEL of 50 ppm determined in the 1st and 2nd two-generation studies.

The overall NOAEL for parental toxicity, reproduction and offspring toxicity was 50 ppm (equivalent to 4.3/5.5 mg/kg bw in males or females).

Developmental toxicity studies were conducted in rats and rabbits.
In a developmental toxicity study in FB30 strain rats, the NOAEL for maternal toxicity was 3 mg/kg bw/day based on reduction of maternal body weights and for fetotoxicity and teratogenicity at 10 mg/kg bw/day the highest test dose.

In a subsequent developmental toxicity study in Wistar rats the maternal NOAEL was 0.5 mg/kg bw/day based on cholinergic signs, muscle fasciculations at 1.5 mg/kg bw/day. The NOAEL for developmental toxicity in rats was 5.0 mg/kg bw/day based on the absence of fetotoxicity or teratogenicity at the highest dose tested.

In a developmental toxicity study in New Zealand White rabbits the NOAEL for maternal toxicity was 3 mg/kg bw/day based on observed clinical signs at the 10 dose level and for fetotoxicity and teratogenicity at 10 mg/kg bw/day the highest test dose.

In a dermal route developmental toxicity study in Chinchilla rabbits the NOAEL was 50 mg/kg bw/day for maternal and foetal toxicity based on reduction in food consumption and body weight gain in dams and birth weight in foetuses.
In this study there was an increased incidence of delayed ossification and non-ossification of the number 5 sternebrae noted (litter basis) that was considered treatment-related. However, a comparison with historical control data on a fetal basis (report 2nd addendum page 7) demonstrated that the incidence of non-ossification, as well as delayed ossification is well within the historical control range. Therefore, the finding is considered to be incidental. Overall, there was no teratogenicity at the highest test dose (250 mg/kg bw/day).
Overall, the NOAELs in the rat were 0.5 mg/kg bw for maternal toxicity and 5 mg/kg bw for developmental toxicity, while in the rabbit they were 3 mg/kg for maternal toxicity and 10 mg/kg bw for developmental toxicity.
Methiocarb is not a primary reproductive toxin.

Table 5.6- 1: Summary of reproductive and developmental studies*

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL (mg/kg bw/day)	Main findings observed at LO(A)EL	Reference
Rat multi-generation (diet) 0-30-100-300 ppm	M F	15 (300 ppm)	>15 (300 ppm)	No significant maternal, fetal or reproductive toxicity at the highest dose tested	[redacted] 1970 M-010170-01-1
Rat, 1-generation (diet) dose-range finder 0-100-300-900 ppm 0-6.1/8.1-18.5/22.4-52.4/76.5 mg/kg bw/day	M F	6.1 8.1 (100 ppm)	18.5 22.4 (300 ppm)	Maternal: retarded body weight gain, ↓ plasma AChE in week 4 Offspring: retarded body weight (up to -21%) and body weight gain reproduction: no effects	[redacted]., 2002 M-035507-01-1

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL (mg/kg bw/day)	Main findings observed at LO(A)EL	Reference
Rat 2-generation (diet) 0-50-150-500 ppm F0: 0-4.3/5.5-12.5/15.4-41/52.1 mg/kg bw/day F1: 0-4.2/6-13.5/18.6-43.5/61.3 mg/kg bw/day	M F	4.3 5.5 (50 ppm)	12.5 18.6 (150 ppm)	Maternal: ↓ body weights during lactation (F1) offspring: ↓ litter size (F1) reproduction: ↓ lactation index (F2&F2b)#	[redacted] 2002 M-036790-01-1
Rat 2-Generation (diet) 0-50-150-500 ppm F0: 0-4.6/6.9-14.8/21.3-55.1/83.9 mg/kg bw/day F1: 0-7.6/5.6-22.3/16.3-89/69 mg/kg bw/day	Dam Fetal	4.6 (50 ppm) 14.8/21.3 (150 ppm)	21.3 (150 ppm) 55.10/83.9 (500 ppm)	↓ RBC AChE offspring: ↓ body weight (F1&F2), clinical signs, ↓ lactation index (F1) ↓ plasma & RBC AChE, slightly delayed vaginal opening and balano-preputial separation in F2 due to lower pup weights ↑ incidence of pups with poor condition (food pasted mouth/nose and empty stomach or intestine)	[redacted] U., 2002 M-064945-01-1
Rat, special sub-chronic (diet)	Dam Fetal	50 ppm	150 ppm	Offspring: ↓ plasma AChE in both sexes	[redacted] 2003 M-088195-01-1
Rat oral (gavage) developmental	Dam Fetal	3 10	10 > 10	Maternal: significant ↓ body weight	[redacted]., 1971 M-009391-02-1
Rat oral (gavage) developmental	Dam Fetal	0.5 5	5 >5	Maternal: cholinergic signs and muscle fasciculation	[redacted] 2002 M-038693-01-1
Rabbit oral (gavage) developmental	Dam Fetal	10 10	30 >30	Maternal: clinical signs (increased respiratory rate, loss of coordination, muscular tremors, marked transient ↓ body weight	[redacted] 1981 M-010210-01-1
Rabbit dermal developmental	Dam Fetal	50 50	250 250	Maternal: ↓ food consumption, ↓ body weight gain Offspring: ↓ birth weight no teratogenicity	[redacted] 1992 M-009985-03-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

M: male F: female ↓: decrease(d) ↑: increase(d)
D: dam bw: body weight

The lactation indices were generally lower in F2/F2b pups in all groups including control in comparison to F1 and HCD.

Comparison of the study results CLP criteria according to REGULATION (EC) No 1272/2008[7]

According to REGULATION (EC) No 1272/2008 a substance is considered to have a reproductive toxicity potential if it causes adverse effects on sexual function and fertility[13], or on the development[14]. A reproductive toxicity potential is also considered, if there are effects on or via lactation.
No structural malformations were observed in any of the reproductive and developmental toxicity studies on methiocarb.

In the different generational studies on methiocarb findings indicative of reproductive / developmental toxicity, i.e. increased prenatal loss, reduced litter size, reduced viability, and a reduced lactation index, were observed. However, these findings were not consistently observed in all studies, generations and at comparable dose levels.
For evaluation if these findings are related to methiocarb treatment, the incidences of these parameters in the dose-range finding, as well as 1st and 2nd two-generation study were taken into account in a weight of evidence approach. In addition, the incidences of the findings were compared with available historical control data (HCD)[11]. In order to give an overview maternal, reproduction and offspring data were sorted by dose (mg/kg bw/day) and generation for all studies in Table 5.6- 2 and Table 5.6- 3 below in comparison to the respective historical control data (HCD)[11] (see M-540041-01-1).

Prenatal loss
Only marginally increased incidences of prenatal loss close to the upper higher limit of the historical control data were observed at 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder as well as at 500 ppm or 89 mg/kg bw/day in the 2nd generation of the second two-generation study. All other values in the dose range finder as well as in all generations of the first two-generation study as well as in the 1st generation of the second two-generation study with doses up to 83.9 mg/kg bw/day were in the range of the historical control data. Since this borderline finding was not consistent over generations and doses, it is not considered to fulfill the requirements for a clear treatment related effect.

Litter size
Mean litter size was also marginally reduced at 150 and 500 ppm or 15.4 and 52.1 mg/kg bw/day in the 1st generation of the first two-generation study as well as at 500 ppm or 89 mg/kg bw/day in the 2nd generation of second two-generation study. All three values were close to the lower limit of the historical control values indicating maximally a borderline effect. Furthermore, no effect on litter size was observed in the 2nd generation (F2 and F2b) of the first two-generation study as well as in the 1st generation of the second two-generation study up to the high dose of 500 ppm. Apart from these three borderline cases a slight reduction in litter size (below the lower limit of the historical controls) was only seen at 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder. However, the fact that no effect was seen at the higher dose of 500 ppm or 83.9 mg/kg bw/day in the first generation of

[13] Adverse effects on sexual function and fertility => any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behavior, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems
[14] Adverse effects on development of the offspring => Developmental toxicity includes, in its widest sense, any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy, or as a result of parental exposure. These effects can be manifested at any point in the life span of the organism. The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency

the second two-generation study shows that also a possible marginal effect on litter size is not consistent over doses and generations and, as such, no clear treatment related effect.

Viability

A marginal reduction of pup viability on lactation day 4 (LD 4) was observed at the high dose of 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder. However, the value was at the lower limit of the historical control data (i.e. 87.4%). In the 2nd two-generation study there was no effect on pup viability at all up to and including to the high dose of 500 ppm or 89 mg/kg bw/day. Based on these data it is improbable that there is a treatment related effect of methiocarb on pup viability. In the 1st two-generation study there was also no effect on pup viability in the F1 and F2 pups. In F2b pups, however, reduced viability indices were noted for all dose groups including controls. Although there seemed to be a very slight dose relationship, it is not clear, if in this single case an additional influence, i.e. a (subclinical) infection of the pups or an unspecific reduction in nursing capability in the F1 animals, contributed to this finding. It is noticeable that pups in these groups displayed more frequently severe clinical signs indicative of a poor general condition. They were thin and had a food pasted mouth/nose and empty stomach (no milkspot). Taken all these facts together, an effect of methiocarb alone on pup viability on lactation day 4 is not probable due to the missing consistency over doses and generations.

Lactation index

The lactation index was reduced at the high dose of 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder with a value of 71.43% below the lower limit of the respective historical control data (for the 1st generation: 85.1 - 100%, for the 2nd generation: 51.7 -100%). The parameter was also statistically significantly reduced in the 1st generation of the 2nd two-generation study at the high dose of 500 ppm or 83.9 mg/kg bw/day with a value of 53.1 %. All other values in both studies including the lactation index of the 2nd generation of the second two-generation study at 500 ppm or 89 mg/kg bw/day of 75.93% were in the range of the respective historical control data.
In the 1st generation of the first two-generation study the lactation index was in the range of the historical control values. However, starting with the F2 pups the lactation index was overall low in F2 and F2b pups in the first two-generation study. Values below the lower limit of the historical controls (51.7 – 100%) were noted in F2 pups at 50 and 500 ppm (with 46.23 and 42.7%) as well as in all doses including controls in F2b pups (46.23 – 37.3 – 34.04 – 28.68 % at 0 – 50 – 150 – 150 ppm (0 – 6.0 – 18.6 – 61.3 mg/kg bw/day)). Pups in these groups displayed more frequently severe clinical signs indicative of a poor general condition (thin pups, pups with a food pasted mouth/nose and empty stomach (no milkspot)). With also the control groups showing low viability (F2b) and lactation indices in the F2 and F2b pups, it is possible that other influences like for example a (subclinical) infection of the young pups or an unspecific reduction in nursing capability in the F1 animals may have contributed to pup deaths. Since a clear treatment related effect cannot be derived from these data, the lactation indices of the 2nd generation of the first two-generation study should not be taken as the basis for the evaluation of a compound related effect.
Overall, there seemed to be a limited effect on the lactation index at the high maternally toxic dose of 83.9 mg/kg bw/day in the 1st generation of the second two-generation study. However, again, this finding was not consistent between doses and generations (i.e., there was no effect at 89 mg/kg bw/day in the 2nd generation of the second two-generation study).

In conclusion, neither of the possible effects on prenatal loss, litter size, pup viability on lactation day 4 or the lactation index was consistently observed over doses and generations. Therefore, a specific effect of methiocarb on these parameters can be ruled out. What could be possible is that methiocarb contributes to pup death as a secondary, non-specific consequence of maternal toxicity.
Therefore, according to the CLP criteria a classification of methiocarb for reproductive toxicity is not justified.

Classification/labelling regarding reproductive toxicity for methiocarb:
- Regulation (EC) No 1272/2008 (CLP): none

Table 5.6- 2: Summary of maternal toxicity, prenatal loss, litter size, lactation index, and viability in generation studies on methiocarb (sorted by dose [mg/kg bw/day])

Dose [ppm in diet]	DRF	1st	2nd	1st	2nd	DRF	1st	2nd	DRF	1st	DRF	2nd
	control			50	50	100	150	150	300	500	900	500
F0 dams (mg/kg bw)	0	0	0	5.5	6.9	8.1	15.4	21.3	22.4	32.1	76.5	83.9
F1 dams# (mg/kg bw)	0	0	0	6.0	7.6	--	18.6	22.3	--	61.3	--	89
Maternal toxicity in dams												
Body weight and body weight gain		F0, F1		F0, F1: N.E.	F0, F1: N.E.	F0: N.E.	F0: bw gain ↓ up to −20%**; during gestation, up to −44% during lactation (not stat. sign) F1 (F2): N.E. F1 (F2b): N.E.	F0, F1: N.E.	F0: bw: ↓ up to −19%* during gestation, −11% during lactation bw loss during gestation; during lactation bw gain reduced up to only 4% of controls	F0: bw gain ↓ up to −16%; during gestation, up to −48% during lactation (not stat. sign) F1 (F2): only slightly ↓ F1 (F2b): only slightly ↓	F0: bw; sign ↓ up to −13**, −17**, −16%** during premating, gestation, lactation bw gain sign ↓ −44%** gestation, −72%** during lactation, partly bw loss during premating, gestation & lactation	F0: bw ↓ up to −8%**; during lactation F1: ↓ from week 1 onwards; up to −16%**
Plasma AChE				F0, F1: N.E.	F0, F1: N.E.	N.E.	F0: −11% F1: −11%	F0: −13% F1: −6%	−26%*	F0: −23%* F1: −37%*	F0:−44%**	F0: −23%* F1: −39%**
RBC AChE				F0, F1: N.E.	F0, F1: N.E.	N.E.	F0, F1: N.E.	F0, F1: N.E.	N.E.	F0: −8% F1: N.E.	F0: −22%	F0: N.E. F1: −24%**
Brain AChE				F0, F1: N.E.	F0, F1: N.E.	N.E.	F0: −9% F1: N.E.	F0, F1: N.E.	N.E.	F0: −14% F1: N.E.	N.E.	F0, F1: N.E.

Dose [ppm in diet]	DRF	1st	2nd	1st	2nd	DRF	1st	2nd	DRF	1st	DRF	2nd
	control			50	50	100	150	150	300	500	900	500
F0 dams (mg/kg bw)	0	0	0	5.5	6.9	8.1	15.4	20.3	22.4	52.1	76.5	83.9
F1 dams# (mg/kg bw)	0	0	0	6.0	7.6	--	18.6	22.3	--	61.3	--	89
Prenatal loss [mean/litter]												
F1	0.7	0.71	1.08	0.52	0.33	0.88	1.48	1.09	0.43	1.3	**1.63**	1.04
F2	--	nd	0.76	nd	1.60	--	nd	1.53	--	nd	--	**2.09***
F2b	--	--	--	nd	--	--	nd	--	--	nd	--	--
HCD: F1: 0.6 – 1.6; F2: 0.8 – 2.0												
Litter size (live pups)												
F1	10.89	12.05	11.52	11.09	11.24	9.63	**9.78****	10.91	10.57	**9.68****	**8.86**	10.75
F2	--	11.13	**11.88**	10.79	10.30	--	10.73	10.58	--	10.13	--	**9.35****
F2b	--	9.74	--	10.36	--	--	10.35	--	--	9.91	--	--
HCD: F1: 9.86 – 12.75; F2: 9.3 – 11.28												
Viability LD 4												
F1	97.76	92.3	90.26	92.49	92.6	93.54	96.01	94.47	94.72	97.05	**86.92**	92.18
F2	--	95.89	97.33	92.89	92.1	--	96	98.45	--	91.34	--	95.33
F2b	--	**86.05**		**82.25**	--	**82.95**			--	**76.75**	--	
HCD: F1: 87.4 – 100; F2: 87.4 - 100												

Dose [ppm in diet]	DRF	1st	2nd	1st	2nd	DRF	1st	2nd	DRF	1st	DRF	2nd
	control			50	50	100	150	150	300	500	900	500
F0 dams (mg/kg bw)	0	0	0	5.5	6.9	8.1	15.4	20.3	22.4	52.1	76.5	83.9
F1 dams# (mg/kg bw)	0	0	0	6.0	7.6	--	18.6	22.3	--	61.3	--	89
Lactation index LD 21												
F1	**78.7**	**72.14**	**76.2**	**80.34**	86.2	**77.23**	**82.35**	**78.8**	96.43	**76.62**	**71.43**	**53.1****
F2	--	61.78	77	**46.23**	82.4	--	58.14	78.48	--	**42.7**	--	75.93
F2b	--	**43.87**		**37.3**		--	**34.04**			**28.68**	--	
HCD for LD 21: F1: 85.1 – 100, F2: 51.7 – 100; no data for F2b												

* Statistically significantly different at p<0.05 ** statistically significantly different at p< 0.01; ↓ = reduced
DRF: Dose range finding study M-035507-01-1
1st: main two-generation study M-036790-01-1
2nd: repeated two-generation generation study M-064945-01-1
Figures printed in bold exceed the historical control range.
N.E. = no effects

Dose levels given for F1 rearing F2 pups. There are no data on dose levels in mg/kg bw/day available for F1 dams rearing F2b.

Nd = no data, F1 dams were mated twice

Table 5.6- 3: Observation in pups during lactation

Dose [ppm in diet]	DRF	1st	2nd	1st	2nd	DRF	1st	2nd	DRF	1st	DRF	2nd
	Control			50	50	100	150	150	300	500	900	500
F0 dams (mg/kg bw)	0	0	0	5.5	6.9	8.1	15.4	21.3	22.4	52.1	76.5	83.9
F1 dams (mg/kg bw)#	0	0	0	6.0	7.6	--	18.6	22.3	--	61.3	--	89
Pup weight LD 0-28												
F1	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	Reduced bw –14% LD 7, stat sign LD14-28 up to –21%	LD 0: bw –14% (f only), afterwards N.E.	Reduced bw LD 7-28, stat. sign LD 14-28, up to -19%	LD 4 – LD28: bw reduced up to –15/–25% (m/f), partly stat. sign
F2	--	N.E.	N.E.	N.E.	N.E.	--	LD14: bw reduced –11% not stat. sign	N.E.	--	LD 4, LD 7 bw reduced up to –17% on LD 7 (not stat. sign.); afterwards no effect	--	LD 28: bw stat. sign reduced: –11% / –9% (m/f)
F2b	--	N.E.	--	N.E.	--	--	LD 14: bw reduced –13% (f)	--	--	LD 4 – LD14: bw reduced up to –22%/ –13% (m/f);	--	--

Dose [ppm in diet]	DRF	1st	2nd	1st	2nd	DRF	1st	2nd	DRF	1st	DRF	2nd
	Control			50	50	100	150	150	300	500	900	500
F0 dams (mg/kg bw)	0	0	0	5.5	6.9	8.1	15.4	21.3	22.4	52.1	76.5	83.9
F1 dams (mg/kg bw)#	0	0	0	6.0	7.6	--	18.6	22.3	--	61.3	--	89
Pup – clinical observation: small (incidence %)												
F1	8.8	3.3	0.0	3.0	0.0	13.1	2.1	0.0	1.8	4.6	23.4	1.5
F2	--	0	0.7	1.1	0.2	--	0.0	2.3	--	0.9	--	3.2
F2b	--	2.6	--	3.9	--	--	0.8	--	--	1.8	--	--
Pup – clinical observation: thin (incidence %)												
F1	0.98	7.7	6.9	7.1	2.1	3.6	4.0	6.4	1.3	9.6	17.2	13.1
F2	--	3.5	4.0	6.9	4.7	--	4.2	6.2	--	4.2	--	15.3
F2b	--	9.7	--	8.2	--	--	10.3	--	--	9.9	--	--
Pup – clinical observation: mouth/nose pasted with feed (incidence %)												
F1	3.9	2.4	2.1	0.74	3.8	2.4	0.33	8.8	17	0.5	31.3	13.1
F2	--	12.3	3.0	14.1	3.3	--	7.5	2.3	--	10.6	--	14.9
F2b	--	15.9	--	12.6	--	--	13.6	--	--	17.1	--	--
Pup – clinical observation: labored breathing (incidence %)												
F1	0.98	0.4	5.9	0.0	3.4	2.4	0.1	5.6	10.7	0.0	14.1	0.2
F2	--	0.4	2.4	1.1	2.8	--	0.0	0.4	--	1.7	--	4.2
F2b	--	3.1	--	1.3	--	--	1.4	--	--	1.8	--	--

Dose [ppm in diet]	DRF	1st	2nd	1st	2nd	DRF	1st	2nd	DRF	1st	DRF	2nd
	Control			50	50	100	150	150	300	500	900	500
F0 dams (mg/kg bw)	0	0	0	5.5	6.9	8.1	15.4	21.3	22.4	52.1	76.5	83.9
F1 dams (mg/kg bw)#	0	0	0	6.0	7.6	--	18.6	22.3	--	61.3	--	89
Pup – clinical observation: no milk spot (incidence %)												
F1	0.0	0.0	9.3	0.0	3.8	0.0	0.0	6.8	0.0	0.0	0.0	12.4
F2	--	8.2	2.0	7.6	3.3	--	4.2	3.9	--	6.8	--	8.4
F2b	--	9.	--	7.8	--	--	9.4	--	--	12.2	--	--
Pup – clinical observation: cold to touch (incidence %)												
F1	0.98	0.0	0.0	0.0	0.4	0.0	0.33	0.8	1.3	0.0	2.4	3.1
F2	--	2.3	0.3	3.8	2.8	--	1.2	0.8	--	3.4	--	2.3
F2b	--	5.1	--	0.9	--	--	5.6	--	--	6.8	--	--

DRF: Dose range finding study M-035507-01-1
1st: main 2-generation study M-036790-01-1
2nd: repeated 2-generation study M-064945-01-1
Dose levels given for F1 rearing F2 pups. There are no data on dose levels in mg/kg bw/day available for F1 dams rearing F2b.

CA 5.6.1 Generational studies

In the previously evaluated two-generation study with methiocarb a relative low lactation index of control F2 pups compared to those of F1 controls was observed. Therefore, a further two-generation study was conducted in a different rat sub-strain, which had been shown previously to have a better lactation performance. In this new study the obtained acetylcholinesterase data for the F1 generation were questionable. Therefore, an additional special sub-chronic study was conducted in order to collect reliable data on acetylcholinesterase inhibition in F1 rats which were treated with methiocarb during their whole pre- and postnatal development, lactation up to weaning and then over a post-weaning period of twelve weeks. Both studies are summarised in the following.

Report:	KCA 5.6.1/04 [redacted] か; [redacted]; 2002; M-064945-01-1
Title:	H 321 - Two-generation study in Wistar rats (supplementary study to the two-generation study T4069306)
Report No.:	AT00047
Document No.:	M-064945-01-1
Guideline(s):	US-EPA OPPTS 870.3800; OECD 416; JMAFF (1985)
Guideline deviation(s):	not applicable
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:	H 321
Synonym (common name):	Methiocarb
Description:	White light-beige powder
Lot/Batch no:	00313/2121
Purity:	98.9-99.3%
Stability of test compound:	guaranteed for study duration; expiry date: 2002-01-31
2. Vehicle:	Plain diet
3. Test animals:	
Species:	rat
Strain:	Wistar: CrlGlxBrlHan:WI
Sex:	Males and females Females were nulliparous and non-gravid
Age:	5-6 weeks
Weight at study start:	Males: 126 g (range: 111-152 g) Females: 109 g (range: 96-128 g)
Source:	[redacted], Germany
Acclimatization period:	1 week
Diet:	NAFAG 9441 G4 ([redacted] [redacted] *ad libitum*
Water:	tap water (municipal water supply), *ad libitum*
Housing:	During the acclimatization period and study rats were housed singly under conventional conditions in Makrolon® cages. During the mating periods females were co-housed overnight with their males. As bedding material low-dust soft-wood shavings were used. When parturition was near cages of females were provided

with a special nesting material such as coarse wood shavings. Both, bedding and nesting material were supplied by [redacted], and tested for contaminants on a random basis.

B. Study design and methods

1. Animal assignment and treatment:

Dose:

Dose (ppm)	**0**	**50**	**150**	**500**
Actual doses (mg/kg bw/day)				
F0 males	0	4.6	14.8	55.1
F0 females	0	6.9	21.2	83.9
F1 males	0	5.6	16.3	69.0
F1 females	0	7.6	22.3	89.0

Duration: F0 animals were dosed prior to (about 10 weeks) and during their mating (up to 3 weeks), during the resultant pregnancy (about 22 days), and up to weaning of their F1 offspring (28 days).

F1 offspring (selected animals) were dosed during their growth into adulthood (about 13 weeks), mating (up to 3 weeks), during resulting pregnancy (about 22 days), and up to weaning of F2 pups (28 days).

Application route: *Ad libitum* in the diet up to necropsy

Dietary preparation: H 321 was blended with NAFAG 9441 G4. To minimize dust formation 1% peanut oil (DAB 10) was added. The amounts of test item were calculated on the basis of an assumed 100% content of H 321. The diet mixtures were prepared once a week.

Group size: 25/sex/group

Observations: Analysis on homogeneity and stability of test substance in the diet, clinical signs, mortality, detailed clinical examinations (general health status, behaviour, condition of fur, examination of orifices and excretory products), body weight, food intake, reproductive parameters (estrus cycle, insemination rate, duration of pregnancy, pup data), necropsy (macroscopic examination, organ weights, histopathology), cholinesterase activity (plasma, RBC, brain), examination of sperm, developmental milestones (F1)

Statistics: Dunnett-Test in connection with a variance analysis for body and organ weights of parent animals; an adjusted Welsh test for cholinesterase data; the Kruskal-Wallis-Test with a Steel-Test for food consumption data. These calculations were performed using SAS® routine on a HP 3000 computer system.

For parametric data of the reproductive parameters the two-tailed Fisher's exact probability test (in case of a positive CHI-SQUARE test with a significance levels of $\alpha = 5\%$) was used. A Fisher's exact CHI-SQUARE test was also used for sperm motility and morphology data. For nonparametric data of the reproductive parameters the Dunnett-Test (in case of a positive ANOVA test with a significance levels of $\alpha = 5\%$)

was used. These calculations were performed using a HP Vectra PC connected with an Alpha computer TASC (Grosse) system.
The sperm and spermatid count data were evaluated with the t-test using the Excel program.
The mean pup weight of each individual litter was used as the basis for calculating the pup weight means of the dose groups. The litter size calculation was based on the number of female animals with living pups.

II. Results and discussion

A. Analysis of the test substance in the diet

The analytical data revealed that the homogeneity of the feed mixtures is confirmed in NAFAG 9441 G4 diet. The stability (15 days) of the test item in the NAFAG 9441 G4 diet was confirmed for the concentration range used in special mixtures. The content checks including a test of homogeneity on actual, randomly taken diet samples (five time points) assure that during the study appropriate and equal mixture procedures were followed.

B. F0 generation

Clinical signs and mortality

No test substance-related effects on the appearance, health or behavior were observed in male or female F0 animals at levels of up to 500 ppm. One 50 ppm male showing several signs of poor general condition had to be killed prescheduled and one 150 ppm female died without exhibiting clinical symptoms during week 9.

Body weights of F0 parent animals

The body weight development of male and female F0 animals receiving up 150 ppm H 321 was not significantly different from control values.
At 500 ppm F0 males generally gained significantly less body weight (−9% week 16). High dose F0 females had significantly lower body weights during the lactation period (maximal −8% on day 4 of lactation – week 15).

Table 5.6.1/04- 1: Summary of body weights of F0 generation (means, g)

	males				Females			
Dose (ppm)	0	50	150	500	0	50	150	500
Week								
0	127	128	126	124	108	110	108	109
1	170	170	165	153**	130	134	131	126
2	212	211	206	189**	148	153	149	144
3	251	249	245	227**	164	171	167	159
4	281	278	277	258**	180	184	181	172
5	304	300	301	281**	189	195	192	182
6	329	322	324	303**	200	206	204	193
7	348	339	341	319**	209	215	212	203
8	364	353	355	333**	215	219	218	207
9	377	364	367	345**	220	226	225	215
10	390	374	37	357**	225	230	229	218
11	388	371	378	354**	222	224	222	214

Dose (ppm) Week	males 0	50	150	500	Females 0	50	150	500
12	399	378	389	367*	242	245	244	235
13	406	384	397	373*	268	271	268	258*
14	419	400	409	384**	326	330	323	311
15	426	407	415	390**	251	254	253	240
15 (day 4)	--	--	--	--	266	269	264	245**
16	436	415	424	397**	271	275	271	252**
17	--	--	--	--	259	263	266	252
18	--	--	--	--	268	271	267	255*
19	--	--	--	--	247	250	249	243

* Statistically significantly different from control ($p < 0.05$)

** Statistically significantly different from control ($p < 0.01$)

Food consumption and test substance intake of F0 parent animals

Food intake in F0 males at 50 ppm and F0 females up to 150 ppm was not affected. At 150 ppm F0 males consumed 10% more food. This was not considered an adverse effect though group means were marked as statistically significant in few weeks. At 500 ppm males consumed 15% and females 20% more food per animal. A clear increase (+24 to 25%) in food intake of 500 ppm males and females was also true, if the intake data per kg body weight is considered.

The test substance intake roughly corresponds to the theoretical dose levels up to 150 ppm. As a consequence of the increase in food consumption per kg body weight there was a somewhat higher test substance intake in high dose males and females than expected from the theoretical dose factor.

Table 5.6.1/04- 2: Mean daily and cumulative intake of food and test substance during the premating period (F0; 70 days)

	Dose ppm	males 0	50	150	500	females 0	50	150	500
Food intake g/animal	total	1725	1783	1892	1971	1717	1804	1814	2063
	per day	24.6	25.5	27.0	28.2	24.5	25.8	25.9	29.5
Food intake g/kg body weight	total	6137	6464	6920	7709	9488	9702	9932	11743
	per day	87.7	92.3	98.9	110.1	135.5	138.6	141.9	167.8
Test substance intake mg/animal	total	-	89	284	988	-	90	272	1032
	per day	-	1.3	4.1	14.1	-	1.3	3.9	14.7
Test substance intake mg/kg body weight	total	-	323	1038	3855	-	485	1490	5871
	per day	-	4.6	14.8	55.1	-	6.9	21.3	83.9

Gross pathological examinations in F0 parent animals

No significant gross pathological findings were observed in male or female F0 animals at levels of up to and including 500 ppm. Concerning uterine implantation sites counted during necropsy no toxicologically relevant discrepancies between the numbers of implantation sites and those of delivered pups occurred. If controls and treated pups are compared (see Table 5.6.1/04- 3). Accordingly there was no change in prenatal loss up to 500 ppm.

Table 5.6.1/04- 3: Summary of implantation sites and prenatal loss in F0 females

Dose ppm	0	50	150	500
No. of implantation sites	316	244	276	284
No. of pups at birth	289	237	251	259
Prenatal loss (means per litter)	1.08	0.33	1.09	1.04

Organ weights in F0 parent animals

There were significantly reduced absolute liver weights in high-dose males. This organ difference is clearly secondary to body weight reduction found in 500 ppm males. Also the significantly increased relative spleen and testes weight mean of 500 ppm males is based on the differences in body weight. Among the remaining organ weights no remarkable or dose-dependent changes were obvious.

Table 5.6.1/04- 4: Mean organ weights of F0-generation

		males				females			
	Dose ppm	0	50	150	500	0	50	150	500
Terminal body weight	(g)	441	423	437	401*	249	253	249	244
Brain weight	absolute (mg)	2060	2079*	2109**	2009	1912	1941	1927	1933
	relative (mg/100 g bw)	458	501*	487	506**	770	771	777	796
Pituitary weight	absolute (mg)	12	12	12	11	14	14	14	14
	relative (mg/100 g bw)	3	3	3	3	6	6	6	6
Adrenal weight	absolute (mg)	66	68	65	64	82	78	84	84
	relative (mg/100 g bw)	15	17	15	16	33	31	34	35
Thyroid weight §	absolute (mg)	13	13	13	14	11	11	12	12
	relative (mg/100 g bw)	3	3	3	3	4	4	5	5
Liver weight	absolute (mg)	14535	14895	14551	13050*	11419	11011	10053*	10238
	relative (mg/100 g bw)	3294	3548	3333	3269	4589	4364	4048*	4208
Spleen weight	absolute (mg)	722	694	736	718	595	570	573	547
	relative (mg/100 g bw)	165	166	169	180*	240	225	231	225
Kidney weight	absolute (mg)	2738	2938	2839	2698	1942	1881	1889	1893
	relative (mg/100 g bw)	624	717	652	677	782	747	760	779
Testes/ Ovaries weight	absolute (mg)	3583	3478	3488	3568	183	170	185	184
	relative (mg/100 g bw)	819	830	803	896*	73	67	74	76
Epididymides §/ Uterus weight	absolute (mg)	827	744*	724**	808	657	636	819	673
	relative (mg/100 g bw)	188	177	166**	202	264	253	329	277
Seminal vesicle weight	absolute (mg)	1356	1279	1348	1252				
	relative (mg/100 g bw)	310	302	311	312				
Prostate weight	absolute (mg)	1016	814**	834**	1019				
	relative (mg/100 g bw)	233	195**	192**	255				

§ Unilateral determination
* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Spermatological evaluations in F0 males

There were no remarkable changes in sperm motility and sperm morphology between control and high-dose males. The mean frequency of sperm abnormalities in samples of the 500 ppm group was comparable with that at 0 ppm. One high-dose male had no sperms and only few spermatids. This finding is considered as incidental. The number of spermatids and sperms counted in the right testis or epididymis was not affected. In summary there were no statistically significant differences among these parameters. Therefore, no sperm analyses were done in 50 and 150 ppm dose groups.

Table 5.6.1/04- 5: Evaluation of sperm motility and morphology and spermatid counts (mean values of 25 (0 ppm) and 24 (500 pp) males/dose)

Dose ppm	Sperm motility and morphology: Sperm motility 1st min (%)	5th min (%)	Difference (%)	Abnormal sperms %	Spermatid counts, Mean number of: Spermatids per mg testis	Sperms per mg epididymis
0	80	73	–7	0.56	50944	840148
500	79	73	–6	0.38	50547	1060696

Estrous cycle staging in F0 females

Estrus staging was done over 19 days beginning three weeks before F0 rats were cohoused for mating. As can be seen from Table 5.6.1/04- 6 below there was no adverse effect on the estrus cycle parameters up to 500 ppm.

Table 5.6.1/04- 6: Evaluation of the oestrus cycle in F0 females

Dose ppm	0	50	150	500
Mean cycle length in days	4.01	4.00	4.08	4.12
Mean number of cycles in 19 days	3.76	3.92	3.75	3.68
No. of females cycling normally	25	25	25	25

Histopathological investigations in F0 rats

There was no evidence of a substance-induced effect on any organ.

Determination of cholinesterase activity

Table 5.6.1/04- 7 summarizes the results of the cholinesterase activity measurements. As can be seen there are no biological relevant (i.e. < –20%) changes / reductions of cholinesterase activities up to 150 ppm (males) or 500 ppm (females) either in the plasma, erythrocytes or brain. The significantly lower mean activity of the brain cholinesterase for 150 ppm males does not reflect a treatment effect since, a dose dependency is lacking. In addition, the activity is only reduced by about 7% and therefore not biologically relevant. At 500 ppm there was a significantly lower activity of the plasma and erythrocyte cholinesterase in males. However, the values were still within the 2s-range of controls from previous studies.

Table 5.6.1/04- 7: Summary of cholinesterase activities in F0 rats (mean values)

AChE activity	Dose ppm	males				females			
		0	50	150	500	0	50	150	500
Plasma#	kU/L	0.37	0.38	0.33	0.28*	1.63	1.63	1.42	1.25
Erythrocytes#	kU/L	0.74	0.76	0.69	0.55**	0.75	0.75	0.81	0.81
Brain##	U/g	9.25	9.04	8.60*	9.24	10.00	10.03	9.86	10.13

5 weeks after study begin ## At necropsy
* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Parameters of Reproduction in F0 parent animals

The insemination, fertility and gestation indices as well as the mean duration of pregnancy did not differ to a toxicologically relevant extent from the control data at levels of up to 500 ppm. There were some F0 females (1-0-1-0 with ascending dose) which had been found to be sperm-positive after the first day of co-housing, but failed to become pregnant. According to experience this could happen, if an inexperienced male, co-housed with a female for the first time, inseminated the female outside the estrus. This assumption is obviously correct, since none (0-0-0-0) of these animals had any pups when remated with the same male for one week following the three week co-housing period.
The mating performance was not affected by the treatment at levels of up to 500 ppm.
There were no toxicologically relevant test substance related effects on life birth indices, the total number of born pups, the mean litter size and the percentages of males born up to and including 500 ppm.

Table 5.6.1/04- 8: Data on mating, fertility, gestation (F0) and litter parameters at birth (F1)

Dose ppm	0	50	150	500
No. of inseminated females on days of mating period Day 1-4	21	22	22	25
Day 8-11	2	2	1	0
Day 15-18	0	0	0	0
Insemination index %	100	96	100	100
Fertility index %	100.0	87.5	95.8	96.0
Gestation index %	100	100	100	100
Gestation length days	22.09	22.05	22.36	22.17
Co-housed females n	25	25	24	25
Litters alive n	25	21	23	24
Pups (total) n	289	237	251	259
Pups (dead) n	1	1	0	1
Live birth index %	99.6	99.6	100.0	99.7
Males %	53.8	54.4	52.6	10.91
Mean litter size[1)] n	11.52	11.24	10.91	10.75

[1)] Viable pups only n = number

Clinical observations in F1 pups

No remarkable clinical signs were observed in F1 pups during the four week lactation period at levels of up to 150 ppm. The higher number of pups showing a nose and/or mouth pasted with food at 150 ppm considered to be incidental since the number of litters affected is comparable with that of controls and is not noted again in 150 ppm F2 pups. However, at 500 ppm more pups being cold, small/thin and/or pasted with food at nose and mouth region were found. These effects are considered to be treatment-related.

Body weights of F1 pups

As shown in Table 5.6.1/04- 9 the birth weights of pups of the treatment groups were comparable with those of controls. The body weights during lactation were not toxicologically relevantly reduced up to 150 ppm in both sexes. At 500 ppm a mostly significantly body weight depression due to the treatment was noted in pups of both sexes with a maximum on day 7 (males -15%; females -25%).
The significantly increase in birth weight means of 150 ppm pups may be based on the fact, that incidentally parturition of several litters were completed after the second daily clinical inspection on that day and was therefore documented one day later.

Table 5.6.1/04- 9: Mean pup weight at birth and during lactation (F1)

	males				females			
Dose ppm	**0**	**50**	**150**	**500**	**0**	**50**	**150**	**500**
D0	6.02	6.19	6.47*	6.01	5.71	5.94	6.16*	5.63
D4[#]	9.80	10.17	9.92	8.68*	9.64	9.56	9.67	8.12**
D7	14.87	15.78	14.49	12.60**	14.76	14.94	14.60	11.05**
D14	31.03	32.03	31.52	28.77*	30.87	30.17	30.68	26.73*
D21	50.45	51.88	51.82	46.50*	49.66	48.87	49.91	45.11*
D28	82.06	84.37	83.24	73.61**	78.61	77.13	78.70	68.94**

[#] After culling
* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Viability, rearing and lactation indices of F1 pups

The viability and rearing indices were not affected in any dose. The lactation indices were not dose-dependently changed up to 150 ppm. A significantly lower lactation index was observed at 500 ppm methiocarb.

Table 5.6.1/04- 10: Viability, rearing and lactation indices (F1)

Dose ppm		**0**	**50**	**150**	**500**
Viability index (%)	D4	90.26	92.60	94.47	92.18
Rearing index (%)	D28	100.0	95.2	100.0	91.7
Lactation index (%)	D21	76.2	86.2	78.8	53.1**

** Statistically significantly different from control ($p < 0.01$)

Gross pathological changes in F1 pups or weanlings

There were no treatment-related macroscopical alterations in F1 pups up to 500 ppm necropsied during the lactation period up to and including weaning.
No skeletal deviations were determined in the F1 pups, which died before day four postpartum, were killed in the process of litter reduction on postpartum day four, or were necropsied unscheduled during lactation at levels of up to 500 ppm.

Organ weights of F1 weanlings

There were no statistically significant changes in mean absolute and relative organ weights up to 500 ppm.

Table 5.6.1/04- 11: Organ weights of F1 weanlings

		males				females			
	Dose (ppm)	**0**	**50**	**150**	**500**	**0**	**50**	**150**	**500**
Body weight	(g)	84.8	86.8	84.8	74.9	79.2	77.9	79.3	70.4*
Brain	absolute (mg)	1545	1568	1598	1552	1522	1519	1522	1466
	relative (mg/100 g bw)	1953	1829	1899	2112	1943	1965	1932	2126
Spleen	absolute (mg)	302	325	295	262	272	276	259	245
	relative (mg/100 g bw)	348	375	349	350	346	354	330	348
Thymus	absolute (mg)	327	362	344	322	325	347	330	295
	relative (mg/100 g bw)	379	417	406	432	413	444	416	414
Uterus	absolute (mg)					131	126	115	96
	relative (mg/100 g bw)					167	163	144	136

* Statistically significantly different from control ($p < 0.05$)

C. F1 generation

Clinical signs and mortality

No test substance-related effects on the appearance or behaviour were observed in male or female F1 parent animals at levels of up to 500 ppm. Two 50 ppm males died during the blood sampling. Therefore, there was no increase in mortality up to 500 ppm.

Body weights of F1 parent animals

There were no test substance-related effects on body weights of F1 rats up to 150 ppm. At 500 ppm significantly reduced body weights were noted in males (max. –15% week 20) and females (max. –12% week 10) indicating that this dose exceeded the maximal tolerated dose (MTD).

Table 5.6.1/04- 12: Summary of body weights of F1 generation parents (means, g)

Dose (ppm) Week	males 0	50	150	500	Females 0	50	150	500
0	78	81	80	73	75	75	75	69
1	115	115	112	97**	101	103	98	88**
2	151	154	149	127**	125	128	124	110**
3	194	196	188	159**	146	149	144	128**
4	234	235	228	193**	164	166	160	144**
5	269	271	264	227**	178	180	176	157**
6	302	302	296	254**	191	192	187	170**
7	328	323	315	276**	201	201	197	177**
8	349	342	336	296**	210	209	206	187**
9	366	361	356	341**	216	217	214	194**
10	378	373	370	328**	226	225	220	199**
11	392	387	383	341**	229	230	225	203**
12	404	396	393	348**	234	234	229	208**
13	413	404	404	359**	238	237	234	213**
14	410	402	402	359**	234	234	232	210**
15	417	409	407	367**	255	256	254	229**
16	426	420	417	377**	276	280	276	251**

Dose (ppm) Week	males 0	50	150	500	Females 0	50	150	500
17	437	428	426	385**	335	338	330	301**
18	443	440	433	393**	261	264	259	238**
18	--	--	--	--	276	274	273	243**
19	455	446	441	399**	279	279	276	245**
20	450	453	450	384**	260	264	273	245
21					275	278	272	247**
22					260	263	256	236**

* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Food consumption and test substance intake of F1 parent animals
There was no treatment-related adverse effect noted on food intake on treated parental F1 animals up to 150 ppm. There were some statistically significant differences at 50 and 150 ppm among the means of weekly food intake data. Since the differences to control values were minor and a dose-correlation does not exist in every case the food intake data of the 50 and 150 ppm group do not indicate an adverse effect. At 500 ppm F1 rats ingested slightly more (10 to 13%) food. Because of the reduced body weights in this group this resulted in a clear (25 to 40%) increase in food intake per kg body weight compared to control rats.
From 50 to 150 ppm the theoretical dose interval of the test substance intake was roughly fulfilled. Because of the higher food intake data per kg body weight at 500 ppm a higher dose factor is calculated than theoretically expected in this group.

Table 5.6.1/04- 13: Mean daily and cumulative intake of food and test substance during the premating period (F1; 91 days)

	Dose	males				females			
	ppm	0	50	150	500	0	50	150	500
Food intake	total	2468	2691	2645	2789	2361	2516	2433	2603
g/animal	per day	27.1	29.6	29.1	30.6	25.9	27.6	26.7	28.6
Food intake	total	8980	10169	9904	12565	12930	13790	13525	16198
g/kg body weight	per day	98.7	111.7	108.8	138.1	142.1	151.5	148.6	178.0
Test substance intake	total		135	397	1394	-	126	365	1302
mg/animal	per day	-	1.5	4.4	15.3	-	1.4	4.0	14.3
Test substance intake	total	-	508	1486	6282	-	689	2029	8099
mg/kg body weight	per day	-	5.6	16.3	69.0	-	7.6	22.3	89.0

Gross pathological examinations in F1 parent animals
There were no test substance related gross pathological findings in parent F1 males and females up to 500 ppm.
Concerning uterine implantation sites counted during necropsy no toxicologically relevant discrepancies between the numbers of implantation sites and those of delivered pups occurred, if controls and treated pups of groups 50 and 150 ppm are compared (see Table 5.6.1/04- 14below). Accordingly there was no dose-dependent change in prenatal loss up to 150 ppm. For controls a relative low mean for the prenatal loss was calculated if control values of F0 females were considered. At 500 ppm there was a remarkable discrepancy between the number of implantation sites and the number of born pups resulting in an increase in prenatal loss in this dose group.

Table 5.6.1/04- 14: Evaluation of implantation sites in F1 females

Dose ppm	0	50	150	500
No. of implantation sites	316	243	296	263
No. of pups at birth	297	211	258	215**
Prenatal loss (Means per litter)	0.76	1.60	1.52	2.09

* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Organ weights in F1 parent animals

There were no remarkable changes in absolute or relative organ weights at 50 or 150 ppm. Statistical significant differences observed in these groups are of no relevance, since dose dependencies are absent in any case. At 500 ppm mostly significantly lower absolute weights for the liver, spleen, and kidneys (in both sexes) as well as epididymides and prostate were recorded than in the other groups. There was also a significantly elevated mean relative testis weight in this group. However, all these statistical significant differences are attributable to the deviations in body weights (relative organ weights are comparable to controls) and are therefore not toxicologically relevance.

Table 5.6.1/04- 15: Organ weights in F1 parent animals

		males				females			
	Dose ppm	0	50	150	500	0	50	150	500
Body weight	absolute (g)	464	458	456	405**	259	259	255	237**
Brain	absolute (mg)	2060	2117	2082	2028	1926	1964	1922	1880
	relative (mg/100 g bw)	448	466	461	504**	748	762	758	796*
Pituitary	absolute (mg)	10	11	12**	10	14	14	13	12
	relative (mg/100 g bw)	2	3ns	3**	3ns	5	6	5	5
Adrenals	absolute (mg)	59	55	59	55	76	74	71	72
	relative (mg/100 g bw)	13	12	13	14	30	29	28	30
Thyroids §	absolute (mg)	10	13**	15**	10	13	11	11	12
	relative (mg/100 g bw)	2	3**	3**	2	5	4	4	5
Liver	absolute (mg)	15132	14901	15170	12760**	10706	10620	10083	9523*
	relative (mg/100 g bw)	3269	3254	3321	3148	4146	4109	3940	4027
Spleen	absolute (mg)	801	721**	753	711**	638	619	611	588
	relative (mg/100 g bw)	174	158**	166	176	248	762	763	761
Kidneys	absolute (mg)	2806	2723	2865	2474**	1945	1968	1945	1801*
	relative (mg/100 g bw)	610	597	631	614	754	762	763	761
Testes/ Ovaries	absolute (mg)	3808	4028	3898	3677	174	166	168	161
	relative (mg/100 g bw)	832	884	862	913*	67	65	66	68
Epididymides §/ Uterus	absolute (mg)	802	801	756	732*	758	676	703	662
	relative (mg/100 g bw)	175	176	167	182	296	265	277	280
Seminal vesicle	absolute (mg)	1415	1379	1389	1278				
	relative (mg/100 g bw)	309	302	307	317				
Prostate	absolute (mg)	1000	1060	1080	827**				
	relative (mg/100 g bw)	218	231	238	205				

§ Unilateral determination

Determination of developmental milestones in F1 weanlings

In all F1 weanlings selected for further treatment the age and body weight at which balano-preputial separation and vaginal opening had occurred was recorded. The calculated mean values are given in Table 5.6.1/04- 16 below. Maturation of external sexual organs was not influenced by the treatment with H 321 up to 150 ppm. At 500 ppm delayed occurrence of balano-preputial separation and vaginal opening were noted, which is considered to be the result of the severely reduced body weights at this dose and is therefore not considered as a primary repro-toxicological effect.

Table 5.6.1/04- 16: Developmental milestones in F1 weanlings

	Dose ppm	0	50	150	500
Balano-preputial separation	mean age (days)	42.9	43.2	43.2	46.9**
	mean body weight (g)	170.3	171.6	169.3	152.4**
Vaginal opening	mean age (days)	33.6	32.9	34.0	35.2*
	mean body weight (g)	101.0	99.0	102.6	93.0*

* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Spermatological evaluation in F1 males

Spermatological evaluation in 500 ppm males revealed no toxicological relevant effect. One high-dose male had no sperms and spermatids. This finding is considered as incidental.
In summary no statistical significant differences were observed among these parameters. Therefore, investigations of 50 and 150 ppm males were not done.

Table 5.6.1/04- 17: Evaluation of sperm motility and morphology and spermatid and sperm counts (F1) (mean values of 25 (0 ppm) and 24 (500 pp) males/dose)

Dose ppm	Sperm motility and morphology: Sperm motility: 1st min	5th min	Difference	Abnormal sperms	Spermatid counts: Mean number of: Spermatids per mg testis	Sperms per mg Epididymis
	%			%		
0	73	68	−5	0.44	48617	991098
500	79	74	−5	0.45	48600	1148084

Estrus cycle staging in F1 females

Estrus staging was done over 19 days beginning three weeks before F1 rats were co-housed. As shown in Table 5.6.1/04- 18 below there was no adverse effect on estrus cycle parameters up to 500 ppm.

Table 5.6.1/04- 18: Evaluation of the oestrus cycle in F1 females

Dose ppm	0	50	150	500
Mean cycle length in days	4.01	4.09	4.17	4.22
Mean no. of cycles in 19 days	3.56	3.56	3.68	3.72
No. of females cycling normally	25	24	25	22

Histopathological investigations in F1 rats

There were no treatment-related histopathological findings observed in any organs examined. The ovarian follicle staging revealed not compound-related effect.

Determination of Cholinesterase activity

There were no toxicologically relevant changes (i.e. changes > 20%) of cholinesterase activities in plasma up to 150 ppm and in brain up to 500 ppm (see Table xxx below).

However, there was a noticeable low control mean for the plasma cholinesterase in F1 females (1.37 kU/L), if compared to that of F0 females (1.63 kU/L) indicating the relative wide variation of Cholinesterase measurements.

The cholinesterase activity in the erythrocytes was significantly reduced in males from 50 ppm onwards (–25.0% / –29.1 % / –35.8%) and in 500 ppm F1 females (–23.6%). If F0 and F1 rats are compared relatively high control means for the erythrocyte cholinesterase activity were evident for F1 rats (F1: 1.48/1.44 kU/L (m/f) vs. F0: 0.74/0.75 kU/L (m/f)).

Table 5.6.1/04- 19: Determination of cholinesterase activity in F1 rats

		males				females			
	Dose ppm	**0**	**50**	**150**	**500**	**0**	**50**	**150**	**500**
Plasma	kU/L	0.40	0.40	0.39	0.34	1.37	1.62*	1.29	0.83**
Erythrocytes#	kU/L	1.48	1.11**	1.05**	0.95**	1.44	1.30	1.29	1.10**
Brain##	U/g	11.01	10.69	10.15*	10.15	10.75	10.58	10.12	10.51

7 weeks after the first pup had been weaned; ## At necropsy

* Statistically significantly different from control ($p < 0.05$)

** Statistically significantly different from control ($p < 0.01$)

Parameters of Reproduction in F1 parent animals

At levels of up to 500 ppm the insemination, fertility and gestation indices as well as the mean duration of pregnancy did not differ from the pertinent control figures to a toxicologically relevant extent.

The mating performance was also not affected by the treatment at levels of up to 500 ppm.

Furthermore none of the litter parameters shown in Table 5.6.1/04- 20 were changed to a toxicologically relevant extent up to 150 ppm. At 500 ppm the number of pups born and the mean litter size was significantly reduced.

Table 5.6.1/04- 20: Data concerning mating, fertility, and gestation (F1) and litter parameters at birth (F2)

Dose ppm		**0**	**50**	**150**	**500**
N females inseminated on	D1-4	25	20	24	23
days of mating period	D8-11	21	22	20	22
	D15-18	2	0	1	1
Insemination index	%	100.0	100.0	100.0	100.0
Fertility index	%	100.0	88.0	100.0	92.0
Gestation index	%	100.0	90.9	96.0	100.0
Gestation length	days	22.00	22.06	22.19	22.09
Co-housed females	n	25	25	25	25
Litters alive	n	25	20	24	23
Pups (total)	n	297	211	258	215**
Pups (dead)	n	0	5*	4	0
Live birth index	%	100.00	95.30	98.29	100.00
Males	%	48.35	42.00	45.85	47.53
Mean litter size[1)]	n	11.88	10.30	10.58	9.35**

[1)] Viable pups only n= number

* Statistically significantly different from control ($p < 0.05$)

** Statistically significantly different from control ($p < 0.01$)

Clinical observations in F2 pups

There were no treatment-related clinical signs observed in F2 pups up to 150 ppm during the four week lactation period, if the number of affected litters is considered.

At 500 ppm there were clearly more litters with pups being pasted with food around the nose/mouth area, thin, cold and/or blue discolored. In the high-dose group there were also more litters with pups showing tremor, respiratory sounds, or labored breathing, and/or rough fur. This was considered to be due to treatment.

Table 5.6.1/04- 21: Summary of clinical signs in F2 pups during lactation

Dose ppm Clinical sign	0	50	150	500
Respiratory sounds	5/2	9/3	8/2	13/7
Laboured breathing	7/3	6/4	1/1	9/6
Rough fur	0/0	0/0	0/0	15/3
Blue discoloration	1/1	5/4	1/1	20/8
Paleness	2/1	0/0	0/0	0/0
Cold to touch	1/1	6/4	2/2	5/5
Nose and/or mouth pasted with feed	9/2	7/5	6/3	32/11
Thin	12/5	10/6	16/9	33/15
Tremor on whole body	0/0	0/0	0/0	11/6

x/y pups / litters affected

Body weights of F2 pups

Body weights of F2 pups are summarized in Table xxx. As shown the mean birth weights were not significantly affected up to 500 ppm. During lactation no adverse effect on body weights could be detected at 50 and 150 ppm. At 500 ppm significant depression of pup weights was established on day 28 in males (-11%) and females (-9%).

Table 5.6.1/04- 22: Mean pup weight at birth and during lactation (F2)

	males				females			
Dose ppm	**0**	**50**	**150**	**500**	**0**	**50**	**150**	**500**
Day 0	5.65	6.04*	6.05*	5.83	5.45	5.78	5.82	5.65
Day 4#	9.63	10.12	10.19	9.38	9.52	9.85	9.99	9.31
Day 7	14.84	15.39	15.03	13.28	14.90	14.75	15.26	13.82
Day 14	30.59	31.00	30.35	28.11	30.31	30.00	30.72	28.61
Day 21	49.89	51.37	50.30	46.22	49.45	49.01	50.07	46.14
Day 28	84.30	86.56	83.32	75.12**	79.20	78.43	79.37	72.10**

After culling
* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Viability, rearing and lactation indices of F2 pups

The viability and rearing indices were comparable between control and treated F2 pups. Up to the dose of 500 ppm there was no dose-dependent reduction in the lactation indices observed.

Table 5.6.1/04- 23: Viability, rearing and lactation indices (F1)

Dose ppm		0	50	150	500
Viability index (%)	D4	97.33	92.50	98.45	95.33
Rearing index (%)	D28	100.0	95.0	95.8	100.0
Lactation index (%)	D21	77.00	82.24	78.47	75.93

Gross pathological changes in F2 pups or weanlings
There were no treatment-related gross pathological findings observed in F2 pups necropsied during the lactation period up to weaning in any dose group.
There were no skeletal deviations observed in F2 pups, which died before day four postpartum, were killed in the process of litter reduction on postpartum day four, or were necropsied unscheduled during lactation at levels of up to 500 ppm.

Organ weights of F2 weanlings
No significant or dose-correlated differences in absolute or relative brain, spleen, thymus and uterus weights occurred between control weanlings and treated rats up to 150 ppm (Table 5.6.1/04- 24). At 500 ppm relative brain weights (both sexes) were significantly increased and absolute spleen weights of males were significantly decreased. Both findings are considered to be the consequence of differences in body weights.

Table 5.6.1/04- 24: Organ weights of F2 weanlings

		males				females			
	Dose ppm	0	50	150	500	0	50	150	500
Body weight	(g)	87.5	88.0	86.8	77.5**	82.7	79.2	83.8	74.2**
Brain	absolute (mg)	1549	1593	1549	1518	1510	1510	1518	1477
	relative (mg/100 g bw)	1783	1822	1808	1993**	1842	1920	1825	2034**
Spleen	absolute (mg)	343	341	341	290*	302	285	308	273
	relative (mg/100 g bw)	392	389	394	380	366	361	368	373
Thymus	absolute (mg)	359	356	376	324	346	338	358	336
	relative (mg/100 g bw)	411	405	434	425	418	428	426	458
Uterus	absolute (mg)					134	102**	114	111*
	relative (mg/100 g bw)					161	128*	137	152

* Statistically significantly different from control ($p < 0.05$)
** Statistically significantly different from control ($p < 0.01$)

Table 5.6.1/04- 25: Summary of study results

	F0 rearing F1				F1 rearing F2			
Dose ppm	**0**	**50**	**150**	**500**	**0**	**50**	**150**	**500**
Parental animals								
Body weight				↓				↓
Food intake				↑				↑
AChE plasma				↓M				↓F
AChE erythrocytes				↓M				↓
Lactation				↓			↓M	
Vaginal opening and balano-preputial separation								→S
Pups								
Prenatal loss								↑
Number of pups born								↓
Litter size								↓
Pup weights				↓				↓
Clinical signs				+				+

↓ = statistically significantly decreased ↑ = = statistically significantly increased
→ = delayed + = present
M = males only; F = females only, S = secondary to lower body weight

III. Conclusion

Based on the study results the dietary concentration of 50 ppm H 321 is established as the no observed adverse effect level (NOAEL) for the parent animals, whereas 150 ppm is the NOEL for the reproduction parameters.

Report: KCA 5.6.1/05[redacted]; 2003; M-088195-01-1
Title: H 321 - Special subchronic study in Wistar rats (A feeding study where F1 rats received H 321 during their pre- and postnatal development and the up to post-weaning (week twelve)
Report No.: AT00341
Document No.: M-088195-01-1
Guideline(s): not applicable, special study
Guideline deviation(s): not applicable
GLP/GEP: yes

I. Materials and methods

A. Materials

1. Test material: H 321
Synonym (common) name: Methiocarb
Description: White light-beige powder
Lot/Batch no.: 00313/2121
Purity: 98.9-99.5%
Stability of test compound: guaranteed for study duration; expiry date: 2003-03-06

2. Vehicle: diet

3. Test animals:

Species:	rat
Strain:	Wistar:CrlGlxBrlHan:WI
Sex:	Males and females Females were nulliparous and non-gravid
Age:	About 13-15 weeks
Weight at study start:	Not recorded
Source:	[redacted], Germany
Acclimatisation period:	1 week
Diet:	[redacted]3888.9.25 ([redacted]AG, Kaiseraugst, Switzerland), *ad libitum*
Water:	tap water (municipal water supply), *ad libitum*
Housing:	The F0 and weaned F1 rats were housed singly under conventional conditions in Makrolon® cages Type IIa except during the mating, gestation and lactation periods of F0 rats where Type IIIh were used. As bedding material low-dust soft-wood shavings were used. When parturition was near cages of females were provided with a special nesting material such as coarse wood shavings. Both, bedding and nesting material were supplied by [redacted], Germany, and tested for contaminants on a random basis (the results are held on file at BAYER AG).

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0, 50, 150 and 500 ppm
Duration:	F0 animals were dosed during their mating (2 weeks), the resultant pregnancy (about 22 days), and during lactation up to weaning of their F1 offspring (28 days). Weaned F1 offspring (incidentally selected animals) were dosed for at least 12 weeks.
Application route:	Oral diet
Dietary preparation:	H 321 was blended with [redacted]3888.9.25 diet. To minimize dust formation 1% peanut oil (DAB 10) was added. The amounts of test item were calculated on the basis of an assumed 100% content of H 321. The diet mixtures were prepared once a week.
Group size:	F0: 10/sex/group F1: 15/sex/group
Observations:	Analysis of test substance in the diet, clinical signs, mortality, detailed clinical examinations (general health status, behaviour, condition of fur, examination of orifices and excretory products), no of pups alive, necropsy, acetylcholinesterase (ACHE) activity in plasma, and RBC (at least 8 and 11 weeks after weaning) and in brain at necropsy of F1 rats.
Statistics:	An adjusted Welsh test was done on acetylcholinesterase data using SAS® routine on a HP 3000 computer system.

II. Results and discussion

A. Analysis of the test substance in the diet

The analytical data received prior to the study revealed that the homogeneity and stability of the feed mixtures are given in the mixture amount and concentration range used.

The content checks including a test of homogeneity (low and high concentration only) on actual, randomly taken diet samples (three time points) assure that during the study appropriate and equal mixture procedures were followed and concentrations were within defined limits.

B. Clinical signs and mortality of F0 and weaned F1 rats

No test substance-related effects on the appearance, health or behaviour were observed in male or female F0 and weaned F1 animals at levels of up to 500 ppm.

One F0 female of the 150 ppm dose group was killed prescheduled due to difficult parturition and one F1 female also at 150 ppm died during blood sampling. Therefore, the mortality was unchanged up to 500 ppm.

C. Determination of Acetylcholinesterase activity

Plasma AChE-activity

In male rats plasma AChE activities were slightly reduced at 50 and 150 ppm, and remarkable reduced at 500 ppm at both time points. All values were statistically significantly ($p \leq 0.01$) reduced compared to controls. Considering the individual values of this parameter (not shown here) it is obvious that in week 8 only one 50 ppm male had a plasma AChE activity (i.e. 0.32 kU/L) that was slightly below the corresponding control range (0.35 to 0.67 kU/L). In week 11 none of the 50 ppm males had a plasma AChE-activity value outside the control range.

In contrast to this at 150 and 500 ppm several individual AChE-activity values were outside of the actual control range at both time points. Therefore, the statistically significantly reduced mean value of the 50 ppm males is not considered biologically relevant. However, an adverse effect on the plasma AChE activity is obvious for mid- and high-dose males.

In females significantly and biologically relevant decreased plasma AChE-activity was observed at 150 ppm (week 11) and at 500 ppm (week 8 and 11).

Thus the dose level of 50 ppm can be considered as the NOAEL for plasma AChE-activity.

Erythrocyte AChE-activity

The erythrocyte AChE-activity of males was not significantly changed up to 150 ppm, but was inhibited biologically significantly at 500 ppm at both time points. In females a significantly lower mean erythrocyte AChE-activity was evident in all treatment groups in week 8, whereas at study termination no adverse effect on this parameter was noted up to 500 ppm.

Since the statistically significant reduction of erythrocyte AChE in females at week 8 was not reproducible at week 11, and since the erythrocyte AChE activities measured in both sexes at 50 ppm in week 11 were higher than the AChE activities measured at 0 ppm, a remarkable inhibition of the erythrocyte activity can be not confirmed for the 50 ppm level for females.

The plasma and erythrocyte AChE activities are summarised in the following table and Figure 5.6.1/05- 1 and Figure 5.6.1/05- 2.

Table 5.6.1/05- 1: Acetylcholinesterase activities in plasma and erythrocytes of F1 rats

			males				females			
		Dose ppm	0	50	150	500	0	50	150	500
Plasma	Week 8	kU/L	0.54	0.43**	0.41**	0.35**	2.17	1.92	2.14	1.58*
		%		–20.4	–24.1	–35.2		–11.5	–1.4	–27.2
	Week 11	kU/L	0.55	0.42**	0.40**	0.32**	2.62	2.17	1.96*	1.86*
		%		–23.6	–27.3	–41.8		–17.2	–25.2	–29.0
Erythrocytes	Week 8	kU/L	1.62	1.61	1.49	1.21**	1.50	1.14*	1.19*	0.94**
		%		–0.6	–8.0	–25.3		–24.0	–20.7	–37.3
	Week 11	kU/L	1.09	1.15	0.92	0.77**	1.08	1.14	1.07	1.02
		%		+5.5	–15.6	–29.4		+5.5	–0.9	–5.6

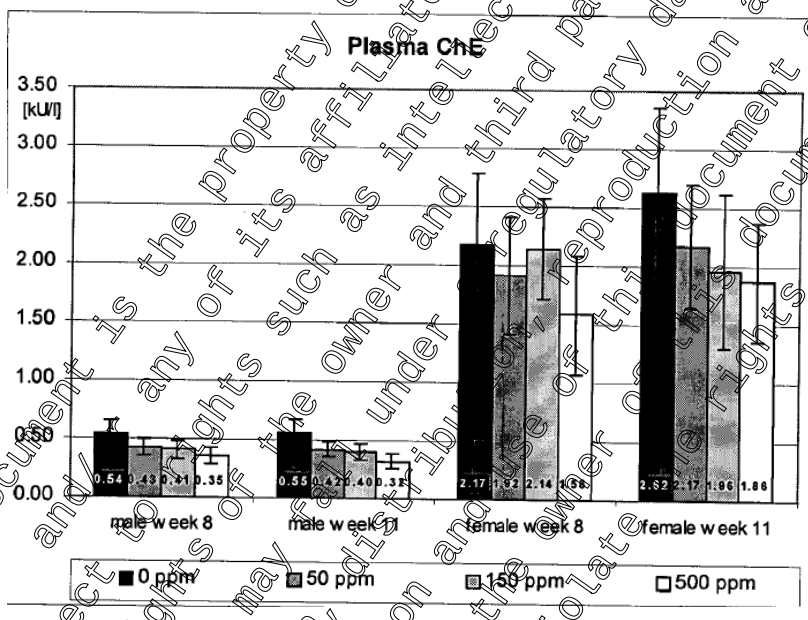


Figure 5.6.1/05- 1: Acetylcholinesterase activities in plasma

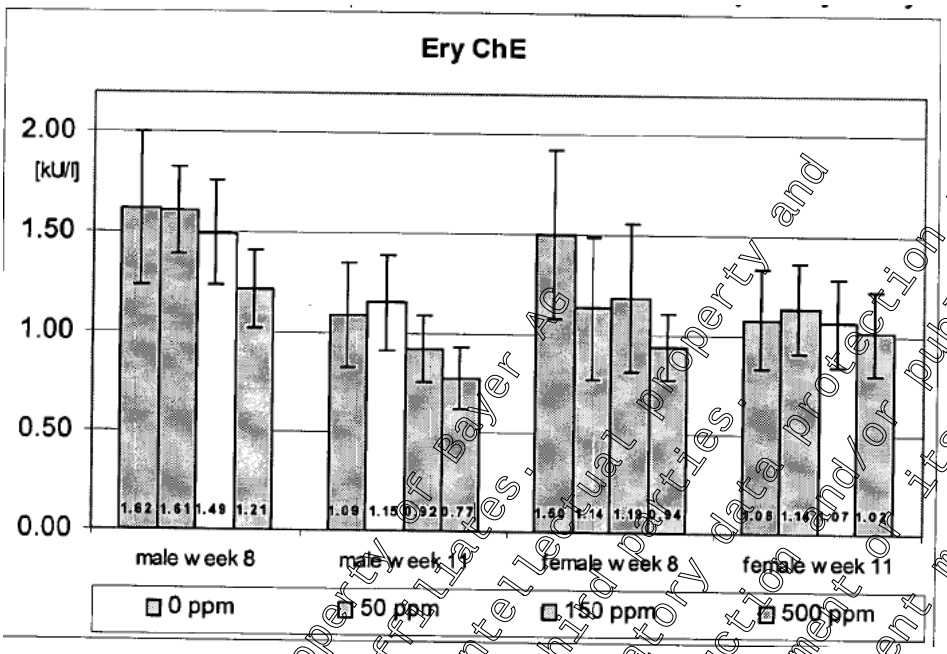


Figure 5.6.1/05- 2: Acetylcholinesterase activities in erythrocytes

Brain AChE activity

As shown in Table 5.6.1/05- 2 and Figure 5.6.1/05- 3 no statistically significantly changed brain acetylcholinesterase activities occurred up to 150 ppm in males, whereas at 500 ppm slightly, but significantly lower activities were measured than at 0 ppm. In females minimal, but significantly lower means occurred at 50 and 150 ppm, which is considered to be incidental and not of toxicological relevance. In 500 ppm females slightly ($p \leq 0.01$), but statistically significantly reduced activities of the brain acetylcholinesterase were measured. However, the observed changes are below 20%, and thus not considered adverse. Therefore in this study the NOEL for the brain acetylcholinesterase is confirmed at 150 ppm.

Table 5.6.1/05- 2: Acetylcholinesterase activities in the brain of F1 rats

	Dose	males				females			
	ppm	0	50	150	500	0	50	150	500
Brain	U/g	11.91	11.61	11.77	10.54**	11.82	11.19**	11.18**	10.15**
	%		-2.5	-1.2	-11.5		-5.3	-5.4	-14.1

From the Figure 5.6.1/05- 1 to Figure 5.6.1/05- 3 the very wide variation of individual values for all three AChE activities, especially in the control group is visible. It is also obvious, that the AChE activity means of the 50 ppm group were only marginal lower than those of the control group or, if moderate differences exist, between these groups dose-relation is mostly lacking.

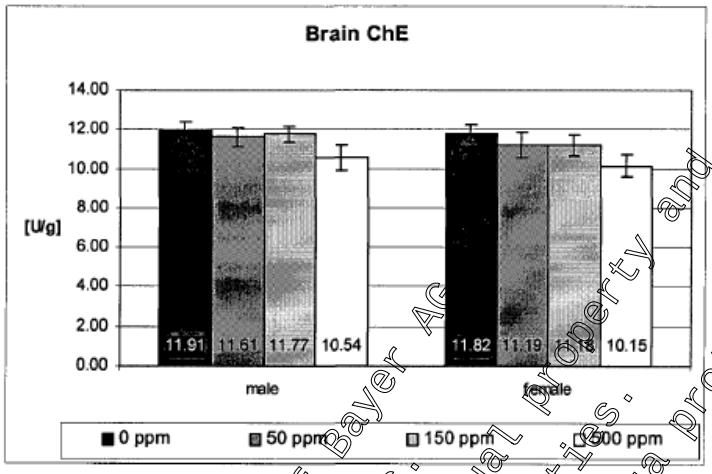


Figure 5.6.1/05- 3: Acetylcholinesterase activities in brain

III. Conclusion

At 150 and 500 ppm several males and females of this study exhibited mostly reproducible plasma acetylcholinesterase values outside the actual control range clearly indicating an adverse effect on this parameter from 150 ppm onwards in both sexes. Therefore an inhibitory effect on the plasma acetylcholinesterase at > 50 ppm is concluded.
The lack of any erythrocyte acetylcholinesterase inhibition up to 500 ppm in females in week 11 and the fact that at study termination at 50 ppm higher means were evident in males and females than in corresponding controls an inhibitory effect of H 321 on the erythrocyte acetylcholinesterase cannot be established for the 50 ppm concentration, which is, therefore, established as the NOEL for this parameter.
There was no biologically relevant reduction of the brain acetylcholinesterase activity up to 500 ppm in both sexes.
Thus, the dietary concentration of 50 ppm H 321 is established as the overall NOAEL for the acetylcholinesterase activity as shown in previous studies.

CA 5.6.2 Developmental toxicity studies

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.7 Neurotoxicity studies

Summary of neurotoxicity
No specific acute and repeated dose neurotoxicity studies are available for methiocarb.

However, methiocarb is a carbamate and as such an inhibitor of acetylcholinesterase, which acts on the nervous system of insects and vertebrates as well. Carbamates reversibly inhibit the enzyme acetylcholinesterase (AChE), which is responsible to hydrolyse the neurotransmitter acetylcholine (ACh) at the postsynaptic membrane. Inhibition of AChE leads to accumulation of ACh in synapses and neuromuscular junctions, resulting in muscarinic and nicotinic symptoms. At the muscarinic nerve endings this excites increased tear flow and salivation, increased bronchial secretion, bronchospasms (leading to dyspnoea), increased gastric and intestinal secretion, contractions and spasms (leading to colic, vomiting, diarrhoea), myosis of the eye and a decrease in blood pressure caused by bradycardia and a decreased tonus of the blood vessels. At the vegetative ganglia and at the neuromuscular plate excess of ACh causes nicotinergic effects like stiffness of muscles in face and neck, tremor, muscle

fasciculations, tonic-clonic cramps. Furthermore, after crossing of the blood-brain barrier, also central effects like seizures, respiratory depression and CNS depression occur.
The typical clinical signs, mortality at higher doses, as well as acetylcholinesterase inhibition were observed in almost all of the available acute and repeated dose toxicity studies with methiocarb. The measurements of AChE inhibitions in rat (gavage) studies revealed peak effects at 20 min for plasma and RBC AChE inhibition, and at 2 h post-dosing for brain AChE inhibition. Recovery to control levels was within 6 to 24 h after dosing.
In dog (capsule) studies of AChE inhibition peaked 1 hour after application, and resolved within 5 hours after application.

Detailed assessments of neurotoxicity were performed in two sub-chronic toxicity studies in rats and dogs. These included neurobehavioral investigations. In the rat study (M-088469-01-1) the functional observational battery (FOB) investigations consisted of observations in the home cage[15], observations during handling[16], open field observations[17], and manipulative tests[18]. In the dog study (M-030181-01-1) neurological examinations consisted of the assessment of mental status/behaviour[19], gait characteristics[20], postural status and reactions[21], and spinal/cranial reflex tests[22]. These investigations, except that there was **no automatic** assessment of motor activity in the rat study, and no *in situ* fixation for histopathology, and no histopathological examination of some PNS tissues (dorsal root ganglia, dorsal and ventral root fibres proximal tibial nerve (at knee), tibial nerve calf muscle branches, skeletal muscle (calf muscle)), fully cover the investigations of acute and subacute neurotoxicity screening studies according to OECD guideline 424.
Both subchronic studies were already evaluated during the EU process for Annex I listing. For details please refer to the Monograph and baseline dossier for methiocarb. Details regarding the results of neurotoxicity assessments conducted in these studies are summarised in the following.
In the 13-week dietary study in Wistar rats (M-088469-01-1) with doses of 0, 100, 300 and 900 ppm there were no treatment-related mortalities and no clinical signs of toxicity observed at any dose level. Body weight gain was decreased in females from 300 ppm and above and in males at 900 ppm (up to 21% and 29% for males and females, respectively). General behaviour, posture and respiration were normal at any dose level. Functional observational battery (FOB) tests provided no indications for treatment-related effects in both sexes up to the highest dose tested (i.e. 900 ppm). AChE activity measurements revealed a slight, not biologically significant reduction of brain AChE activity (-9%) in high dose females at the end of the treatment period. Grip strength measurements performed at the termination of the study showed in males relative changes in forelimb grip strength of −10%, −23%, and −14% (increasing dose levels) and for hind limbs of −4%, −25%, and −14%. In females relative changes in forelimb strength were −9%, −15, −29%, and for hind limbs −1%, −3%, −17%. At the end of the recovery period the relative changes were −4% in males for forelimbs and hind limbs, and in females −2% for forelimbs and 0% for hind limbs. These not clearly dose-related effects occurred at dose levels of ≥ 300 ppm which have to be regarded as close to or already above the maximum tolerated dose (MTD). Therefore, the observed changes in grip strength were not regarded as specific neurotoxic effects.

[15] posture, piloerection, gait abnormalities, involuntary motor movements, vocalizations
[16] ease of removing, reaction to being handled, muscle tone, palpebral closure, pupil size, lacrimation, salivation, nasal discharge, stains
[17] piloerection, respiratory abnormalities, posture, involuntary motor movements, stereotypy, bizarre behavior, gait abnormalities, vocalizations, arousal, rearing, defecation, urination
[18] pupil response, approach response, touch response, auditory response, tail pinch response, righting reflex, forelimb and hindlimb grip strength
[19] arousal, motor activity, startle response, auditory response, cowering/apprehension, aggression
[20] ataxia, knuckling, stiff/stilted, hypermetria, hypomettria, stride length, stride width, proprioceptive placing
[21] reluctant to stand, tremors, fasciculations, wide-based stance, flat-footed, head position, tail position, back position, head tilt, hemihop, hemiwalk, hemistanding, wheel-barrow, visual placing, tactile placing, withdrawal response
[22] patellar, bicep, tricep, gastrocnemius, pupillary- direct/indirect, perineal

Overall, except for the slight AChE inhibition in brain in the high-dose group, no specific dose-related neurotoxic effects were observed in both sexes up to 900 ppm.

In the 90-day dietary study in beagle dogs (M-030181-01-1) with doses of 0, 10, 50, and 250 ppm increased incidence of vomiting was observed at the highest dose group, while no other relevant dose-related clinical signs were observed. Also in the high-dose group food consumption and body weight gain was reduced. Plasma and RBC cholinesterase activities were statistically and biologically relevant decreased at the high dose, while there was no decrease of the brain AChE. Neurological examination of mental status / behaviour, gait characteristics, postural status and reactions, and spinal/cranial reflex tests conducted on all animals by a veterinarian at pre-treatment and just prior to termination revealed no treatment-related neurological defects.

In addition, there are also short-term studies in rat and dog available which were conducted for the determination of AChE inhibition [redacted]., 1973, M-009378-01-1 (KCA 5.3.1/01 & KCA 5.8.2/13 [redacted]., 1981, M-009348-01-1 (KCA 5.3.1/02), [redacted]., 1981, M-009577-01-1 (KCA 5.3.1/03)).
After acute exposure (M-009378-01-1) rats exhibited typical signs of AChE inhibition (trembling, cramps) at methiocarb doses of ≥ 10 mg/kg bw. The symptoms appeared within 5 min to 10 min after application, and were resolved 2 h later. The maximum dose-related levels of cholinesterase inhibition were recorded 20 minutes after application of ≤25 mg/kg bw methiocarb, and 20 minutes to 2 hours after application of 50 mg/kg bw. Brain AChE inhibition peaked after 2 hours at dose levels of 10 and 20 mg/kg bw methiocarb. Thereafter, brain AChE activity increased again.
In the sub-acute experiments (M-009378-01-1, M-009348-01-1), rats exhibited biologically significantly decreased AChE 20-30 min after gavage application of 2 or 10 mg/kg bw. Brain AChE was also inhibited at 10 mg/kg bw two hours after application.
In dogs (M-009577-01-1) repeated applications of methiocarb at 0.05 and 0.5 mg/kg bw/day by capsules caused signs of toxicity and significant AChE inhibition in plasma and RBC at 0.5 mg/kg bw/day. Maximum depression of AChE was observed 1 to 3 hours after application, with full recovery within 6 hours. For further details please refer to KCA 5.3.1 & KCA 5.8.2

Taken all this together the main neurotoxic effect of methiocarb is AChE inhibition in plasma, RBC and brain. Clinical signs indicative of neurotoxicity are cholinergic signs like tremors, salivation, lacrimation, decreased locomotor- and motoractivity, decreased grip strength muscular fasciculations were observed after acute and repeated exposure, but there was no cumulative effect on the AChE activity observed. In addition, in none of the studies with methiocarb there were any histopathological findings indicative of neurotoxicity observed.
Except for the inhibition of AChE and correlated symptoms, there are no further signs indicative of neurotoxicity observed in any of the toxicity studies. The sub-chronic rat and dog studies cover all neurobehavioral investigations that are necessary for neurotoxicity screening. It is concluded that the neurotoxic potential of methiocarb is adequately assessed in the available toxicity studies. Therefore, further neurotoxicity screening studies are considered to be not necessary.

Methiocarb did not show any evidence of a potential to cause **delayed neurotoxicity** in strains of domestic hens. The acute oral LD_{50} in White Leghorn hens was 380 mg/kg bw. Pre-treatment with atropine sulphate provided protection against the neurotoxic effects and hens survived repeated dosing at the 380 mg/kg bw dose level.

To assess that there are no further neurotoxic signs to be expected after methiocarb exposure, data of 9 further N-methylcarbamates (carbofuran, oxamyl, methomyl, pirimicarb, thiodicarb, carbaryl, formetaneate, aldicarb and propoxur) were assessed. The data were taken from publically available

sources, i.e. mainly EFSA conclusions, and US-EPA evaluations[23]. Based on the available neurotoxicity data all these N-methyl carbamates showed the same or comparable effects like methiocarb. They inhibited AChE in plasma, RBC and brain. The peak of the AChE inhibition was shortly after application (i.e. 15-30 min post dosing). Full recovery was in general within 24 hours. Also due to the AChE inhibition, reduced locomotor, and motoractivity was observed in parallel to the AChE inhibition. Available FOB evaluations revealed besides the mentioned effects also decreases of fore and hind limb strength, reduced extensor trust, tail and toe pinch response, and increased hind limb splay. Like for methiocarb, there were also no histopathological neurotoxicity findings observed for these N-methylcarbamates. Furthermore, none of these carbamates induced delayed polyneuropathy in hens.

In addition, three publications emerged from public literature between 2007 and 2010, which described AChE and neurobehavioral effects of methiocarb after acute exposure in rats *in vivo*. All three of them were based on non-GLP studies, which had some deficiencies but were considered to be reliable with restrictions. These publications provide relevant but supplemental information on the neurotoxicity (time-course and dose-response of AChE inhibition, neurobehavioral effects) of methiocarb. One publication showed a clear correlation between AChE inhibition and reduced motor activity (M-510973-01-1). Furthermore, they provide neurotoxicity data of six other N-methylcarbamates. The presented results for AChE inhibition (RBC, brain) are in accordance with the available GLP studies for methiocarb. The obtained results do not change existing endpoints or the risk assessment for methiocarb.

Overall, except for the designated effect of methiocarb to inhibit AChE activity, with related cholinergic signs, there is no evidence for treatment-related neurotoxic effects. Since the AChE inhibition is only transient (i.e. reversible), and not accumulative, it can be concluded that methiocarb exhibits no potential to permanently impair the central or peripheral nervous system (i.e. methiocarb does not induce significant functional changes in the central or peripheral nervous system, signs of CNS depression, effects on the senses (sight, hearing, smell), and histopathological damage to the brain). In addition, methiocarb did not induce delayed polyneuropathy.

Table 5.7- 1: Summary of neurotoxicity testing*

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
Hen, delayed neurotoxicity				LD_{50} = 380 mg/kg bw	[redacted]; [redacted], B.,

[23] US EPA Reregistration Eligibility Decision for Aldicarb, September, 2007
EFSA Scientific Report (2006) 80, 1-71, Conclusion on the peer review of carbaryl, 12 May 2006 (revised version of 10 July)
EFSA Scientific Report (2006) 90, 1-88, Conclusion on the peer review of carbofuran, 28 July 2006;
EFSA Scientific Report (2009) 310, 1-132 - Conclusion regarding the peer review of the pesticide risk assessment of the active substance Carbofuran, 16 June 2009
EFSA Scientific Report (2006) 69, 1-78, Conclusion on the peer review of formetanate, 24 April 2006
US EPA - Reregistration Eligibility Decision for Formetanate Hydrochloride, September 24, 2007
EFSA Scientific Report (2006) 83, 1-73, Conclusion on the peer review of methomyl, 23 June 2006
EFSA Scientific Report (2005) 26, 1-78, Conclusion on the peer review of oxamyl, 14 January 2005
EFSA Scientific Report (2005) 43, 1-76, Conclusion on the peer review of pirimicarb, 10 August 2005
US EPA Reregistration Eligibility Decision (RED) – Propoxur; EPA738-R-97-009, August 1997
EFSA Scientific Report (2005) 55, 1-76, Conclusion on the peer review of thiodicarb, 14 December 2005
US EPA Reregistration Eligibility Decision (RED) – Thiodicarb; EPA738-R-98-022, December 1998

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
0-25-50-100 mg/kg bw					1978 M-009572-01-1
13-week,dietary rat 0-100-300-900 ppm 0-7.34/10.0-22.72/30.71-67.59/90.74 mg/kg bw/day	M F	7.3 10.0 (100 ppm)	22.7 30.7 (300 ppm)	Reduced bw gain in females no significant AChE inhibition at 900 ppm (–9% for brain AChE in females at termination)	[redacted], 2001 M-088469-01-1
90-day, dietary dog 0-10-50-250 ppm 0-0.30/0.25-1.32/1.33-6.46/5.91 mg/kg bw/day	M F	1.3 1.3 (50 ppm)	6.46 5.9 (250 ppm)	Vomiting, plasma and RBC AChE inhibition, reduced food consumption, reduced bw gain	[redacted], 2000 M-030181-01-1
Rat acute oral (gavage) 0-1-10-25-50 mg/kg bw	M F	10 10	50 --	Trembling, cramps, plasma, RBC and brain AChE inhibition	[redacted], 1973 M-009378-01-1
Rat 4-week,oral (gavage) 0-0.5-2.0 mg/kg bw/day	F	0.5	2	Tremors (during the first days only), ↓ ≥ 25% of plasma and erythrocyte AChE activity (during the first week only)	[redacted] 1981 M-009348-01-1
Dog, 29-day, oral (capsule) 0-0.05-0.5 mg/kg bw/day	M F	0.05 0.05	0.5 0.5	Cholinergic signs, slight ↓ of erythrocyte AChE activity (2 h post application)	[redacted], 1981 M-009577-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Comparison of the study results with CLP criteria for Specific Target Organ Toxicity – Single exposure (STOT SE) or Repeated Exposure (STOT-RE) according to REGULATION (EC) No 1272/2008[7]

According to CLP criteria a classification for STOT-SE need to be considered if the substance causes non-lethal target organ toxicity after a single exposure (i.e. significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed and not already covered by acute toxicity, skin corrosion / irritation, eye damage / irritation, respiratory or skin sensitization, genotoxicity, carcinogenicity and reproductive toxicity).

Based on the results of the studies with acute exposure of methiocarb, there were no significant toxic effects observed at non-lethal dose levels. Thus, classification of methiocarb for STOT-SE category 1 or 2 is not warranted.
There is also no indication of transient effects like respiratory tract irritation (RTI) and narcotic effects (NE) after single exposure to methiocarb. Therefore, classification of methiocarb for STOT-SE category 3 is not warranted.
According to CLP criteria a classification for STOT-RE need to be considered if the substance causes non-lethal target organ toxicity after a repeated exposure (i.e. significant health effects that can impair

function, both reversible and irreversible, immediate and/or delayed and not covered by acute toxicity, skin corrosion / irritation, eye damage / irritation, respiratory or skin sensitization, genotoxicity, carcinogenicity and reproductive toxicity).
Based on the results of the studies with repeated exposure of methiocarb, there were no significant toxic effects observed at non-lethal dose levels. Thus, classification of methiocarb for STOT-RE category 1 or 2 is not warranted.

Classification/labelling regarding STOT-SE or STOT-RE for methiocarb:
- Regulation (EC) No 1272/2008 (CLP): none

CA 5.7.1 Neurotoxicity studies in rodents

Publications

N-methyl carbamates produced AChE inhibition that peaked within minutes to hours after a single oral dose and recovered by 24 h. The dose-response of AChE inhibition did not correlate well with decreased motor activity in most cases.

Report: KCA 5.7.1/03 [redacted]; 2007; M-504906-01-1
Title: Time course of cholinesterase inhibition in adult rats treated acutely with carbaryl, carbofuran, formetanate methomyl, methiocarb, oxamyl or propoxur.
Report No.: M-504906-01-1
Document No.: M-504906-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: no

1. Materials and methods

A. Materials

1. Test material: Methiocarb

Source: [redacted], PA, USA
Description: Not reported
Lot/Batch no: Not reported
Purity: 99%
Stability of test compound: expiry date: not reported
Additional test substances: 1) Carbaryl
2) Carbofuran
3) Formetanate
4) Methomyl
5) Oxamyl
6) Propoxur
In this summary detailed results are only presented for Methiocarb.

Source: [redacted], PA, USA
Purity: ≥ 99%

Stability:	expiry date: not reported
2. Vehicle:	For methicarb, and carbaryl, carbofuran, and propoxur treatment: corn oil; for formetanate, methomyl and oxamyl: distilled water
3. Test animals:	
Species:	Rat
Strain:	Long Evans [Crl: (LE) BR]
Age:	90 days
Sex:	Male
Weight at dosing:	Approx. 400 g
Source:	[redacted] Laboratories, [redacted], NC, USA
Acclimatisation period:	1 week
Diet:	*Ad libitum*, name of diet not reported
Water:	*Ad libitum*
Housing:	Individually
B. Study design and methods	
1. Animal assignment and treatment:	
Dose:	Methiocarb: 1 x 25 mg/kg bw Carbaryl: 1 x 30 mg/kg Carbofuran: 1 x 0.5 mg/kg bw Formetanate: 1 x 10 mg/kg bw Methomyl: 1 x 3 mg/kg bw Oxamyl: 1 x 1 mg/kg bw Propoxur: 1 x 20 mg/kg bw The dose levels were chosen after literature review.
Application route:	oral gavage
Application volume:	1 ml/kg bw
Group size:	5 males/dose/time point; 1/time point for control
Observations:	Mortality, acetylcholine esterase (AChE) activity in brain and red blood cells (RBC)
Examination time points:	0.5, 1, 2, 4 and 24 h (time points based on preliminary experiments that were not reported!) Blood and brain tissue sampling were collected at the specified time points
Tissue/blood preparation for assays:	At the specified time point rats were decapitated under CO_2 anaesthesia for blood and brain collection. Whole brain was collected and immediately placed on dry ice. Trunk blood was collected in a heparinized tube and centrifuged at 1000 x g for 10 min to separate plasma and RBC. The RBC fraction was diluted 1:3 (1 part RBC plus two parts 0.1 M sodium phosphate buffer, pH 8.0 containing 1% Triton). Brain and diluted RBC were stored at −80°C until assayed. The brain tissue was thawed on ice and prepared on the day of analysis. The brain was weighed and diluted (weight/volume) with 0.1 M sodium phosphate buffer, pH 8.0 containing 1% Triton. The final dilution was 1:3. The

	brain tissue was homogenized on ice using a Polytron (Polytron PT3100, probe 3012/2TM, 20,000 rpm, [redacted], NY) for 20 s. Further dilution of both red blood cells and brain homogenate was necessary for analysis on the automated analyzer. The previously diluted (1:3) red blood cells were diluted (on ice) 1:8 for a final dilution of 1:24. The 1:3 brain homogenate was further diluted (on ice) 1:5 for a final dilution of 1:15.
AChE Assay:	Each sample was analyzed on the same day using both the spectrophotometric and radiometric cholinesterase assays (described below). Both of the assays measured total cholinesterase activity (acetyl- and butyryl-cholinesterase activity).
Spectrophotometric method:	Total cholinesterase activity was determined for brain and red blood cells using a Hitachi 911 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN) according to a method described by Hunter and co-workers (Hunter et al., 1997; Toxicol. Methods 7, 43–53). This variation of the Ellman method (Ellman et al., 1961; Biochem. Pharmacol. 7, 88–95) has a total assay volume of 355 µL, consisting of 5 µL of tissue, 300 µL of chromogen/buffer (0.3 mM 5,5′-dithio-bis (2-nitrobenzoic acid); final DTNB concentration in assay 0.25 mM) and 50 µL of substrate (8.45 mM acetylthiocholine iodide; final concentration in assay 1.2 mM). There is a 5-min pre-incubation period and the reaction is conducted at 37 °C.
Radiometric method:	The radiometric assay was essentially as described by Johnson and Russell (1975; Anal. Biochem. 64, 229–238), with a total reaction volume of 100 µL consisting of up to 80 µL of tissue and 20 µL of substrate (6.0 mM acetylcholine iodide and 0.1 µCi of [^{3}H]acetyl-choline iodide [76.0 mCi/mmol, Perkin Elmer Life Sciences, Boston, MA] per 20 µL; final substrate concentration was 1.2 mM). The assay was conducted at 26 °C using 20 µL of 1:3 brain homogenate incubated for 1 min or 80 µL of 1:3 red blood cells incubated for 3 min. After the reaction was stopped and scintillant was added, activity was counted within 24 h of the assay in a Beckman scintillation counter. Counting efficiency, as determined by an external quench standard, was approximately 62%.
Statistics:	All statistical analyses were conducted using StatView® (Version 5.0.1, SAS Institute, Inc.). All data are presented as means ± SE. For comparison of the two assay methods, all data for each compound for brain and red blood cell cholinesterase activity were converted to % control (i.e., divided by the mean of the control values) and compared using a repeated measures ANOVA with assay method as the independent variable and brain and red blood cell cholinesterase activity as the dependent variables. To analyse the time course data for each compound, a repeated measure ANOVA was conducted with time as a

dependent variable and tissue cholinesterase (brain and red blood cells were the repeated measures) as the dependent variable. Where there was a significant interaction of time by tissue, step-down analyses were conducted; otherwise the data were collapsed to identify treatment effects.

II. Results and discussion

A. Comparison of the assay methods

A comparison of the data from all seven of the N-methyl carbamate pesticides showed that the Ellman method (spectrometric assay) under the conditions used here in general, underestimated the cholinesterase inhibition.

The Ellman assay reported more activity in the sample than did the radiometric assay. This is true for both the brain and red blood cell cholinesterase activity.

Interestingly, the Ellman assay as performed under these conditions did not detect any brain cholinesterase activity lower than approximately 38% of control, whereas the radiometric assay indicated that many brain samples had activity less than 30% of control.

A comparison between red blood cell and brain cholinesterase inhibition showed excellent correlation if the radiometric assay was used for analysis, but that correlation became non-existent if the spectrophotometric assay under the conditions performed here was used for analysis. The authors conclude that this is most likely reflects the fact that if one does not have control of the key variables that affect reactivation, the data may become more variable and would tend, therefore, to exhibit less of a correlation between two measures.

B. Mortality

One animal of the methiocarb treated group died 2 hours after dosing.

C. Time course for AChE activity

The time course for brain and red blood cell cholinesterase activity in methiocarb treated rats is presented in Figure 5.7.1/03- 1. The dose of 25 mg/kg bw produced approximately 60–75% cholinesterase inhibition in both brain and red blood cells by 0.5 h after dosing. There was no interaction between tissue and time, so the data were collapsed and compared at each time. This showed that the brain and red blood cell cholinesterase activity did not reach control levels until between 4 and 24 h after dosing.

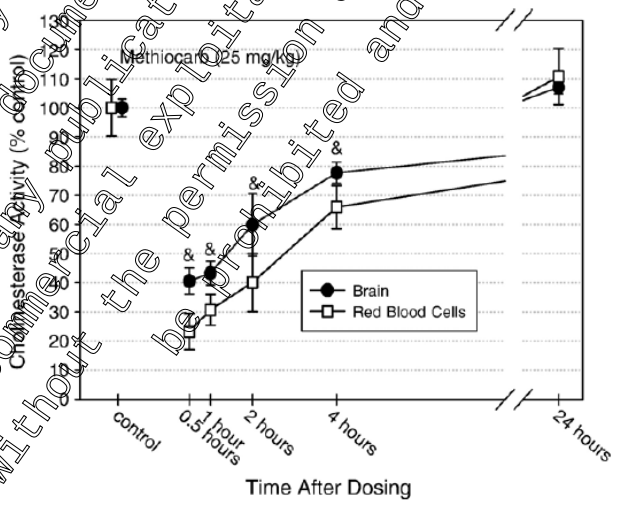


Figure 5.7.1/03- 1: **Time course of brain and red blood cell cholinesterase inhibition in adult male rats dosed orally with methiocarb in corn oil.**

A repeated measures ANOVA did not indicate an interaction between tissue (brain or red blood cell) and time (p=.18).

& Brain and red blood cells are different from control. n=5 / time point, except n=4 at 2 and 24 h (one animal died). Brain control activity = 5959 ± 181 nmol acetylcholine hydrolyzed/min/g tissue. Red blood cell control activity = 449 ± 44 nmol acetylcholine hydrolyzed/min/ml red blood cells. All data are presented as means ± SE.

The results for the other six carbamates showed comparable results. Initiation of cholinesterase inhibition was rapid (within an hour) and short acting. Within 24 h, both red blood cell and brain cholinesterase inhibition had returned to control levels or above.

III. Conclusion

The authors concluded that the potencies of the seven tested carbamates spanned more than an order of magnitude, their toxicity profiles were quite similar in that (1) the spectrophotometric assay using traditional, unmodified conditions was likely to underestimate the cholinesterase inhibition in tissues from these carbamate-treated animals; (2) there was a close correlation between red blood cell cholinesterase inhibition and brain cholinesterase inhibition; (3) all of these seven N-methyl carbamate pesticides were rapidly acting with the time of peak cholinesterase inhibition as early as 30 min after dosing; and (4) recovery from the cholinesterase inhibition was also rapid, usually within hours, and by 24 h after an acute dose of each compound, there was no remaining inhibition of brain or red blood cell cholinesterase activity.

Bayer conclusion:

This non-GLP rat study that was conducted with methiocarb at a dose in the range of known effect levels provides supplemental information on choline esterase inhibition after acute methiocarb exposure. The reported results do not change existing endpoints and do not change the risk assessment for methiocarb.

The reliability evaluation of the publication is given below.

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study, conducted according to scientific principles with minor reporting and methodical deficiencies. Only one dose tested, group size smaller than recommended for neurotoxicity studies (only 5 rats and only 1 control), no information provided if animals were fasted before application, clinical signs not reported, justification for dose level not provided.
Relevance of study:	**Relevant: Supplemental information that does not change existing endpoints and does not lead to a more conservative risk assessment.**

References cited in the publication:

Hunter et al., 1997; Automated instrument analysis of cholinesterase activity in tissues from carbamate-treated animals: a cautionary note. Toxicol. Methods 7, 43–53.
Ellman et al, 1961; A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95
Johnson and Russell, 1975; A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. Anal. Biochem. 64, 229–238

Report:	KCA 5.7.1/04 [redacted]; 2007; M-510973-01-1
Title:	Comparison of acute neurobehavioral and cholinesterase inhibitory effects of N-methylcarbamates in rat.
Report No.:	M-510973-01-1
Document No.:	M-510973-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

1. Test material: Methiocarb

Synonyms:	
Source:	[redacted], PA, USA
Description:	Not reported
Lot/Batch no:	Not reported
Purity:	≥ 99%
Stability of test compound:	expiry date: not reported
Additional test substances:	carbaryl, carbofuran, formetanate, methomyl, oxamyl, propoxur
Source:	[redacted], PA, USA
Purity:	≥ 99%
Stability:	expiry date: not reported

2. Vehicle: For methicarb, and carbaryl, carbofuran, and propoxur treatment: corn oil; for formetanate, methomyl and oxamyl: distilled water

3. Test animals:

Species:	Rat
Strain:	Long Evans
Age:	Approx. 97 days
Sex:	Male
Weight at dosing:	Not reported
Source:	[redacted] Laboratories, [redacted], NC, USA
Acclimatisation period:	Almost 1 week
Diet:	Not reported
Water:	Not reported
Housing:	Individually

B. Study design and methods

1. Animal assignment and treatment:

Dose:	Methiocarb: 0, 0.5, 2, 5, 12, 25 mg/kg bw/day
Application route:	oral gavage
Number of applications:	2 single applications on 2 consecutive days, or 1 single exposure (not clearly stated)

Application volume:	1 mL/kg bw
Group size:	10 males/dose
Dosing solution analysis:	All dosing solutions were analysed.
Observations:	Clinical signs, Tox Score (ranked, global description of degree of overt cholinergic signs, including, but not limited to, lacrimation, miosis, fasciculations, smacking, tremors, polyuria, and diarrhoea (any of these alone or in combination)). Rats were scored as 1, normal; 2, some effects that were not very obvious; and 3, severe and obvious effects, motor activity assessment, AChE assay (brain and RBC)
Examination time points:	Tox Score: approx. 10-12 min after application Motor activity assessment: 15 min after application (session length was 20 min) Tissue sampling: 35-40 min after application from 5 males/dose
Motor activity assessment:	Activity was monitored in a photocell-based chamber shaped like a figure eight (Reiter, 1983). A set of eight photocells spread throughout the chamber measured horizontal activity, and a bank of photocells placed 14 cm above the flooring measured vertical activity. Assessment was made on 10 males/dose group
Tissue/blood sampling and preparation for assays:	At the end of the motor activity measurement, half of the rats (n = 5/dose group) were removed and immediately decapitated under CO_2 anaesthesia for blood and brain collection. The other half of the rats were returned to their home cages and were not used further. Whole brain was collected and immediately placed on dry ice. Trunk blood was collected in a heparinized tube and centrifuged at 1000 x g for 10 min to separate plasma and red blood cells (RBC). The RBC fraction was diluted 1:3 (1 part RBC plus two parts 0.1 M sodium phosphate buffer, pH 8.0 containing 1% Triton). Brain and diluted RBC were stored at −80°C until assayed.
Acetylcholinesterase (AChE) Assay:	The brain tissue was thawed on ice and prepared on the day of analysis. The brain was weighed and diluted (w/v) with 0.1 M sodium phosphate buffer, pH 8.0 containing 1% Triton. The final dilution was 1:3. The brain tissue was homogenized on ice using a Polytron (model PT3100, probe 3012/2TM, 20,000 rpm, [redacted], NY) for 20 s. Special care was taken to limit reactivation of the brain and RBC carbamylated AChE. Tissue dilution was kept to a minimum, and tissues were not further diluted until the exact moment of adding the substrate at the beginning of the assay. Tissues were kept on ice until the exact moment of

the assay, and the time between homogenization (brain) and assay was minimized (preliminary experiments indicated that brain homogenate diluted 1:3 kept on ice for 90 min did not show any significant reactivation).

The radiometric assay was essentially as described by Johnson and Russell (Johnson and Russell, 1975), with a total reaction volume of 100 µL with a final substrate concentration of 1.2 mM acetylcholine iodide spiked with 0.1 µCi of [^{3}H]acetylcholine iodide (76.0 mCi/mmol, Perkin Elmer Life Sciences, Boston, MA). The assay was conducted at 26°C using an incubation of 1 min for brain homogenate and 3 min for RBC. After the reaction was stopped and scintillant was added, activity was counted within 24 h of the assay in a Beckman scintillation counter. Counting efficiency, as determined by an external quench standard, was approximately 62%. On the day of each assay, reference standards (serial dilutions of control rat brain homogenate kept frozen at −80°C) were analyzed immediately before the experimental tissues to ascertain that the assay was performing correctly. These reference values varied no more than 10% over the course of the experiments

Statistics:

Two-way analyses of variance were conducted with a grouping factor of dose and either type of activity (horizontal and vertical) or tissue (brain and RBC) as within-subject factors. Significant interactions were followed by stepdown analyses of individual dose-response data for each measure, with Dunnett's t-test to determine which dose groups were significantly different from control. In addition, paired t-tests were used at each dose level to compare horizontal versus vertical activity and RBC versus brain AChE activity on a percent control basis; Tukey's correction of the p-value was used for these multiple tests.

Pearson's product moment correlation coefficients (r) of within-subject data were calculated for combinations of horizontal, vertical, brain, and RBC AChE data. A t-test was used to test for differences of dependent correlations from the same sample (Blalock, 1972). This t-test was used to compare the correlation of brain or RBC AChE activity versus horizontal activity and brain or RBC AChE activity versus vertical activity.

Benchmark dose modelling software (version 1.4.1; USEPA, 2007) was used to calculate doses estimated to produce a 10% decrease (BMD_{10}; i.e., 90% of control) in each end point. In all cases, the Hill model was used, with a correction for non-equal variances when they were present.

II. Results and discussion

A. Analyses of dosing solutions

The average difference between the nominal and actual concentration for each dose for each carbamate was 9.2%. Each carbamate was administered on two consecutive days with new dosing solutions made up each day. The average concordance between the day 1 and day 2 dosing solutions was 4.5%.

The results for the methiocarb dosing solutions are presented in the following table.

Table 5.7.1/04- 1: Results of dosing solution analyses (mean, n = 2)

Methiocarb concentration in corn oil (mg/mL; nominal / actual)					
0 / 0	0.5 / 0.6	2.0 / 2.0	5.0 / 4.5	12.0 / 11.8	25.0 / 25.4

B. Mortality

There were no mortalities at any dose level of methiocarb or the other carbamates.

C. Tox Score, clinical signs

There were no severe signs of toxicity (e.g. convulsion or respiratory distress) observed in any dose group for any carbamate.

At doses of ≥ 5 mg/kg bw Tox Scores of greater than 1 were observed in few animals. However, the incidence did not exceed 30% (individual data not shown in publication).

D. Motor activity measurements and AChE assays

Methiocarb showed a differentiation between RBC and brain AChE activity at the lowest dose due to an increase in RBC AChE activity. The differentiation between AChE activity and motor activity effects were most clear at the higher three doses. For example, vertical activity was about 5% of control levels while brain AChE activity was at about 55% (i.e., 45% inhibition). While a few rats in the three higher dose groups had Tox Scores greater than '1', the incidence did not exceed 30%. Correlations between AChE activity and motor activity were generally high (~0.9, see Table 5.7.1/04-2). Brain AChE activity was significantly more predictive than RBC only for horizontal activity. The BMD10 value for RBC AChE was highest (5.9 mg/kg), but BMD10 values for the other endpoints (motor activity, brain AChE) had similar values (0.4 – 1.1 mg/kg).

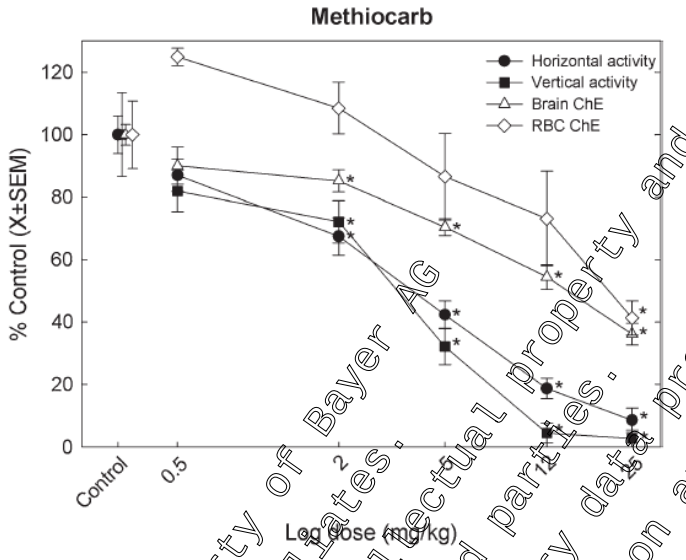


Figure 5.7.1/04- 1: **Effects of methiocarb on motor activity (horizontal and vertical) and AChE activity (brain and RBC), expressed as percent control. * indicates doses significantly different from control ($p < 0.0001$); n =5/dose for AChE measurements, n = 10/dose for motor activity**

D. Combined data of all tested carbamates

When the data of all seven carbamates were combined into a single correlation analysis (see Table 5.7.1/04- 2), the correlations between brain AChE and both forms of activity were significantly higher than the correlations with RBC AChE activity.

Table 5.7.1/04- 2: Within-Subject Pearson Correlation Coefficients between Brain and Blood AChE Activity and Horizontal and Vertical Motor Activity, n = 25 for Each Chemical (5/Dose and 5 Doses). All Correlations Were Significant (all p Values < 0.01)

Test substance	AChE	Motor activity	
		Horizontal	Vertical
Methiocarb	Brain	0.914	0.880
	RBC	0.808	0.793
Carbaryl	Brain	0.576	0.641
	RBC	0.639	0.707
Carbofuran	Brain	0.750	0.701
	RBC	0.683	0.580
Formetanate	Brain	0.829	0.633
	RBC	0.776	0.610
Methomyl	Brain	0.771	0.627
	RBC	0.793	0.514
Oxamyl	Brain	0.922	0.883
	RBC	0.817	0.866

Test substance	AChE	Motor activity	
		Horizontal	Vertical
Propoxur	Brain	0.887	0.807
	RBC	0.679	0.593
All carbamates combined	Brain	0.758	0.737
	RBC	0.637	0.580

With Pearson correlation coefficients generally greater than 0.8, these experiments show a clear correspondence between motor activity and AChE inhibition for all of these carbamate pesticides. Correlation analyses indicated that brain AChE activity was significantly better than RBC AChE when correlated with horizontal activity for propoxur, oxamyl, and methiocarb and for vertical activity only for propoxur. Comparing all carbamates (see Figure 5.7.1/04-2 below) reveals considerable overlap in the data. For almost all carbamates, a linear relationship between decreasing activity and AChE activity, i.e., no threshold was evident (except for formetanate where motor activity was not altered until brain AChE was around 50% of control levels (suggesting a threshold).

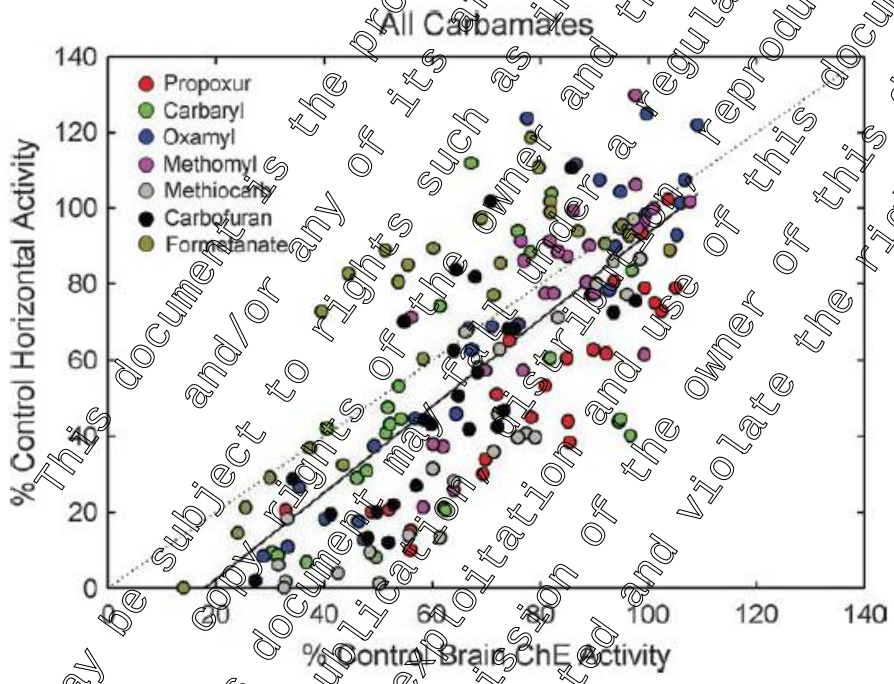


Figure 5.7.1/04- 2: **Scattergram of brain AChE activity versus horizontal activity (both expressed as percent control). Individual data are plotted; solid line shows the regression line; dotted line shows the equi-effective line with a slope of 1.**

III. Conclusion

The authors concluded, that motor activity was a sensitive measure of AChE inhibition for all these carbamate pesticides, and vertical activity showed the greatest magnitude of effect at the highest doses compared to either horizontal activity or AChE inhibition. Brain and RBC AChE activities were generally affected similarly. Pearson correlation coefficients of within-subject data showed good correlation between the behavioural and biochemical end points, with brain AChE inhibition and horizontal activity showing the highest correlation values. Thus, motor activity decreases are highly

predictive of AChE inhibition for N-methyl carbamates, and vice versa.

Bayer conclusion:
This non-GLP rat study that was conducted with methiocarb at doses in the range of known effect levels provides supplemental information on choline esterase inhibition after acute methiocarb exposure. The reported results do not change existing endpoints and do not change the risk assessment.
In addition, the study provides some information on acute neurobehavioral and cholinesterase inhibitory effects of other N-methyl carbamates.

The reliability evaluation of the publication is given below.

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study, conducted according to scientific principles with reporting and methodical deficiencies. Clinical signs were graded according to a scoring system, but details of the scoring system were not reported. Effects in individual animals were not reported, and the number of animals with clinical signs per group was not specified. There was only one time point for assessment of clinical signs, no motor activity assessment were done before administration of test substance, no information provided if animals were fasted before application, justification for dose level not provided. There were also contradictory information if one or two applications were made.
Relevance of study:	**Supplemental information that does not change existing endpoints and does not lead to a more conservative risk assessment.**

Report:	KCA 5.7.1/05 [redacted]; [redacted]; [redacted]; [redacted]; 2010; M-495813-01-1
Title:	Time-Course, Dose-Response, and Age Comparative Sensitivity of N-Methyl Carbamates in Rats.
Report No.:	M-495813-01-1
Document No.:	M-495813-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

1. Test material:	Methiocarb
Synonyms:	Mesurol
Source:	[redacted] Inc., [redacted], PA, USA
Description:	Not reported
Lot/Batch no:	Not reported
Purity:	≥99%

Stability of test compound:	not reported
Additional test substances:	Carbaryl Carbofuran Formetanate Methomyl Oxamyl Propoxur
Assay reagent:	[^{3}H] acetylcholine (Ach) iodide (76 Ci/mmol) (Perkin Elmer Life Science, Boston, USA)

2. Vehicle/Positive controls:

Vehicle:	Corn oil (for methiocarb)
Positive control:	None

3. Test animals:

Species:	Rat
Strain:	Long-Evans
Age:	PND 17 (for dose-response study with methiocarb)
Sex:	Male
Weight at dosing:	Measured, but not reported
Source:	See details below
Details on test animals:	Long-Evans hooded timed-pregnant were obtained from [redacted] Laboratory ([redacted], NC, USA). Pregnant rats were allowed to deliver naturally; day of birth is considered PND0. On PND4 (time-course) or PND2 (dose response), all pups were grouped by sex and redistributed to the dams, assuring that littermates were spread across litters. All litters were culled to eight pups, with six males in each. Only males were used in these studies. Pups in each litter were dosed in a split-litter design, i.e., with no more than one pup within a litter receiving the same treatment. For the time-course studies, all six males in a litter were dosed, and for the dose-response studies, five males in a litter were dosed to assure that no more than one pup in a litter received the same dose. General observations of the litters indicated that dams did not treat the pups differently based on their dosing conditions.
Acclimatisation period:	Not relevant
Diet:	Purina Formulab Diet #5008, *ad libitum*
Water:	filtered tap water, *ad libitum*
Housing:	On hardwood chip bedding (Beta-Chip) in an AAALAC International-accredited animal facility with regulated temperature (72°C ± 2°C) and humidity (50 ± 20%). Pregnant rats also had either a cotton pad (Nestlet) (time-course studies) or Enviro-Dri (dose-response studies) in each cage to serve as nesting material.

B. Study design and methods

1. Animal assignment and treatment:

Dose:	Time-course:	12 mg/kg bw
	Dose response:	0, 0.5, 2, 5, 10 mg/kg bw
	Based on a range-finding study to determine which doses produces moderate but not extreme signs of toxicity.	
Application route:	oral gavage	
Application volume:	2 mL/kg	
Treatment schedule:	Time-course:	Single dose on PND17
	Dose response:	Single dose on PND17
Group size:	Time-course:	n = 6/time point (n = 30 for methiocarb, n = 18 for control)
	Dose response:	n = 10/dose
Observations:	Clinical signs, motor activity, choline esterase activity in brain and blood	
Time-course experiment:	Nominal time points for the time-course study were 15, 45, 90, 180, or 1440 min (24 h); in practice, precise times were 15–20, 45–55, 90–95, 180–190, and 1440–1450 min after dosing. Vehicle-treated control rats were included only at 45, 180, and 1440 min. Acetylcholine esterase (AChE) activity in brain and blood was assessed after sacrifice.	
Dose-response experiment:	Motor activity was assessed 15 min after dosing. Immediately after the activity session, rats were euthanized for tissue collection (40–45 min after dosing). Choline esterase activity in brain and blood was assessed after sacrifice.	
Motor activity:	In the dose-response study, fifteen minutes after dosing, rats were placed in activity chambers shaped like a figure eight. Photobeams spaced around the chamber detected movement as counts for a total of 20 min.	
Acetylcholine esterase (AChE) assay	At the appropriate time, rats were decapitated quickly under CO_2-induced anaesthesia. Trunk blood was collected in heparinized tubes. The whole brain was removed from the skull, split sagitally (including cerebellum), and placed in dry ice. Whole blood was spun at 1000 × g for 10 min, and RBCs were collected and diluted with chilled 0.1 $MNaPO_4$, pH 8.0/1.0% Triton buffer at a 1:2 dilution (one to two parts), and then placed in dry ice. Tissues were stored at -80°C until the day of assay. A radiometric assay was used to determine brain and blood AChE activity. On the day of assay, brain tissues and RBC were thawed on ice (about 20 min). Brains were diluted in two volumes of chilled 0.1 M sodium phosphate buffer (pH 8.0) with 1% Triton X-100 and homogenized for 20–30 s. RBC samples were used directly as prepared on the day of collection. The assay was conducted with a small reaction volume to minimize tissue dilution. The final acetylcholine (ACh) iodide concentration was 1.2 mM. Reactions took place in a water bath at 26°C; incubation times were 30 s for brain and 2 min for RBC. The reaction was then stopped using acid buffer. A toluene-based scintillant was added, the vials were shaken to allow	

extraction of the labelled [^{3}H] acetate, and [^{3}H] activity was counted within a few hours in a Beckman scintillation counter. All samples were run in duplicate; duplicates >20% apart were not used (this only occurred in 0.5% of the total samples assayed). Negative values after blank subtraction were set to zero.

Statistics: To assess differences between treated groups at each of the specific time points on the day of dosing, data for carbamate-treated rats at each time point on the day of dosing were first subjected to two-way ANOVAs with tissue (brain and RBC) as one factor and time as a repeated (within-subject) measure. Since the overall tissue-by-time interactions were highly significant, each tissue was then analyzed separately. Where the resultant overall F-test was significant ($p < 0.05$), it was followed by Tukey's Honestly Significant Difference (HSD) test to provide multiple comparisons across time points while maintaining alpha protection.

To assess persistent differences between the treated and control groups 24 h after dosing, the 24-h treated and control groups for each tissue were compared separately (simple t-test).

For all AChE analyses, data were analyzed using the actual values (i.e., not percent of control). Motor activity dose-response data were analyzed using a one-way ANOVA across dose groups. Dunnett's t-test was used as a protected multiple comparison procedure to provide comparisons between the control and each dose group.

To evaluate motor activity changes as a function of AChE inhibition, correlations were assessed by fitting a linear regression to the data and noting the r^2 value as a measure of goodness of fit. Finally, to better assess comparability across studies, a *post hoc* analysis of the control motor activity data was conducted using a one-way ANOVA followed by Tukey's HSD test for pairwise comparisons across control groups. Dose-response data for AChE activity were analyzed using two approaches (1) traditional ANOVAs to evaluate significant dose-response differences between brain and RBC and to establish dose groups that were significantly different from control and (2) dose-response functions by fitting the data and determining point estimates for doses producing specific levels of AChE inhibition. As with the time-course data, initial two-way ANOVAs were conducted with tissue (brain and RBC) as a within-subject factor and dose as the second factor. Following significant tissue-by-dose interactions, one-way ANOVAs for each tissue were then conducted with Dunnett's t-test to determine dose groups that were different from control. To obtain point estimates, data were fitted to a four-parameter logistic curve using PROAST 17.04 software. For estimation of the benchmark dose (BMD), the models are reparameterized so that the BMD is one of the model's parameters; this allows calculation of confidence intervals around that estimate. Point estimates for 10% (BMD10) and 50% (BMD50) inhibition of AChE were calculated with 95% confidence

intervals.

II. Results and discussion

A. Mortality
In the time-course study one out of six methiocarb-treated rats died prematurely at 12 mg/kg bw.

B. Clinical signs
In general, high-dose pups occasionally displayed mild tremors; however, systematic observations were not possible since the animals were not in view while in the motor activity chambers, and they were euthanized as soon as they were removed.

C. Time-course study
AChE activity for the control groups in the time-course studies was very similar within each study as well as between studies (range, brain 4.98–5.803 µmol acetylcholine (Ach) hydrolyzed/min/mg tissue and RBC 0.678–0.896 µmol/min/mL). There were no marked differences between control AChE activities on the day of dosing compared to the 24-h time point.
For methiocarb, brain and RBC AChE showed considerable inhibition (brain: 60% inhibition and RBC: about 90% inhibition) on the day of dosing. Based on statistically significant differences across the time points, the peak inhibitions for methiocarb were obtained at 15–90 min for brain, and 15–45 min for RBC AChE (based on actual values and not on % control).

According to Figure 5.7.1/05-1 (below) the peak inhibitions for methiocarb were obtained at 45 min for brain, and at 15–45 min for RBC AChE. Recovery of AChE activity to control levels was evident at 24 h (based on % control).

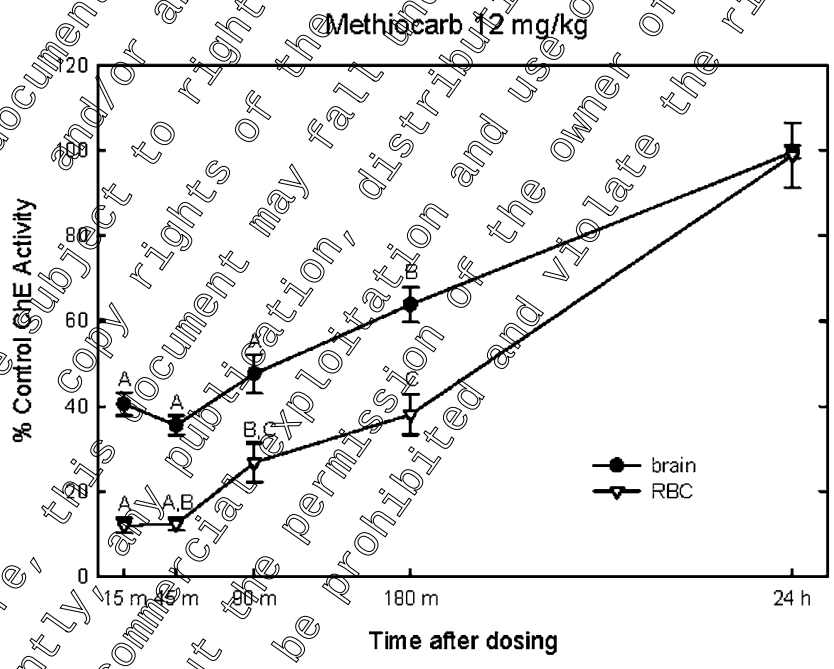


Figure 5.7.1/05-1: Time-course of brain and RBC AChE inhibition for methiocarb in PND17 rats expressed as % control (mean ± SEM). On the day of dosing, dose groups that are not significantly different (as indicated by Tukey's HSD multiple comparisons test) are indicated by the same letters. Asterisk indicates a significant difference between the control and treated group (t-test) at 24 h. Group size: n = 6 per dose per time point.

For the other six carbamates, brain and RBC AChE showed also considerable inhibition (brain, 30–70% inhibition and RBC, 60–90% inhibition) on the day of dosing. Treated groups were not significantly different from each other from 15 to 180 min for carbaryl-induced inhibition of brain and RBC AChE and for carbofuran inhibition of RBC. In contrast, methomyl showed the most rapid recovery during that time frame. The time of peak effect of each carbamate ranged from 15 to 90 min for both compartments. Based on statistically significant differences across the time points, the peak inhibitions were obtained at: carbaryl brain and RBC, 15–180 min; carbofuran brain, 15–45 min and RBC, 15–180 min; formetanate brain, 45–90 min, and RBC, 15–90 min; methomyl brain and RBC, 15 min; oxamyl brain and RBC, 45–90 min; and propoxur brain, 15–90 min, and RBC, 15–45 min. Recovery of AChE activity to control levels was evident at 24 h for all except carbaryl RBC AChE (13% inhibition, p = 0.0173) and methomyl brain AChE (6% inhibition, p = 0.0385). In both cases, the degree of inhibition at 24 h was marginal (see Figure 5.7.1/05- 2 below).

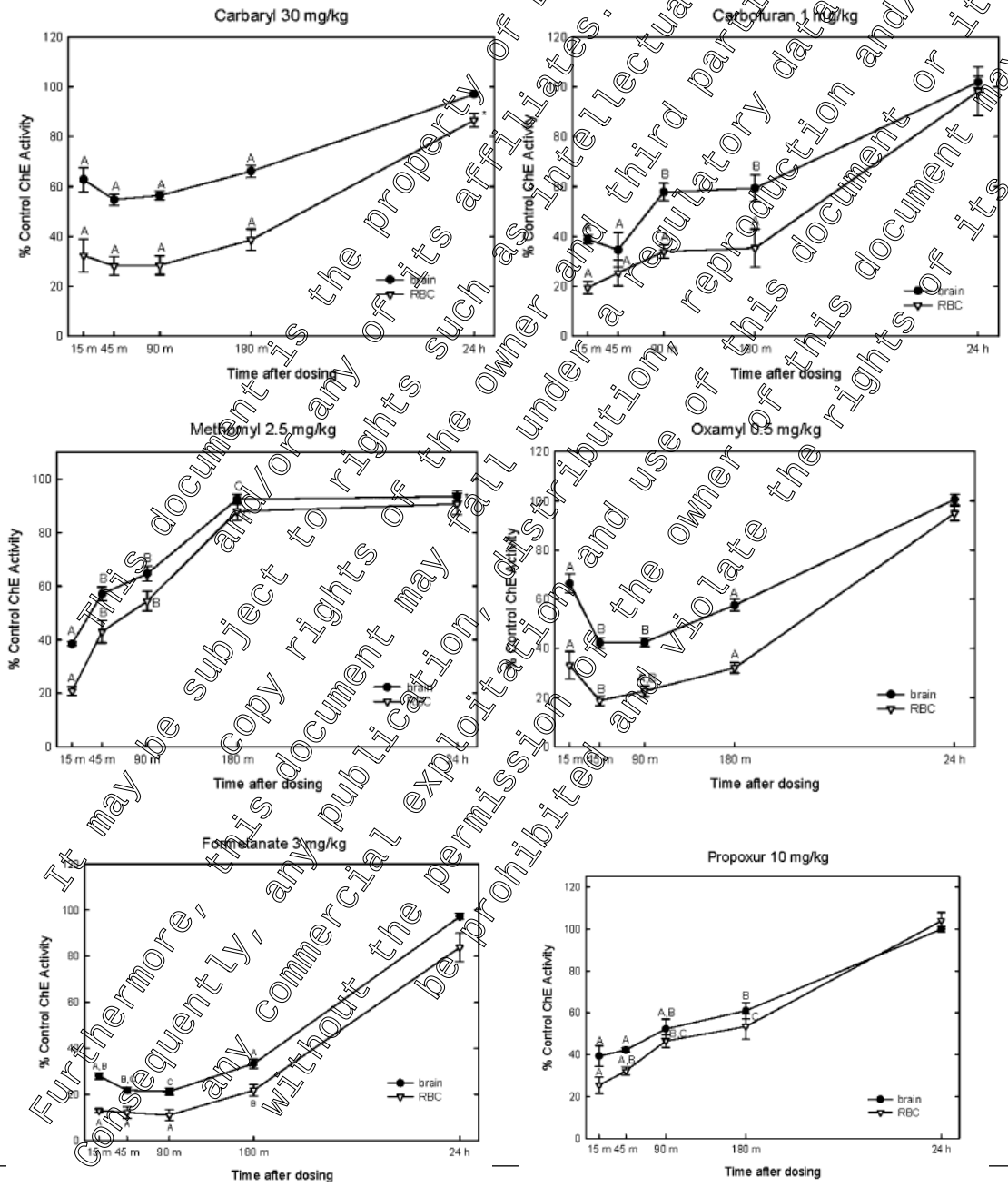


Figure 5.7.1/05- 2: Time-course of brain and RBC AChE inhibition for carbaryl, carbofuran,

methomyl, oxamyl, formetaneate and propoxur in PND17 rats expressed as % control (mean ± SEM). On the day of dosing, dose groups that are not significantly different (as indicated by Tukey's HSD multiple comparisons test) are indicated by the same letters. Asterisk indicates a significant difference between the control and treated group (t-test) at 24 h. Group size: n = 6 per dose per time point.

D. Dose-response study

There was internal consistency across the studies in that the degree of AChE inhibition in the time-course studies was similar to the inhibition produced when the same dose was used in the dose-response studies.

All carbamates showed a monotonic dose-related decrease in brain and RBC AChE activity (data presented only for methiocarb).

Rats treated with the lowest dose of methiocarb showed 15–16% inhibition of brain and RBC AChE, but only the brain was significantly different from control, reflecting the greater variability of the RBC measurement. At the higher doses, RBC and brain inhibition curves diverged, leading to similar BMD10 values for brain and RBC but lower BMD50 values for RBC (see Figure 5.7.1/05- 3 and Table 5.7.1/05- 1).

The two higher doses significantly decreased motor activity, although the magnitude of effect was similar in the two dose groups.

Motor activity depression correlated somewhat with brain AChE inhibition ($r^2 = 0.489$), whereas there was less predictability using the RBC AChE data ($r^2 = 0.357$).

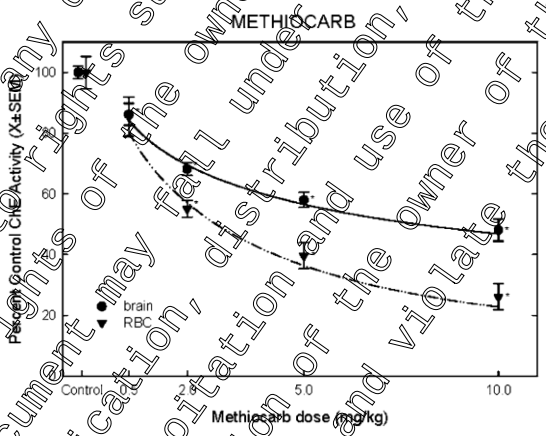


Figure 5.7.1/05- 3: Dose-response of brain and RBC AChE inhibition in PND17 rats expressed as % control (mean ± SEM). Asterisk indicates a significant difference between the control and treated group (Dunnett´s t-test). Group size: n = 6 per dose per time point.

Table 5.7.1/05- 1: Point Estimates for 10% AChE Inhibition (BMD10) and 50% Inhibition (BMD50) of methiocarb in PND17 Rats. Doses are milligrams per kilogram with 95% Confidence Limits in Parentheses

Tissue	BMD10	BMD50
Brain	0.40 (0.23 – 0.58)	3.1 (2.4 – 19.7)
RBC	0.25 (0.086 – 0.47)	1.3 (1.1 – 1.6)

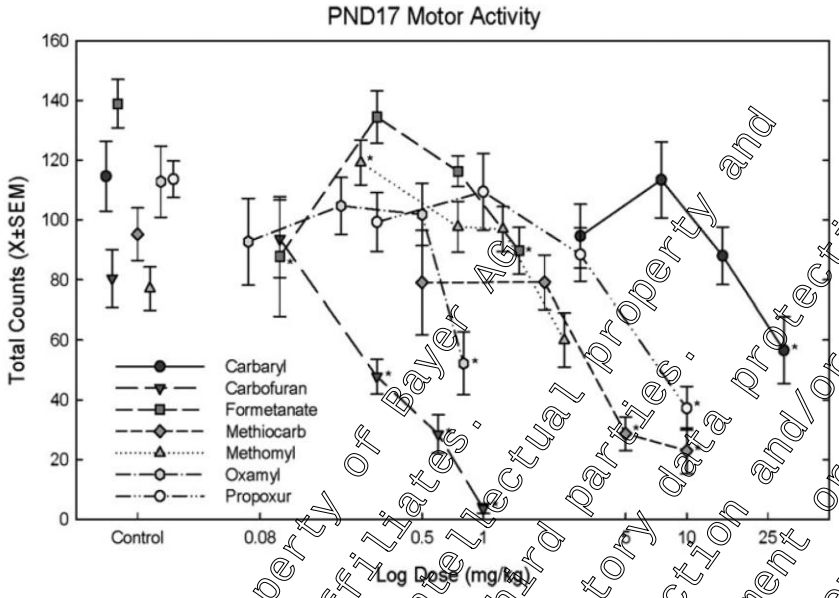


Figure 5.7.1/05- 4: Dose-response of motor activity counts (mean ± SEM) during 20- min session produced by methiocarb and other carbamates in PND17 rats. Asterisk indicates dose groups significantly different from control (Dunnett's t-test). For each carbamate, n = 10 per dose.

III. Conclusion

The authors concluded that N-methyl carbamates produced AChE inhibition that peaked within minutes to hours after a single oral dose and recovered by 24 h. The dose-response of AChE inhibition did not correlate well with decreased motor activity in most cases.

Bayer conclusion:

This non-GLP rat study that was conducted with methiocarb at doses in the range of known effect levels provides supplemental information on acetylcholine esterase inhibition after acute methiocarb exposure. The reported results do not change existing endpoints and do not change the risk assessment for methiocarb.

In addition, the study provides some information on dose-response and acetylcholinesterase inhibitory effects of the six N-methyl carbamates carbaryl, carbofuran, formetanate, methomyl, oxamyl, and propoxur.

The reliability evaluation of the publication is given below.

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study, conducted according to scientific principles with reporting and methodical deficiencies. Clinical signs in individual animals were not reported, number of animals with clinical signs/group not specified, body weights were not reported, no motor activity assessment before administration of test substance, no information provided if animals were fasted before application.
Relevance of study:	**Supplemental information that does not change existing endpoints and does not lead to a more conservative risk**

	assessment.

CA 5.7.2 Delayed polyneuropathy studies

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of methiocarb.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

During the previous EU review, the toxicological properties of several animal, plant and/or soil/groundwater metabolites (methiocarb sulfoxide (M01), methiocarb sulfone (M02), methiocarb phenol (M03), Methiocarb sulfoxide phenol (M04), Methiocarb sulfone phenol (M05), N-hydroxymethyl methiocarb (M06), N-hydroxymethyl methiocarb sulfone (M07) and N-hydroxymethyl sulfoxide (M08)) had already been evaluated based on studies on acute oral toxicity in rats. For methiocarb sulfoxide (M01) sub-acute studies in rats and dogs were also evaluated.
In addition, studies on the plant metabolites methiocarb phenol (M03), methiocarb sulfoxide phenol (M04) and methiocarb sulfone phenol (M05) on acute oral toxicity, and mutagenicity *in vitro* (bacterial reverse mutation assay (Ames test)) were conducted in the confirmatory data phase and peer reviewed on EU level (see Addendum to the DAR of methiocarb[24]).
The results of all available studies on the above mentioned metabolites are provided in the following paragraphs.

Publications
In addition, for methiocarb phenol (M03) one article on the evaluation of estrogenic activity *in vitro* was published in 2011. The publication was considered to be reliable with restrictions. Due to methodically deficiencies and the fact that the *in vitro* concentrations are much higher than in *vivo* concentrations after non-lethal doses, the results of the *in vitro* assays are of no relevance for the human risk assessment of methiocarb and its metabolites.

Methiocarb sulfoxide (M01)
For the minor rat metabolite methiocarb sulfoxide (M01) acute oral toxicity studies in fasted and non-fasted rats, as well as sub-acute oral toxicity studies in rats and dogs are available.
The acute oral LD_{50} of methiocarb sulfoxide in fasted rats was 7-9 mg/kg bw and in a repeat test 6-8 mg/kg bw. In non-fasted rats a LD_{50} of 43 mg/kg bw was determined. Therefore, the metabolite methiocarb sulfoxide (M01) is considered to be more toxic than the parent compound.
In a comparative 28-day oral study for cholinesterase inhibition in the rat with methiocarb and the primary metabolite, methiocarb sulfoxide, the NOAEL for plasma and erythrocyte cholinesterase activity was 0.5 mg/kg bw/day for methiocarb and <0.5 mg/kg bw/day for methiocarb sulfoxide.
In the dog, the NOEL for methiocarb based on plasma cholinesterase inhibition was 0.05 mg/kg bw/day whilst very slight sporadic inhibition was observed for methiocarb sulfoxide indicating a threshold dose level for cholinesterase inhibition. Brain cholinesterase activity was not determined.
The cholinesterase NOEL for methiocarb sulfoxide in 28-day rat study was estimated to be at 0.1-0.2 mg/kg bw/day, whilst in the 29 day oral study in the dog the minimal effect level was 0.05 mg/kg bw/day. The corresponding NOEL for the parent methiocarb was 0.05 mg/kg bw/day.

[24] Methiocarb Addendum – Confirmatory Data (Mammalian Toxicology, Residues and Ecotoxicology) to the Report and Proposed Decision of the United Kingdom made to the European Commission under Article 8(1) of 91/414/EEC, taking into account of confirmatory data specified in the inclusion Directive 2007/5/EC and Review Report for methiocarb (SANCO/10039/2006 rev.3), RMS United Kingdom, April 2011

Overall, the metabolite methiocarb sulfoxide is more toxic than the parent compound and is also a more potent cholinesterase inhibitor.

Table 5.8.1- 1: Summary of studies with methiocarb sulfoxide (M01)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	25-30-40-50-60-100 mg/kg bw	LD_{50} = 43 mg/kg bw	[redacted], 1978 M-010382-01-1
Rat Acute oral (fasted)	2.5-5-10-20 mg/kg bw	LD_{50} = 9/7 mg/kg bw (m/f)	[redacted], C.S., 1976 M-012510-01-1
Rat Acute oral (fasted)	2.5-5-10-20 mg/kg bw	LD_{50} = 6/8 mg/kg bw (m/f)	[redacted], C.S., 1976 M-012503-01-1
Rat 4-week,oral (gavage)	0-0.5-2.0 mg/kg bw/day	NOEL = 0.5 mg/kg bw/day (estimated to be between 0.1 and 0.5 mg/kg bw/day) based on AChE inhibition at ≥ 0.5 mg/kg bw/day	Hixson, E.J., 1981 M-009348-01-1
Dog, 29-day, oral (capsule)	0-0.05-0.2 mg/kg bw/day	NOEL = 0.05 mg/kg bw/day based on slight erythrocyte AChE inhibition, cholinergic signs	[redacted], 1981 M-009577-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Methiocarb sulfone (M02)

For rat, plant, cow and soil metabolite methiocarb sulfone (M02) an acute oral toxicity study in rats has been conducted.

The acute oral toxicity study revealed a LD_{50} > 1000 mg/kg bw showing that M02 is of less acute toxicity than methiocarb.

Table 5.8.1- 2: Summary of studies with methiocarb sulfone (M02)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	100-250-500-1000 mg/kg bw	LD_{50} > 1000 mg/kg bw	[redacted], 1970 M-010387-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Methiocarb phenol (M03)

Two acute oral toxicity studies and an Ames test are available on the rat, plant, animal, soil and water metabolite methiocarb phenol (M03).

Acute oral toxicity studies in rats with LD_{50}-values of > 2000 mg/kg bw (fasted) and > 1000 mg/kg bw revealed that **methiocarb phenol (M03) has a lower acute oral toxicity than the parent compound methiocarb**. In the study of 2007, no mortality, clinical signs or symptoms of AChE inhibition were observed. Furthermore, **methiocarb phenol (M03) was negative for point mutations in the bacterial reverse mutation test.**

In addition, one publication emerged from public literature from 2011 that described estrogenic activity in two *in vitro* test systems. The publication based on non-GLP studies, which due to methodically deficiencies and large differences between the determined effect concentrations and

maximum plasma concentrations that can be obtained after non-lethal doses *in vivo* are considered to be not relevant.

Table 5.8.1- 3: Summary of studies with methiocarb phenol (M03)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	100-250-500-1000 mg/kg bw	LD_{50} > 1000 mg/kg bw	[redacted] 1970 M-010349-01-1
Rat Acute oral (fasted)	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] 2007 M-292380-01-1
Bacterial reverse mutation assay (*S. typhimurium* TA1535, TA100, TA1537, TA98, TA102)	1st assay: 16-5000 µg/plate (plate incorporation) 2nd assay: 16-512 µg/plate (plate incorporation) (+/- S9 mix)	negative (+/- S9 mix)	[redacted] 2007 M-292915-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Methiocarb sulfoxide phenol (M04)

Two acute oral toxicity studies and an Ames test are available on the rat, plant, animal, soil and water metabolite methiocarb sulfoxide phenol (M04).

Acute oral toxicity studies in rats with LD_{50}-values of > 2000 mg/kg bw (fasted) and > 1000 mg/kg bw revealed that **methiocarb sulfoxide phenol (M04) has a lower acute oral toxicity than the parent compound methiocarb**. In the study of 2007, no mortality, clinical signs or symptoms of AChE inhibition were observed. Furthermore, **methiocarb sulfoxide phenol (M04) gave a clear negative result for point mutations in the bacterial reverse mutation test**.

Table 5.8.1- 4: Summary of studies with methiocarb sulfoxide-phenol (M04)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	100-250-500-1000 mg/kg bw	LD_{50} > 1000 mg/kg bw	[redacted]., 1970 M-010323-01-1
Rat Acute oral (fasted)	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted]., 2007 M-292384-01-1
Bacterial reverse mutation assay (*S. typhimurium* TA1535, TA100, TA1537, TA98, TA102)	16-5000 µg/plate (plate incorporation) 16-5000 µg/plate (pre incubation) (+/- S9 mix)	negative (+/- S9 mix)	[redacted] 2007 M-292915-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Methiocarb sulfone phenol (M05)

Two acute oral toxicity studies and an Ames test are available on the rat, plant, animal, soil and water metabolite methiocarb sulfone phenol (M05).

Acute oral toxicity studies in rats with LD_{50}-values of > 2000 mg/kg bw (fasted) and > 1000 mg/kg bw revealed that **methiocarb sulfone phenol (M05) has a lower acute oral toxicity than the parent compound methiocarb**. However, at 2000 mg/kg bw mortality and clinical signs indicative of cholinesterase inhibition were observed. In addition, **methiocarb sulfone phenol (M05) was negative for point mutations in the bacterial reverse mutation test**.

Table 5.8.1- 5: Summary of studies with methiocarb sulfone-phenol (M05)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	100-250-500-1000 mg/kg bw	LD_{50} > 1000 mg/kg bw	[redacted] 1979 M-010375-01-1
Rat Acute oral (fasted)	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] 2007 M-292392-01-1
Bacterial reverse mutation assay (*S. typhimurium* TA1535, TA100, TA1537, TA98, TA102)	16-5000 µg/plate (plate incorporation) 16-5000 µg/plate (pre-incubation) (+/- S9 mix)	negative (+/- S9 mix)	[redacted], 2007 M-294754-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

N-hydroxymethyl methiocarb (M06), N-hydroxymethyl methiocarb sulfone (M07) and N-hydroxymethyl sulfoxide (M08)

For the three metabolites N-hydroxymethyl methiocarb (M06), N-hydroxymethyl methiocarb sulfone (M07) and N-hydroxymethyl sulfoxide (M08) only acute oral toxicity studies in rats are available.

The acute oral LD_{50} of N-hydroxymethyl methiocarb, N-hydroxymethyl methiocarb sulfone and N-hydroxymethyl sulfoxide were determined to be >112, >112 and >160 mg/kg bw, respectively, compared to 33 and 47 mg/kg bw in males and females for methiocarb technical in the same study.

Table 5.8.1- 6: Summary of studies with N-hydroxymethyl methiocarb (M06)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	10.6-15.8-17.9-23.3-39.4-66.7-112.7 mg/kg bw	LD_{50} > 112 mg/kg bw	[redacted], 1979 M-026415-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Table 5.8.1- 7: Summary of studies with N-hydroxymethyl methiocarb sulfoxide (M07)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	2.5-5-10-20-40-80-160 mg/kg bw	LD_{50} > 160 mg/kg bw	[redacted], 1979 M-026415-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Table 5.8.1- 8: Summary of studies with N-hydroxymethyl methiocarb sulfone (M08)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	23.3-39.4-66.6-112 mg/kg bw	LD_{50} > 112 mg/kg bw	[redacted] 1979 M-026415-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Publication(s)
methiocarb phenol (3,5-Dimethyl-4-(methylthio)phenol (DMP), M03)

Report:	KCA 5.8.1/18[redacted],;[redacted];[redacted]; 2011; M-495823-01-1
Title:	Estrogenic activity of alkyl(thio)phenols and 4,4-thiodiphenol formed from degradation of commercial insecticides.
Report No.:	M-495823-01-1
Document No.:	M-495823-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

1. Test material: 3,5-Dimethyl-4-(methylthio)phenol

Synonyms: DMP

Source: Synthesised in course of this study by sulfenylation of 3,5-dimethylphenol using dimethyl disulfide under acidic conditions.

Description: Characterised by NMR, chemical structure was given

Purity: 98% (GC), purified with reverse-phase HPLC

Stability of test compound: Not tested

Additional test substances: Methiocarb (MTC) (98.5% purity), Dr. [redacted], Germany)
3-methyl-4-(methylthio)phenol (MMP) (> 95.0% purity), 4-(methylthio)phenol (MTP, >98.0% purity), and 4,4´-thiodiphenol (TDP, > 98.0% purity) all from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan)

2. Vehicle/positive controls:

Vehicle: Not stated

Positive controls: Reference compound (set to 100% relative activity):
BPA (analytical grade), no source given
Estrogenicity positive control:
17β-estradiol (E2, >97.0% purity), no source given

3. Test system:

Species: Yeast

Strain: *Saccharomyces cervisiae* Y190

Source: Not reported

Details on the test organism: See below

B. Study design and methods

1. Treatment:

Yeast assay:

Details on the assay: The estrogenic activities of the test compounds were examined with the yeast two-hybrid estrogenicity assay system developed by Nishikawa *et al. (1999)* [Toxicol. Appl. Pharmacol., 154, 76–83] and modified using yeast cells (*Saccharomyces cervisiae* Y190); hERα and coactivator TIF2 had been introduced into this system. The expression plasmids for the hormone receptor ligand binding domain and

	pGAAD424-TIF-2 were introduced into the yeast cells that carried the β-galactosidase reporter gene. The assay was adapted to a chemiluminescent reporter gene (for β-galactosidase) method by employing a 96-well culture plate.
Treatment:	To measure the estrogenic activity in this system, aliquots from test chemical solutions (solvent not reported!) were incubated for 4 hr at 30°C, where yeast cells were preincubated overnight at 30°C in a modified synthetic dextrose medium (not containing tryptophan and leucine).
Concentration range for test substances:	E2: 31.3 – 2,000 pM (= positive control) BPA: 313 – 20,000 nM (= reference compound) DMP: 3,125 – 200,000 nM MTC: 3,125 – 200,000 nM
Quantification/luciferase assay:	After the incubation period, a mixed solution of 2 mg/mL of zymolyase and a commercial reporter gene assay kit, which induces chemiluminescence and enzymatic digestion, was added at a 5:3 ratio. This was followed by the addition of a light-emission accelerator reagent. The intensity of the chemiluminescence produced by the released β-galactosidase was measured using a 96-well plate luminometer.
Calculations:	The estrogenic activity was recorded as the effective concentration (EC) × 10, which is defined as the concentration of the test solution required to produce a chemiluminescent signal 10 times stronger than that of the blank control. The relative activity (RA) was calculated on the basis of the following equation: RA (%) = (EC × 10 for BPA)/(EC × 10 for test compound) × 100
Replicates:	The assays were repeated in triplicate for all of the tested compounds

<u>ER-Elisa:</u>

Details on the assay:	The hERα competitive binding assay was conducted using an Estrogen-R (α) Competitor Screening kit (Wako Pure Chemical Industries, Ltd.), in accordance with the manufacturer's protocol.
Treatment:	A mixture of fluorescent-labeled E_2 and the test compounds were added to the wells of a plate coated with hERα, and they were allowed to compete at room temperature for 2 hr. After washing the wells with the wash-solution contained in the kit, the assay solution was added to each well. The fluorescence intensity in each well was read with a microplate reader at an excitation and emission wavelength of 485 and 535 nm, respectively.
Concentration range for test substances:	E2: 1.56 – 50 nM (= positive control) BPA: 625 – 20,000 nM (= reference compound) DMP: 12500 – 400,000 nM TDP: 1250 – 40,000 nM MTP and MMP: 50000 – 1600000 nM
Replicates:	The assay was repeated in triplicate for all of the tested compounds.
Calculations	The inhibition curves for fluorescent-labeled E2 binding were obtained on the basis of a reduction in the fluorescence intensity. IC_{50} values, *i.e.*, the concentrations at which replacement of fluorescent-labeled E_2 was 50%, were

calculated using the inhibition curves.
The relative binding ability (RBA) was calculated as follows:
RBA (%) = (IC_{50} for BPA)/(IC_{50} for test compounds) × 100.

Statistics: All data were expressed as mean ± standard deviation (SD).
Data were not statistically analysed.

II. Results and discussion

A. Estrogenic activity – yeast two hybrid assay

The RA% value of DMP was estimated to be 4% of BPA activity. Although this value is smaller than that of BPA, it appeared that the compound was weakly active. In addition, the EC × 10 of E_2 was estimated 0.3 ± 0.07 nM. On the other hand, dose-response relationship was not observed for MTC in the range of the tested concentrations. Results of the assay with methiocarb were not reported.

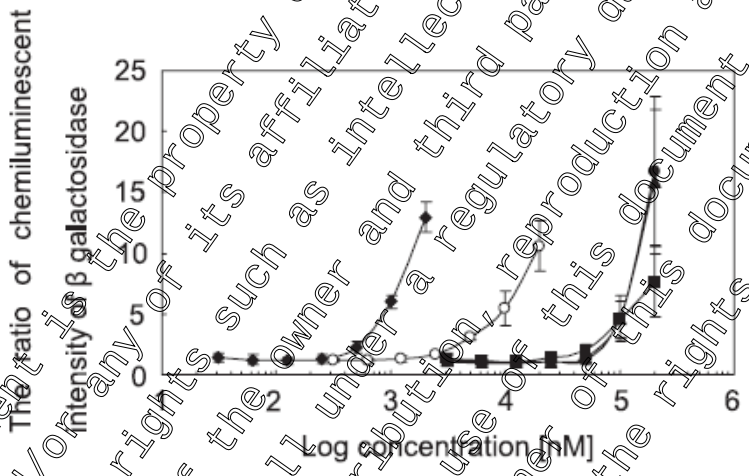


Figure 5.8.1/18- 1: Dose-response curves in Yeast Two-hybrid Assay

● = MTP, ▲ = MMP, ■ = DMP, ◆ = TDP, ○ = BPA (reference compound).
Each point represents the mean of triplicate.

Table 5.8.1/18- 1: Estrogenic activity of relevant test compounds[a]

Group	Estrogenic activity	
	EC × 10 (nM)[b]	RA (%)[c]
DMP	449000 ± 103000	4
BPA	17000 ± 600	100
E_2	0.3 ± 0.07	5666667

DMP = 3,5-dimethyl-4-(methylthio)phenol, BPA = bisphenol-A, E_2 = 17β-estradiol

[a] Only results for DMP, BPA and E_2 are shown. RA-data (%) for E_2 were calculated from the EC x 10-data given in the paper and depicted here for illustrative purposes. Values are presented as the mean ± standard deviation of triplicate

[b] EC = effective concentration; EC × 10 = the concentration of test compounds showing 10% of the activity of blank controls

[c] RA = relative activity (BPA = 100)

Data for methiocarb were not provided (no dose-relationship was observed was observed!)

B. Estrogenic activity – ER-ELISA

In general the RBA-values observed in the ER-ELISA were smaller than the RA-values determined in the yeast two hybrid assay.

The relative binding ability (RBA%) of DMP to hERα was 6% of BPA binding ability. Regarding RBA% and RA% both values were similar for DMP. However, it was also found that the ordering of activities obtained from both bioassays indicated the same trend: TDP > BPA> MTP, MMP, and DMP.

Based on the results the authors determined that *p*S-alkyl phenols possess binding ability to hERα, and that the estrogenic activity observed from the yeast two-hybrid assay is responsible for this ability.

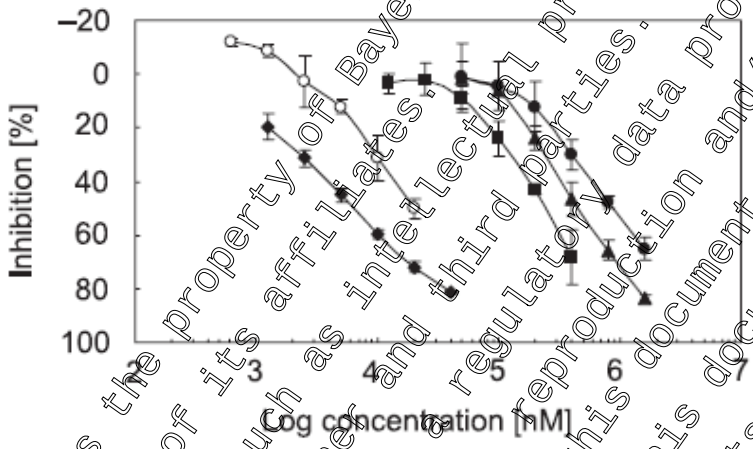


Figure 5.8.1/18- 2: Dose-response curves in ER-ELISA

● = MTP, ▲ = MMP, ■ = DMP, ◆ = TDP, ○ = BPA (reference compound)
Each point represents the mean of triplicate.

Table 5.8.1/18- 2: hERα binding ability of relevant test compounds[a]

Group	hERα binding ability	
	IC_{50} (nM)[b]	RBA (%)[c]
DMP	271000± 14000	6
BPA	17000± 700	100
E_2	25 ± 4	68000

DMP = 3,5-dimethyl-4-(methylthio)phenol, BPA = bisphenol-A, E2 = 17β-estradiol

[a] Only results for DMP, BPA and E_2 are shown; RBA-data (%) for E_2 were calculated from the IC_{50}-data given in the paper and depicted here for illustrative purposes. Values are presented as the mean ± standard deviation of triplicate

[b] IC_{50} = 50% inhibition concentration

[c] RBA = relative binding ability (BPA = 100)

Data for methiocarb were not provided

C. Structural considerations

Regarding bio-isosterism (i.e. description of functional groups related in structure and possessing similar biological effects) it was surmised that an alkylthio-group substituted at the "*para*"-position of a phenol ring plays a key role in the binding abilities of the investigated phenolic compounds.

III. Conclusion

The authors concluded, that DMP, as well as the other tested para-S-alkyl phenols possesses a weak estrogenic activity.

Bayer conclusion

With regard to estrogenicity the yeast two-hybrid estrogenicity assay and the hERα competitive binding assay revealed no relevant information. The authors report the supposed estrogenicity of DMP compared to BPA. When compared to BPA, DMP possesses only weak if any estrogenic activity in the assays, i.e. RA of 4%, with a low binding activity, i.e. RBA of 6%.

The authors fail to report the estrogenic activity/binding ability to the hERα of DMP compared to the positive control estrogen, E2, which is negligible, i.e. 7 × 10^{-5}% and <0.01% for RA and RBA, respectively. In addition, no negative control was used in the assays – only blank controls, and no statistical analysis was conducted.

Furthermore, the observed effect levels for estrogenic activity (measured as EC x 10) and binding ability to hERα (IC_{50}) for DMP were 449 µM and 271 µM, respectively. However, the total maximum plasma concentration of DMP observed after treatment of rats with 0.91 mg/kg bw methiocarb by oral gavage was 0.064 µM (based on a maximum dose-normalized concentration CN_{Max} of 0.111 kg bw/kg blood ([redacted], 2009; M-348264-02-1; see also KCA 5.1.1/05)). After an acute lethal dose of 19 mg/kg bw methiocarb the calculated total plasma concentration of DMP that can be reached is 0.64 µM. This in vivo plasma concentration is about 33.8 times and about 20.4 times lower than the determined EC x 10 and IC_{50} values measured in the in vitro assays, respectively. For details of the calculation, please refer to the box below.

Furthermore, it has to be considered that due to protein-binding in blood the unbound fraction of DMP is even lower than the total concentration. This consideration leads to a further increase of the factors between the tested in vitro and in vivo concentrations of DMP.

Taking these considerations into account, the described weak estrogenic effect observed in the in vitro assays is considered to be of no biological relevance for the in vivo exposure situation in humans.

The reliability evaluation is given below

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	The study was not conducted according to GLP. For the assays there exist no validated test guidelines. The assays were conducted according to accepted scientifically principles. However, there were methodically and / or reporting deficiencies. Methiocarb that was used to synthetize DMP was not purchased from Bayer/BCS, the impurity profile of the used materials is not reported. No negative controls except blanks were included in the assays. A cytotoxicity assessment for the concentration range tested was not conducted. The DMP concentration tested are not relevant to the *in vivo* exposure situation of methiocarb. No statistical analysis was included.
Relevance of study:	**Due to the methodically deficiencies and the large differences between the determined EC x 10 and IC_{50} values and the maximum total plasma concentration that can be reached *in vivo* after non-lethal doses the results of these *in vitro* assays are of no relevance for the human risk assessment of methiocarb and its metabolites.**

From [redacted] 2009; M-348264-02-1; see also KCA 5.1.1/05:**
In this study the maximum dose normalised concentration (CN) of the metabolite methiocarb-phenol (= M03, = DMP) and its conjugates (sulfate and glucuronic acid) was 0.111 kg bw/kg blood detected 0.25 h after application of 1 mg/kg bw methiocarb.

Based on this value the maximum plasma concentration (c_{Max}) of DMP after a lethal dose of 19 mg/kg bw methiocarb (LD_{50}) can be calculated as follows:

CN_{Max} (DMP + conjugates) = 0.111 kg bw/kg blood ≙ 0.111 mg/kg blood
With a dose (D) of 19 mg/kg bw the resulting c_{Max} is
$c_{Max} = CN_{Max} * D$
c_{Max} = 2.109 mg/kg blood
Taken into account a blood density of 1.06 kg/L (i.e. 1 kg blood = 0.943 L) the resulting c_{Max} is
c_{Max} = 2.24 mg/L
with a molecular weight of DMP of 168 g/mol the total maximum concentration of DMP and conjugates in blood is:
c_{Max} = 0.0133 mM (i.e. = 13.3 µM)

Abbreviations
CN = dose-normalized concentration

CA 5.8.2 Supplementary studies on the active substance

Summary of supplementary studies
Supplementary studies for methiocarb were done for specific examinations on cholinesterase inhibition, antidote effects and potentiation. These studies were already evaluated and summarized by the former RMS United Kingdom in Volume 3, Annex B, B.6 of the DAR, July 2005 (public version). Summaries of the evaluation are given below.

Studies on cholinesterase inhibition
The mode of action of methiocarb is inhibition of the AChE. Therefore, measurements of AChE inhibition in plasma, RBC and brain were conducted in most of the repeated dose toxicity studies (for details please refer to the respective section of this dossier).
A specific study for AChE inhibition after acute and repeated 28-day exposure to methiocarb was also conducted. In the acute experiments rats received a single dose of 0, 1, 10, 25 or 50 mg/kg bw. Plasma and RBC AChE were determined at 0 min, 20 min, 1.5 h (only control and high dose), 2 h, 3 h (only control and high dose), and 5 h after application for plasma and RBC AChE, and at 0.5 h, 1 h, 2 h, 3 h, and 5 h after application for brain AChE. Typical symptoms of AChE inhibition (trembling, cramps) were observed at ≥ 10 mg/kg bw. The symptoms appeared within 5 min to 10 min after application, and were resolved 2 h later. The maximum dose-related levels of cholinesterase inhibition were recorded 20 minutes after the application in the dose groups of 25 mg/kg bw and below, and 20 minutes to 2 hours after application in the highest dose group. Two hours after the application, a marked increase in enzyme activity was already noted again in all dose groups except the highest one. In the lower dose groups AChE was only slightly, not biologically significant (<20%) reduced. The brain AChE inhibition peaked after 2 hours at dose levels of 10 and 20 mg/kg bw methiocarb. Thereafter, brain AChE activity increased again.
In the sub-acute experiment rats (M-009378-01-1) received methiocarb daily for 28 days by gavage application at dose levels of 0, 3 or 10 mg/kg bw/day. Plasma and RBC AChE were determined 20 min after application on days 1, 8, 14, 21, 28 and on day 28 five hours after application. Brain AChE was determined 2 h after the final application. Plasma and RBC AChE was biologically significantly decreased at the highest tested dose of 10 mg/kg bw 20 min after application. 5 h after the final application AChE was still slightly but not biologically relevant reduced (i.e. inhibition was <20%). Brain AChE was also inhibited at 10 mg/kg bw two hours after application. In other dose groups brain AChE was slightly reduced (not biologically significant). Cumulative cholinesterase inhibition was not observed during the 4 week treatment.

In another sub-acute study (M-009348-01-1) rats received daily doses of 0, 0.5 or 2 mg/kg bw/day methiocarb by gavage. Plasma and RBC AChE were determined before dosing and 30 min after treatments on days 0, 7, 14 and 21 in one sub-group, and before dosing and 4 hours after dosing on days 4, 11, 18 and 25 in a second sub-group. In rats receiving methiocarb at 2 mg/kg bw/day, a significant reduction of plasma AChE was observed during the first three weeks. RBC AChE was reduced only during the first week. There was a decreasing effect with increasing duration of treatment observed for the plasma AChE. Recovery to normal activity was within four hours after treatment.
In a 29-day study (M-009577-01-1) dogs received daily applications of methiocarb at 0.05 and 0.5 mg/kg bw/day by capsules. Signs of toxicity and significant AChE inhibition in plasma and RBC were observed at 0.5 mg/kg bw/day. Maximum depression of AChE was observed 0 to 3 hours after application, with full recovery within 6 hours.

Table 5.8.2 - 1: Summary of special cholinesterase inhibition studies*

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
Rat, acute oral (gavage) 0-1-10-25-50 mg/kg bw	M F	1 1	10 10	Biological relevant ↓ of plasma, erythrocyte and brain AChE activity; cholinergic symptoms (trembling, cramps)	[redacted] 1973 M-009378-01-1
Rat 4-week, oral (gavage) 0-3-10 mg/kg bw/day	M F	3 3	10 10	↓ of plasma, erythrocyte and brain AChE activity; cholinergic symptoms, No accumulation of AChE inhibition	
Rat 4-week,oral (gavage) 0-0.5-2.0 mg/kg bw/day	F	0.5	2	Tremors during the first days only, (> 25%) of plasma and erythrocyte AChE activity (during the first week only)	[redacted] 1981 M-009348-01-1
Dog, 29-day, oral (capsule) 0-0.05-0.5 mg/kg bw/day	M F	0.05 0.05	0.5 0.5	Cholinergic signs, slight ↓ of erythrocyte AChE activity (2 h post application)	[redacted] 1981 M-009577-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.
↓: decrease(d)

Antidote studies

In investigations of antidote effects in rats, the acute oral LD_{50} of methiocarb was 67 mg/kg bw (without antidote); 467.5 mg/kg bw (with atropine), 188 mg/kg bw (with PAM), 225 mg/kg bw (with BH6), 497.5 mg/kg bw (atropine + PAM) and 512.5 mg/kg bw (atropine + BH6). Atropine was shown to provide significant antidotal protection against the cholinergic effects of methiocarb. PAM and BH6 did not provide significant antidotal effects. Atropine sulphate also provided greater antidotal effect than TEAC. The acute oral LD_{50} of methiocarb was 104.5 mg/kg bw (without antidote); 415 mg/kg bw (with TEAC); 643 (with atropine sulphate); and 580 (with atropine sulphate + TEAC).

Table 5.8.2 - 2: Summary of antidote studies*

Study	Dose levels / concentrations tested	Result	Reference
Rat	10-1000 mg/kg bw	LD_{50} values (mg/kg bw) without antidote: 67 with atropine: 467.5 with PAM: 188 with BH6: 225 with atropine+PAM: 487.5 with atropine+BH6: 512.5	[redacted] 1966 M-010305-01-1
Rat	Not reported	LD_{50} values (mg/kg bw) without antidote: 104.5 with atropine: 3643 with TEAC: 415 with atropine+TEAC: 580	[redacted], 1974 M-015306-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.
PAM = 2-pyridine aldoxime methyl chloride or pralidoxime
BH6 = obidoxime chloride
TEAC = tetraethyl ammonium chloride

Potentiation studies

There were two pre-GLP studies that investigated the potentiating effect of methiocarb with other anticholinesterase insecticides. In the first study with dose levels of methiocarb and the other insecticides at one half of the reported LD_{50} no potentiating effects were observed. In the second study methiocarb was tested in combination with 4 other products again at one half of the reported LD_{50} values. Details of the 4 products were not provided. Only with one product (Dipterex) there was a slight increase in mortality (55%) observed.

Table 5.8.2- 3: Summary of potentiation studies*

Study Doses tested	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL / Results	Reference
i.p rat 15 mg/kg bw methiocarb in combination with other pesticides	na	na	Mortalities were less than 50% (5-45%) for all tested combinations	[redacted]., 1961 M-016511-01-1
i.p rat 15 mg/kg bw methiocarb in combination with other pesticides	na	na	Slight increase in mortality with methiocarb in combination with Dipterex	[redacted] 1961 M-016504-01-1

* New studies, i.e. studies previously not submitted / evaluated on EU level, are written black. Previously evaluated studies are written in light grey.

Publications

Seven publications emerged from public literature between 2009 and 2013. In four publications the potential of methiocarb to cause oxidative damage after acute and repeated *in vivo* exposure of rats was evaluated. The other three reported studies on binding activities to three receptors (i.e. PPAR, AhR and PXR).

The four publications that examine oxidative damage are all based on non-GLP studies. Due to different deficiencies three of the publications are considered to be not reliable and therefore not relevant. The fourth publication on this topic had also some deficiencies and was assessed to be reliable with restrictions. This publication provides supplemental information on potential oxidative stress induced by methiocarb treatment, but has no influence on existing endpoints and the risk assessment for methiocarb.
The three publications providing information on *in vitro* activation / binding activities of methiocarb to three different receptors are also based on non-GLP studies that were conducted according to scientific principles. Due to methodological and reporting deficiencies they were assessed to be reliable with restrictions. They provide supplemental information that has no influence on existing endpoints and do not lead to a more conservative risk assessment.

The summaries of the publications, as well as the reliability and relevance assessment are provided below.

Report:	KCA 5.8.2/08 [redacted]; 2009; M-504908-01-1
Title:	Methiocarb-induced oxidative damage following subacute exposure and the protective effects of vitamin E and taurine in rats.
Report No.:	M-504908-01-1
Document No.:	M-504908-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

1. Test material:	Methiocarb
Synonyms:	Mesurol
Source:	Bayer, [redacted], Turkey
Description:	Not reported
Lot/Batch no.:	Not reported
Purity:	99.8%
Stability of test compound:	expiry date: not reported
Additional chemicals:	All chemicals were analytical reagent grade and chemicals required for all biochemical assays were obtained from [redacted] ([redacted], MO, USA), and [redacted] ([redacted], Germany
2. Vehicle:	For methicarb treatment: corn oil; for Vitamin E: olive oil for taurine: distilled water
3. Test animals:	
Species:	Rat
Strain:	Wistar albino
Age:	12-13 weeks
Sex:	Male
Weight at dosing:	160 ± 20 g

Source:	[redacted], University of [redacted], Turkey
Acclimatisation period:	10 days
Diet:	Standard dry pellet diet, *ad libitum*
Water:	water, *ad libitum*
Housing:	In groups of eight in polypropylene cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	Methiocarb (MC): 28 x 2 mg/kg bw/d Vitamin E (Vit. E): 100 mg/kg bw/d Taurine (T): 50 mg/kg bw/d
Application volume:	0.5 mL/rat (corresponding to about 0.3 mL/100 g bw)
Application route:	Applications were done in the morning (between 9 and 10 a.m.) to non-fasted rats. Methiocarb: oral gavage Vitamin E and Taurine: i.p.
Treatment schedule:	Control group (I):0.5 mL corn oil/animal for 28 consecutive days Methiocarb group (II): 2 mg/kg bw/d in corn oil for 28 consecutive days Vitamin E group (III): 100 mg/kg bw/d in olive oil (0.5 mL/rat) for 48 days; i.p. Vitamin E plus methiocarb group (IV): pre-treatment with 100 mg/kg bw/d for 20 days (i.p), then methiocarb at 2 mg/kg bw/d along with Vitamin E for 28 days Taurine group (V): 50 mg/kg bw/d in distilled water (0.5 mL/rat) for 48 days; i.p. Taurine plus methiocarb group (VI): pre-treatment with 50 mg/kg bw/d for 20 days (i.p), then methiocarb at 2 mg/kg bw/d along with taurine for 20 days
Group size:	8
Observations:	Clinical signs, histology (liver, kidney), lipid peroxidation assay, GSH-assay, measurement of antioxidant enzymes, protein assay
Tissue preparation for assays:	At the end of the treatments, the rats were sacrificed by cervical dislocation. Liver and kidney samples were dissected out and washed immediately with ice-cold physiological saline (0.9% NaCl) and one parts of the both tissues immediately stored at −80°C until analysis. The other parts of the both tissues were taken from the rats for histological analysis. The tissues were homogenized in 0.9% NaCl using an Ultra Turrax tissue homogenizer to make up the 10% tissue homogenate (w/v) and then, one parts of tissue homogenate (10%) centrifuged at 10000 × g at 4°C for 20 min to obtain

	cytosolic fraction. Tissue homogenates (10%) were used to determine levels of GSH and malondialdehyde (MDA). Cytosolic fractions were used to determine activities of antioxidant enzymes.
Lipid Peroxidation Assay:	Quantitative measurement of lipid peroxidation was performed in liver and kidney homogenates (10%) according to the method of Buege and Aust (1976) [Method Enzymol, 52:302-310] based on the formation of thiobarbituric acid reactive substances (TBARS) and expressed as the extent of malondialdehyde (MDA) production. The amount of TBARS was determined in tissue homogenates by using phase HPLC with UV diode-array detection at 532 nm according to the modification of the HPLC method of Draper and Hadley (1990) [Method Enzymol, 186:421-431]. The levels of TBARS were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as nmol MDA per mg of protein.
GSH assay:	Levels of glutathion (GSH) were determined in the liver and kidney homogenates (10%) according to the method of Beutler (1975) [Red cell metabolism. A Manual of Biochemical Methods. Second ed. New York, Grune and Stratton, 71-73] by using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reagent. DTNB was reduced by free -SH groups of GSH to form 5-mercapto-2-nitrobenzoate and its absorbance were measured by spectrophotometric means at 412 nm. Results were expressed as µmol GSH per mg of protein using standard calibration curve.
Measurement of antioxidant enzymes:	Activities of antioxidant enzymes (Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSH-Px), Glutathione reductase (GSH-Rd)) were determined in the cytosolic fractions of liver and kidney homogenates.
SOD assay:	Superoxide dismutase (SOD) activity was determined according to the method of Sun et al. (1988) [Clin Chem, 34:497-500] based on the inhibition of nitroblue tetrazolium (NBT) reduction by using the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the supernatants of 10 000g of ethanol/chloroform (5/3, v/v) extracts of tissue homogenates (10%). One unit of SOD was defined as the enzyme amount causes 50% inhibition in NBT reduction rate. Specific activity was expressed as unit SOD per mg of protein using standard calibration curve.

CAT assay: Catalase (CAT) activity was assayed in the cytosolic fractions of tissue homogenates by the decomposition of hydrogen peroxide according to the method of Aebi (1984) [Method Enzymol, 105:121-126]. This reaction follows a first-order kinetic given by equation k = (2.3/t) (log A0/A1). Specific activity was expressed as k per mg of protein using standard calibration curve

GSH-Px assay: Glutathione peroxidase (GSH-Px) activity was determined in the cytosolic fraction of tissue homogenates according to the method of Pleban et al. (1982) [Clin Chem, 28:311-316] based on the decrease in the absorbance of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm.
Specific activity was expressed as unit GSH-Px per mg of protein using standard calibration curve. One unit of GSH-Px is defined as the amount of enzyme that oxidizes 1 µmol of NADPH per minute.

GSH-Rd assay: Glutathione reductase (GSH-Rd) activity was performed in cytosolic fraction of tissue homogenates by monitoring the oxidation of NADPH in the presence of oxidized glutathione according to the method of Beutler (1969) [J Clin Invest, 48:1957-1966.]. Specific activity was expressed as mU GSH-Rd per mg protein using standard calibration curve.
One unit of GSH-Rd is defined as the amount of enzyme that oxidizes 1 µmol of NADPH per minute.

Protein assay: Contents of proteins were measured in the liver and kidney homogenates (10%) and cytosolic fractions of liver and kidney homogenates according to the method of Lowry et al. (1951) [J Biol Chem, 193:265-275] using bovine serum albumin (BSA) as standard.

Histology: Liver and kidney tissues were fixed in Bouin's solution. The tissues were dehydrated and embedded in paraffin. The paraffin sections of 5 µm thickness were stained with Masson's triple dyes (Masson). All sections were examined under a light microscope (Olympus-CX 41).

Statistics: All data were expressed as mean ± standard deviation (SD). Data were analyzed by ANOVA test using "SPSS version 13.0 for Windows", statistical program and the individual comparisons of groups were obtained by using Scheffe`s multiple comparison procedure. p values of less than 0.05 and 0.001 were selected as the levels of significance

II. Results and discussion

A. Mortality

There were no mortalities in the methiocarb treatment groups.
No information reported for the control groups.

B. Clinical signs

None of the rats showed any signs of morbidity after repeated oral treatment with 2 mg/kg bw/day methiocarb.
No information reported for the control groups.

C. Lipid peroxidation assay

The content of MDA in the rats treated with methiocarb was statistically significantly increased 1.95-fold in liver (p<0.001) and 1.55-fold in kidney (p<0.001) when compared to the control group. Pre-treatment and co-treatment with vitamin E and taurine resulted in a statistically significant decrease in the content of hepatic (p<0.001) and renal (p<0.001, p<0.05, respectively) MDA when compared to the methiocarb group.
Treatment of rats with vitamin E and taurine alone did not affect the levels of MDA when compared to the vehicle control groups.

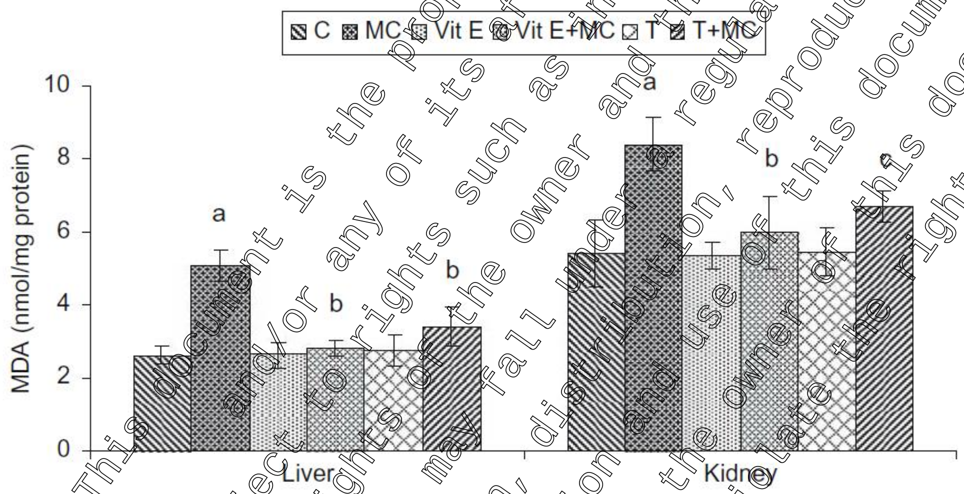


Figure 5.8.2/06- 1: Results of the lipid peroxidation assay – Effects of vitamin E and taurine on MDA content (nmol/mg protein) in methiocarb treated rat liver and kidney. MDA levels (mean ± SD, n = 8)

MC = methiocarb; Vit. E = vitamin E; T = taurine
a	Significantly different from control group (p < 0.001).
b	Significantly different from methiocarb group (p < 0.001).
c	Significantly different from methiocarb treated group (p < 0.05) (one way ANOVA).

D. GSH assay

In contrast to the MDA levels, the level of GSH in the rats treated with methiocarb was significantly decreased in liver (−19%, p<0.001) and kidney (−30%, p<0.001) when compared to the vehicle control group. However, pretreatment with vitamin E and taurine resulted in a significant increase in the level of hepatic (p<0.001) and renal (p<0.001) GSH in comparison with that of the methiocarb group.
Treatment of rats with vitamin E and taurine alone did not affect the levels of GSH when compared to the vehicle control groups.

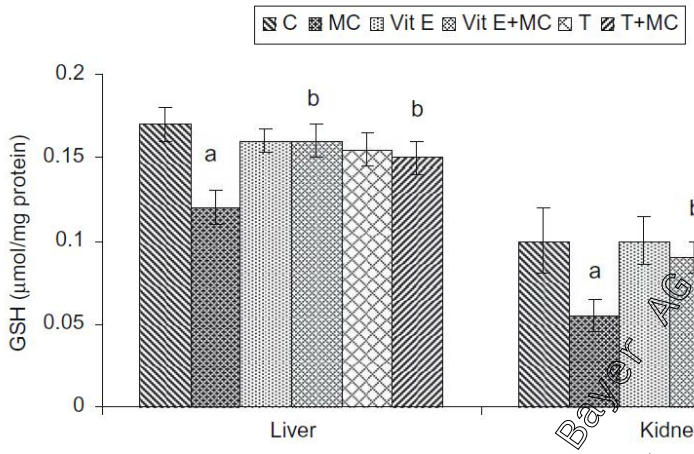


Figure 5.8.2/06- 2: Results of the GSH assay – Effects of vitamin E and taurine on GSH levels (µmol/mg protein) in methiocarb treated rat liver and kidney. GSH levels (mean ± SD, n = 8)

MC = methiocarb; Vit. E = vitamin E, T = taurine
a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.001$) (one way ANOVA).

E. Measurement of antioxidant enzymes

SOD assay

The activity of SOD was statistically significantly decreased in liver (–30%, $p < 0.001$) and kidney (–30%, $p < 0.001$) tissues of the methiocarb treatment group when compared to the vehicle control. However, pre-treatment with vitamin E and taurine resulted in a statistically significant increase in the activity of SOD in liver ($p<0.05$) and kidney ($p<0.05$) in comparison with that of the methiocarb group.

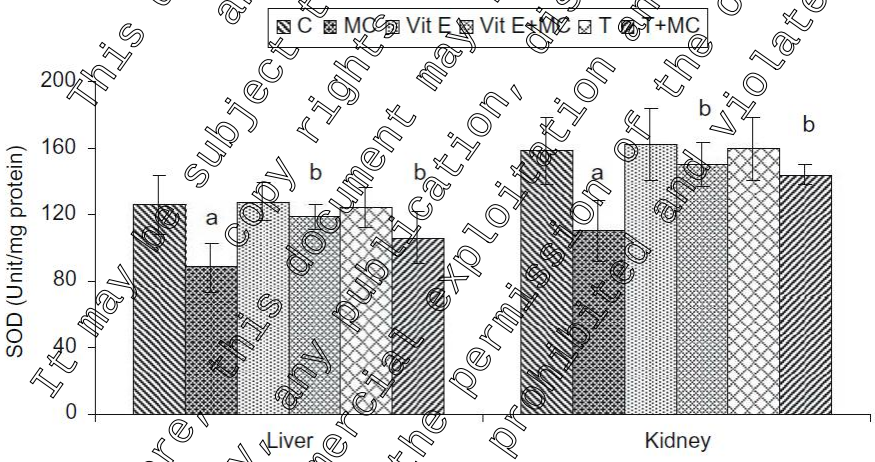


Figure 5.8.2/06- 3: Results of the SOD assay – The effects of vitamin E and taurine on SOD activity (U/mg protein) in methiocarb treated rat liver and kidney. (Values expressed as mean ± SD, n = 8)

MC = methiocarb; Vit. E = vitamin E, T = taurine
a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.05$) (one way ANOVA).

CAT assay

The activity of CAT was statistically significantly decreased in liver (–23%, $p < 0.001$) and kidney (–18%, $p < 0.001$) of the methiocarb treatment group when compared to the vehicle control. However, pre-treatment with vitamin E and taurine resulted in a statistically significant increase in the activity of CAT in liver ($p<0.05$) and kidney ($p<0.001$ and $p<0.05$, respectively) in comparison with that of the methiocarb group.

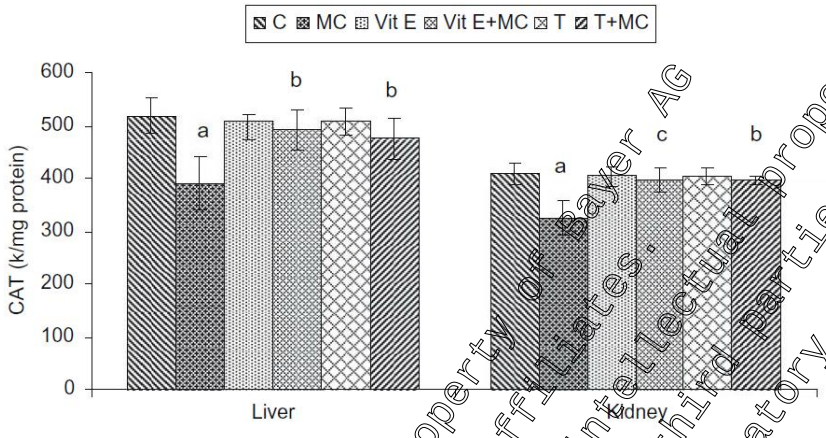


Figure 5.8.2/06- 4: Results of the CAT assay –Effects of vitamin E and taurine on CAT levels (k/mg protein) in methiocarb treated rat liver and kidney (Values expressed as mean ± SD, n = 8)

MC = methiocarb; Vit. E = vitamin E, T = taurine
a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.05$).
c Significantly different from methiocarb treated group ($p < 0.001$) (one way ANOVA).

GSH-Px assay

The activity of GSH-Px in the methiocarb group was significantly decreased in liver (–40%, $p<0.001$) and kidney (–32%, $p<0.001$) when compared to the vehicle control group. However, pre-treatment with vitamin E and taurine resulted in a significant increase in the activity of GSH-Px in liver ($p<0.05$) and kidney ($p<0.05$) in comparison with that of the methiocarb group.

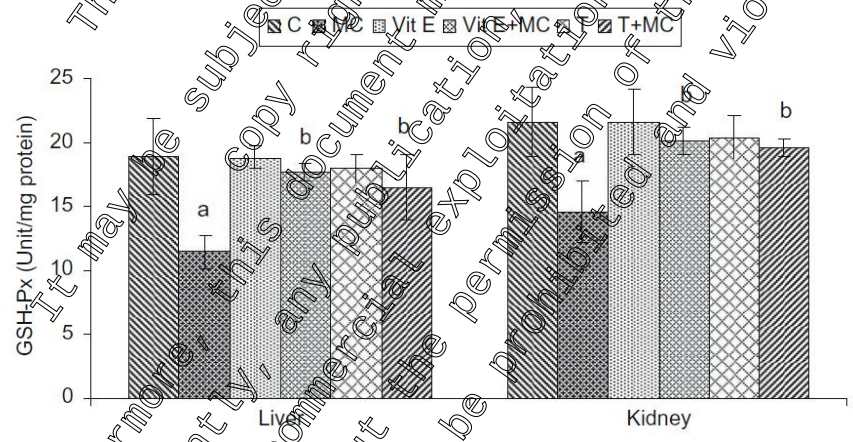


Figure 5.8.2/06- 5: Results of the GSH-Px assay – The effects of vitamin E and taurine on GSH-Px levels (U/mg protein) in methiocarb treated rat liver and kidney. (Values expressed as mean ± SD, n = 8)

MC = methiocarb; Vit. E = vitamin E, T = taurine
a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.05$) (one way ANOVA).

GSH-Rd assay

The activity of GSH-Rd in the methiocarb group remained unchanged in liver and decreased non-significantly in kidney (–8%) when compared to the control group. Pre-treatment with vitamin E and taurine showed no change in the activity of GSH-Rd in liver and kidney when compared to the methiocarb group.

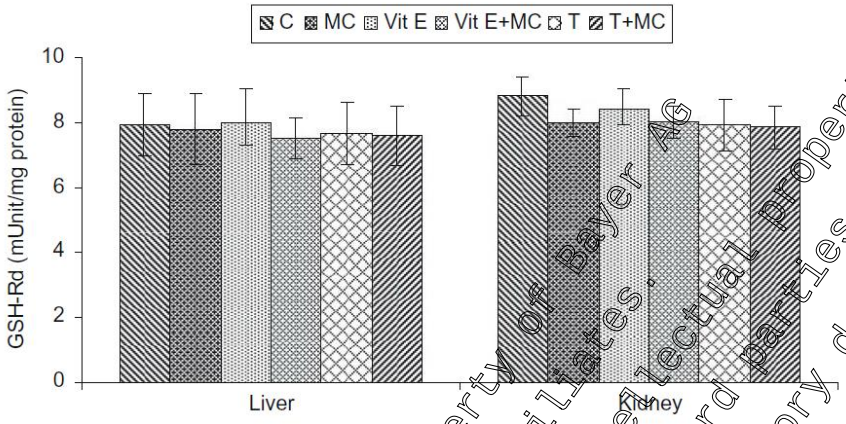


Figure 5.8.2/06- 6: Results of the GSH-Rd assay – The effects of vitamin E and taurine on GSH-Rd levels (mU/mg protein) in methiocarb treated rat liver and kidney. (Values expressed as mean + SD, n = 8)

MC = methiocarb; Vit. E = vitamin E, T = taurine

Furthermore, treatment of rats with vitamin E and taurine alone did not affect the activities of all measured antioxidant enzymes when compared to the vehicle control groups.

D. Histopathology

Liver

There were no histopathological findings observed in the livers of control animals. According to the publication administration of methiocarb produced mild degenerative changes such as little sinusoidal dilatations and small accumulation of collagen fibres in around some veins in the liver tissue. In the presence of vitamin E and taurine, there was no improvement in the liver tissue comparing to the group treated with methiocarb.

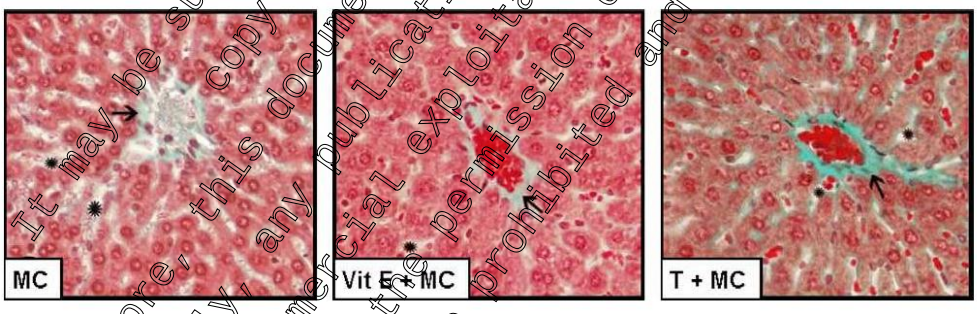


Figure 5.8.2/06- 7: The effects of vitamin E and taurine on histological changes in liver of methiocarb treated rats, with and without pre-treatment of either vitamin E or taurine.

MC = methiocarb; Vit. E = vitamin E, T = taurine; Sinusoidal dilatation (*); accumulation of collagen fibers around veins (→). Masson stain. Original magnification x270

Kidney

According to the authors there were no histopathological findings observed in kidneys of control animals. The cortex had a large number of glomeruli that proximal and distal tubules located around them.

The methiocarb-induced injury consisted of tubulointerstitial changes visible as shortening at the brush border, cytoplasmic debris and desquamated nuclei in the widened lumens of proximal tubules and accumulation of collagen fibers in necrotic areas which localized around especially damaged proximal tubules. Moreover, glomerula showed changes in morphology such as widening between parietal and visceral leaves of Bowman capsules in kidneys treated with methiocarb. Furthermore, few mononuclear cell infiltration was also seen in this group. On the other hand, the animals treated with vitamin E plus methiocarb and taurine plus methiocarb, showed a partly decrease in kidney damage comparing to the group treated with methiocarb.

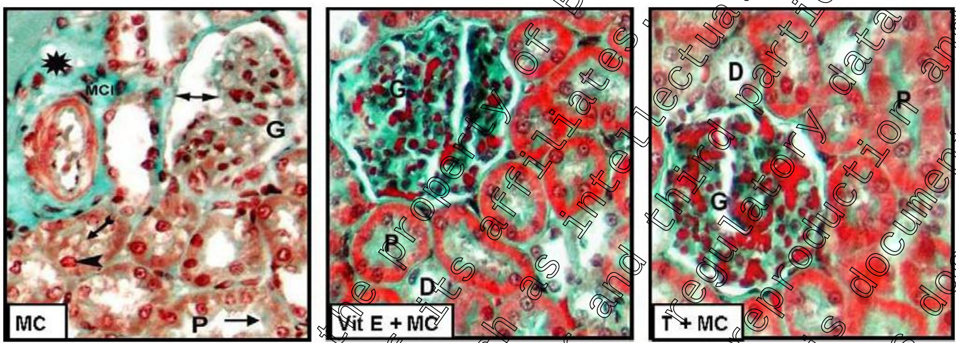


Figure 5.8.2/06- 8: The effects of vitamin E and taurine on histological changes in kidney of methiocarb treated rats. The widening between parietal and visceral leaves of Bowman capsules (↔), the shortening at the brush border (→), desquamated nuclei (➤) and cytoplasmic debris (➡) in the proximal tubules (P), accumulation of collagen fibers in necrotic areas (✸), few mononuclear cell infiltration (MCI) can be seen in kidney tissue of methiocarb treated rat.

MC = methiocarb; Vit. E = vitamin E, T = taurine
Masson stain. Original magnification x270.

III. Conclusion

The author concluded that methiocarb exposure results in the induction of lipid peroxidation and changes in antioxidant system, as well as tissue degeneration in rat liver and kidney. The reported data indicate that reactive oxygen species might be associated with the methiocarb-induced toxicity. Moreover, they proposed that the results of the study showed that pre-treatment of vitamin E and taurine may attenuate methiocarb-induced oxidative damage by decreasing lipid peroxidation and altering antioxidant defence system in rat liver and kidney. But, because of the high antioxidant potential of liver, vitamin E and taurine could not showed the effect for protection in the liver cells with less histological changes whereas they could partly prevent the histological damage in kidney cells.

Bayer conclusion

This non-GLP study is considered to be not reliable (see reliability assessment below). The authors conclusions on the different assays for the assessment of oxidative damage based solely on statistical significances, without taking into account the biological relevance of the observed changes. For example, the authors describe a decrease of GSH-levels of up to –30% after the repeated exposures to methiocarb when compared to control values. However, it is known that enhanced lipid peroxidation

is only seen when the hepatic GSH-content decreases to about 20% of the initial / or control concentration. A change in antioxidant defense enzyme activity not necessarily results in organ damage. Only if the capacity of the antioxidant defense system is exhausted organ damage can occur.
Regarding the histopathological findings, it has to be considered that the animals were not exsanguinated. Thus, the observed sinusoidal dilatation of the liver could be an artefact. Furthermore, described kidney changes, like the alteration of the brush border, are only observable with electron microscopy.
Without positive and negative historical control data it is not possible to draw a conclusion from the reported results.
Overall, it is concluded that the results of this study, besides the fact of its lack of reliability, have no impact on the risk assessment of methiocarb.

The reliability evaluation is given below.

Klimisch evaluation

Reliability of study:	**Not reliable (Klimisch code 3)**
General comment on reliability:	Non-GLP study with methodical and reporting deficiencies: • only one methiocarb dose tested • only one sex tested • results only presented as illustrations; • the results for the enzyme assays are not presented in tabulated form (i.e. no means and no individual number of affected animals/group reported) • the conclusion for the enzyme assays based solely on statistical evaluation, the biological relevance of the measured values is not provided • exact number of animals in individual groups with histopathological findings not reported, • no historical control data provided
Comment on reliability Histopathology:	• animals were sacrificed (non-fasted) by cervical dislocation • animals were not exsanguinated • applied staining method not suitable for the assessment of cytoarchitectural changes
Relevance of study:	**Non relevant due to lack of reliability.**

Report: KCA 5.8.2/09 [redacted]., [redacted]; 2010; M-505197-01-1
Title: Effects of methiocarb on lipid peroxidation and glutathione level in rat tissues.
Report No.: M-505197-01-1
Document No.: M-505197-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: **no**

I. Materials and methods

A. Materials

1. Test material: Methiocarb

Synonyms:	Mesurol
Source:	Bayer, [redacted], Turkey
Description:	Not reported
Lot/Batch no:	Not reported
Purity:	99.8%
Stability of test compound:	expiry date not reported

2. Vehicle: corn oil

3. Test animals:

Species:	Rat
Strain:	Wistar albino
Age:	12-13 weeks
Sex:	Male
Weight at dosing:	160 ± 20 g
Source:	[redacted] University of [redacted], Turkey
Acclimatisation period:	10 days
Diet:	Standard dry pellet diet, *ad libitum*
Water:	water, *ad libitum*
Housing:	In groups of 8 in polypropylene cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	Methiocarb (MC): 1 x 25 mg/kg bw 5 x 10 mg/kg bw/day 28 x 2 mg/kg bw/day
Application volume:	0.5 mL/rat (corresponding to about 0.3 mL/100g bw)
Application route:	Applications were done in the morning (between 9 and 10 a.m.) to non-fasted rats Methiocarb: oral gavage
Treatment schedule:	Control group (1): 0.5 mL corn oil/animal for 1 day (i.g.) Methiocarb group (2): 25 mg/kg bw/d in 0.5 mL corn oil for 1 day (i.g) Control group (3): corn oil for 5 days (i.g.) Methiocarb group (4): 10 mg/kg bw/d for 5 days (i.g.). Control group (5): corn oil for 28 days (i.g.) Methiocarb group (6): 2 mg/kg bw/d for 28 days (i.g.).
Group size:	8
Observations:	Clinical signs, lipid peroxidation assay, GSH-assay, protein content
Sacrifice / sampling time:	24 h after the last treatment
Tissue samples:	Liver, kidney, brain, testis

Tissue preparation for assays:	At the end of the treatments, the rats were sacrificed by cervical dislocation. Tissues were removed immediately, then washed with ice-cold physiological saline (0.9% NaCl) and stored at–80°C until the day of the experiment. Tissues were homogenized in ice-cold 0.9% NaCl, using a tissue homogeniser (Basic Ultra-Turrax T-18, IKA Werke GmbH and Co., Staufen, Germany) to make up the 10% homogenate (w/v). Tissue homogenates (10%) were used for the analysis of protein, LPO, and GSH.
Lipid Peroxidation Assay:	Quantitative measurement of lipid peroxidation was performed in tissue homogenates (10%) according to the method of Buege and Aust (1976) [Method Enzymol, 52:302-310] based on the formation of thiobarbituric acid reactive substances (TBARS) and expressed as the extent of malondialdehyde (MDA) production. The amount of TBARS was determined in tissue homogenates by using reversed-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet (UV) diode-array detection (Thermo Separation Products, Thermo Separation Products, California, USA) at 532 nm, according to the modification of the HPLC method of Draper and Hadley (1990) [Meth Enzymol 186:421–431]. After preparing the samples and carrying out the thiobarbituric acid reactions with the method of Buege and Aust, the reaction products were purified on a reverse-phase C18 cartridge, from which the TBARS complex was recovered in 2 mL of methanol. Aliquot was applied to a LiChrospher RP-18e C18 column (5 µm, 4.6 × 250 mm; Phenomenex, California). A mobile phase of 60% MeOH and 40% phosphate buffer (pH 6.8) and UV detection at 532 nm were used for the optimum conditions. The levels of TBARS were calculated by using 1,1,3,3-tetraethoxypropane as the standard and expressed as nmol MDA/mg protein.
GSH assay:	Levels of glutathion (GSH) were determined in tissue homogenates (10%) according to the method of Beutler (1975) [Red cell metabolism. A Manual of Biochemical Methods. Second ed. New York, Grune and Stratton, 71-73] by using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reagent. DTNB was reduced by free -SH groups of GSH to form 5-mercapto-2-nitrobenzoate and its absorbance were measured by spectrophotometric means at 412 nm. Results were expressed as µmol GSH per mg of protein using standard calibration curve.

Protein content: Contents of proteins were measured in tissue homogenates (10%) according to the method of Lowry et al. (1951) [J Biol Chem, 193:265-275] using bovine serum albumin (BSA) as standard.

Statistics: All data are expressed as mean ± standard deviation. Data were analyzed by the analysis of variance test, using the SPSS version 13.0 for Windows (SPSS, Inc., Chicago, Illinois, USA) statistical program, and the individual comparisons of groups were obtained by using Scheffe's post-hoc analysis at $P < 0.001$ and $P < 0.05$.

II. Results and discussion

A. Mortality

There were no mortalities observed in the methiocarb treatment groups.
No information reported for the other groups.

B. Clinical signs

None of the rats treated with methiocarb at 25 mg/kg bw, 10 mg/kg bw/day or 2 mg/kg bw/day showed any signs of morbidity.
No information reported for the other groups.

C. Lipid peroxidation assay

The MDA levels, an end-product of lipid peroxidation (LPO), in the rats treated with methiocarb for one day were significantly increased in liver (+129%; $P < 0.001$), kidney (+54%; $P < 0.001$), brain (+159%; $P < 0.001$), and testis (+89%; $P < 0.001$), when compared to the vehicle control group.

In the 5-day period of methiocarb treatment, MDA levels were significantly increased in liver (+60%; $P < 0.001$), kidney (+50%; $P < 0.001$), brain (+89%; $P < 0.001$), and testis (+64%; $P < 0.001$), when compared to the vehicle control group.

In the 28-day period of methiocarb treatment, MDA levels were significantly increased in liver (+81%; $P < 0.001$), kidney (+55%; $P < 0.001$), brain (+90%; $P < 0.05$), and testis (+57%; $P < 0.05$), when compared to the vehicle control group.

Thus, the highest increase of MDA levels were observed in the brain after 1-, 5-, and 28-day treatments of methiocarb possibly due to large amounts of polyunsaturated fatty acids in the brain, which are sensitive to free radical attacks. Moreover, in spite of the high rate of oxidative metabolism, the brain has relatively low antioxidant defense systems.

Elevated MDA levels in the testis showed that the testis may be sensitive to peroxidation, and that methiocarb may cause oxidative damage in reproductive organs.

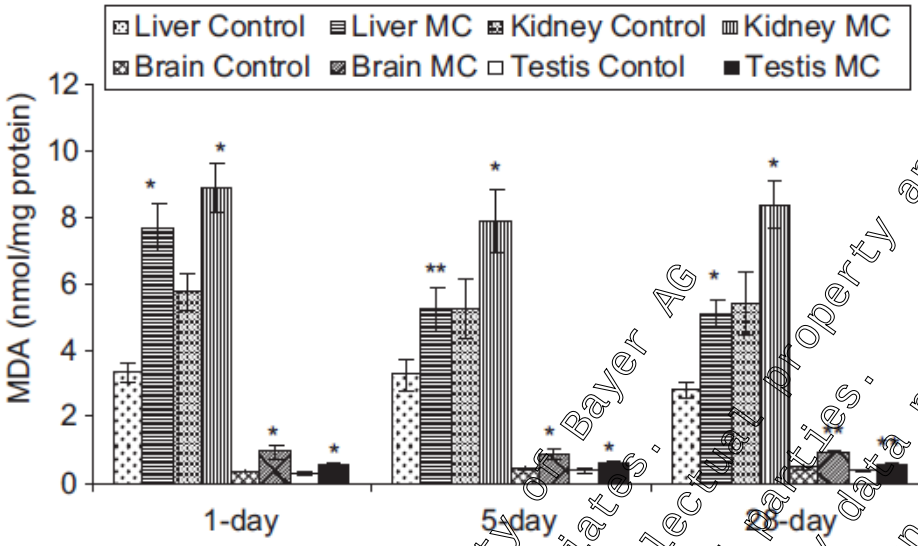


Figure 5.8.2/07- 1: The effects of methiocarb on MDA content (nmol/ mg protein) in rat liver, kidney, brain, and testis. Values are expressed as mean ± standard deviation; n = 8 for each treatment group.

MC = methiocarb
* Significantly different from the control group (P < 0.001).
** Significantly different from control group (P < 0.05) (one-way analysis of variance).

D. GSH assay

In the 1-day period of methiocarb treatment, GSH levels were significantly increased in liver (+25%; P < 0.001), kidney (+33%; P < 0.001), brain (+33%; P < 0.05), and testis (+28%; P < 0.05), when compared to the vehicle control group.

Conversely, in the 5-day period of methiocarb treatment, GSH levels were significantly decreased in liver (–17%; P < 0.001), kidney (–21%; P < 0.001), brain (–30%; P < 0.05), and testis (–25%; P < 0.05), when compared to the vehicle control group.

In the 28-day period of methiocarb treatment, GSH levels were significantly decreased in liver (–19%; P < 0.001), kidney (–30%; P < 0.001), brain (–24%; P < 0.05), and testis (–22%; P < 0.05), when compared to the vehicle control group.

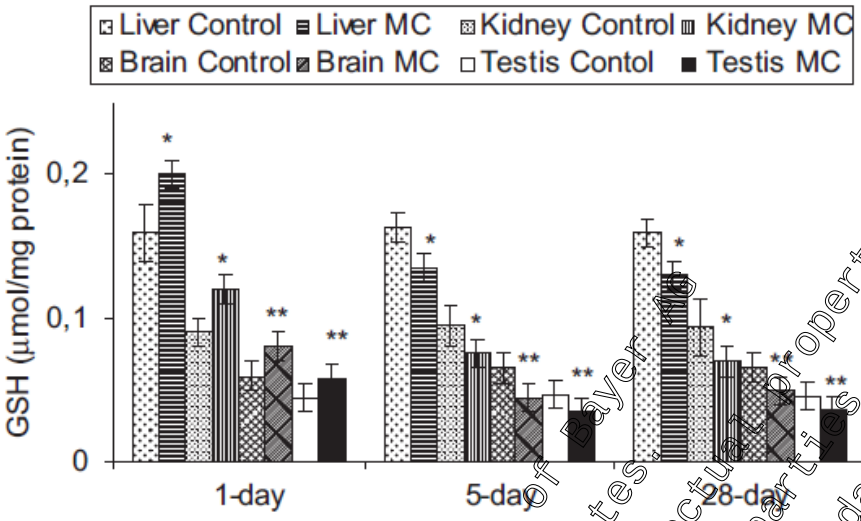


Figure 5.8.2/07- 2: The effects of methiocarb (MC) on levels of GSH (µmol/ mg protein) in rat liver, kidney, brain, and testis. Values are expressed as mean ± standard deviation; n = 8 for each treatment group.

MC = methiocarb; Vit. E = vitamin E; T = taurine
* Significantly different from the control group ($P < 0.001$).
** Significantly different from the control group ($P < 0.05$) (one-way analysis of variance).

In summary, LPO in liver, kidney, brain, and testis after administrations of different doses and time points of methiocarb (1-, 5- and 28-day oral doses of methiocarb treatment) resulted in statistical significant increases in MDA levels in liver, kidney, brain, and testis.
The 1-day high-dose treatment of methiocarb may induce disturbances in the activities of the enzymes regulating GSH metabolism. A considerable decline in GSH levels in the tissues after the 5- and 28-day periods of methiocarb treatment may have been due to its utilization to challenge the common oxidative stress in the influence of ROS generated from methiocarb metabolism.
Free radicals may be produced during the oxidation of methiocarb in the liver by cytochrome P450 oxidases and in the extra hepatic tissues, such as the kidney, by FMOs, resulting in LPO.

III. Conclusion

The authors concluded that 1-day high-dose and 5- and 28-day lower dose treatments of methiocarb may induce lipid peroxidation and produce disturbances in GSH levels in liver, kidney, brain, and testis, supporting that free radicals may be involved in the mechanism of methiocarb-induced toxicity.

Bayer conclusion:
This non-GLP study that was conducted to evaluate the potential of methiocarb to cause oxidative damage has some deficiencies. The results were only presented as illustrations and %-difference, without providing mean and / or individual values. The assessment of the results based solely on statistical analysis, without taking into account the biological relevance of the observed changes. Without positive and negative historical control data it is not possible to evaluate / draw a conclusion from the reported results. Furthermore, it was stated that no clinical signs were observed after methiocarb application. It is hard to believe that especially at the higher dose of 25 mg/kg bw/day no clinical (cholinergic) signs were observed. In the available guideline studies with methiocarb cholinergic symptoms occurred shortly after application of single oral application of ≥ 10 mg/kg bw. In addition, without histopathological evaluation it cannot be determined if an organ damage resulted due to the application of methiocarb and possible caused lipid peroxidation. A change in antioxidant

defence enzyme activity not necessarily results in organ damage. Only if the capacity of the antioxidant defence system is exhausted.
The results provide some indications of oxidative stress induction, but due to the described deficiencies (see also reliability assessment) the biological relevance of the observed findings is questionable / unclear. Overall, the provided information is considered to be supplemental information that does not change existing endpoints and does not affect the risk assessment of methiocarb.

The reliability evaluation is given below.

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study with methodical and reporting deficiencies: • only one dose for each treatment interval tested • only one sex tested • results were only presented as illustrations and %-difference. • no mean and / or individual values were provided • no histopathology was conducted to evaluate if increased or decreased activities of the antioxidant defense enzymes cause organ damage • No historical control data provided
Relevance of study:	**Supplemental information that does not change existing endpoints, and does not affect the risk assessment.**

Report: KCA 5.8.2/10 [redacted]; 2012; M-495849-01-1
Title: Effects of taurine and vitamin E on lipid peroxidation, antioxidant system and histological changes in wistar rat liver and kidney after short-term exposure of methiocarb
Report No.: M-495849-01-1
Document No.: M-495849-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: **no**

I. Materials and methods

A. Materials

1. Test material: Methiocarb
Synonyms: Mesurol
Source: Bayer, [redacted], Turkey
Description: Not reported
Lot/Batch no: Not reported
Purity: 99.8%
Stability of test compound: expiry date: not reported
Additional test substances: 1) Vitamin E (300 mg DL-α-tocopherol acetate)
2) Taurine
Source: 1) [redacted], Turkey
2) [redacted], USA
Purity: Not reported

Stability:	expiry date: not reported
2. Vehicle:	For methicarb treatment: corn oil; for Vitamin E: olive oil for taurine: distilled water
3. Test animals:	
Species:	Rat
Strain:	Wistar albino
Age:	12-13 weeks
Sex:	Male
Weight at dosing:	160 ± 20 g
Source:	Not reported
Acclimatisation period:	Almost 1 week
Diet:	Standard dry pellet diet *ad libitum*
Water:	water, *ad libitum*
Housing:	Polypropylene cages
B. Study design and methods	
1. Animal assignment and treatment:	
Dose:	Methiocarb: 10 mg/kg bw/d Vitamin E: 100 mg/kg bw/d Taurine: 50 mg/kg bw/d
Application volume:	Only reported for control, vitamin E and taurine treated groups, i.e. 0.5 mL/animal
Application route:	Applications were done in the morning to non-fasted rats Methiocarb: oral gavage Vitamin E and Taurine: i.p.
Treatment schedule:	Control group (C):0.5 mL corn oil/animal for 5 consecutive days Methiocarb group (MC): 10 mg/kg bw/d in corn oil for 5 consecutive days Vitamin E group (Vit E): pre-treatment with 100 mg/kg bw/d in olive oil (0.5 mL/rat) for 25 days; i.p. Vitamin E plus methiocarb group (Vit E + MC): pre-treatment with 100 mg/kg bw/d for 20 days (i.p), then methiocarb at 10 mg/kg bw/d along with Vitamin E for 5 days Taurine group (T): pre-treatment with 50 mg/kg bw/d in distilled water (0.5 mL/rat) for 25 days; i.p. Taurine plus methiocarb group (T + MC): pre-treatment with 50 mg/kg bw/d for 20 days, then methiocarb at 10 mg/kg bw/d along with Vitamin E for 5 days
Group size:	8
Observations:	Clinical signs, histology (liver, kidney tissues), lipid peroxidation assay, GSH-assay, measurement of antioxidant enzymes, protein assay

Tissue preparation for assays:	At the end of the treatments, the rats were sacrificed by cervical dislocation. Liver and kidney samples were dissected out and washed immediately with ice-cold physiological saline (0.9% NaCl) and one parts of the both tissues immediately stored at -80°C until analysis. The other parts of the both tissues were taken from the rats for histological analysis. The tissues were homogenized in 0.9% NaCl using an Ultra Turrax tissue homogenizer to make up the 10% tissue homogenate (w/v) and then, one parts of tissue homogenate (10%) centrifuged at 10000 × g at 4°C for 20 min to obtain cytosolic fraction. Tissue homogenates (10%) were used to determine levels of GSH and malondialdehyde (MDA). Cytosolic fractions were used to determine activities of antioxidant enzymes.
Lipid Peroxidation Assay:	Quantitative measurement of lipid peroxidation was performed in liver and kidney homogenates (10%) according to the method of Buege and Aust (1976) [Method Enzymol, 52:302-310] based on the formation of thiobarbituric acid reactive substances (TBARS) and expressed as the extent of malondialdehyde (MDA) production. The amount of TBARS was determined in tissue homogenates by using phase HPLC with UV diode-array detection at 532 nm according to the modification of the HPLC method of Draper and Hadley (1990) [Method Enzymol, 186:421-431.]. The levels of TBARS were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as nmol MDA per mg of protein.
GSH assay:	Levels of glutathion (GSH) were determined in the liver and kidney homogenates (10%) according to the method of Beutler (1975) [Red cell metabolism. A Manual of Biochemical Methods. Second ed. New York, Grune and Stratton, 71-73] by using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reagent. DTNB was reduced by free -SH groups of GSH to form 5-mercapto-2-nitrobenzoate and its absorbance were measured by spectrophotometric means at 412 nm. Results were expressed as µmol GSH per mg of protein using standard calibration curve.
Measurement of antioxidant enzymes:	Activities of antioxidant enzymes were determined in the cytosolic fractions of liver and kidney homogenates. Superoxide dismutase (SOD) activity was determined according to the method of Sun et al. (1988) [Clin Chem, 34:497-500.] based on the inhibition of nitroblue tetrazolium (NBT) reduction by using the xanthine-xanthine oxidase system as a superoxide generator. Catalase (CAT) activity was assayed by the decomposition

of hydrogen peroxide according to the method of Aebi (1984) [Method Enzymol, 105:121-126]. Glutathione peroxidase (GSH-Px) activity was determined according to the method of Pleban et al. (1982) [Clin Chem, 28:311-3160] based on the decrease in the absorbance of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm.

Glutathione reductase (GSH-Rd) activity was performed by monitoring the oxidation of NADPH in the presence of oxidized glutathione according to the method of Beutler (1969) [J Clin Invest, 48:1957-1966.]. Specific activities were expressed as U/mg protein for SOD and GSH-Px, mU/mg protein for GSH-Rd and k/mg protein for CAT using standard calibration curves.

Protein assay: Contents of proteins were measured in the liver and kidney homogenates (10%) and cytosolic fractions of liver and kidney homogenates according to the method of Lowry et al. (1951) [J Biol Chem, 193:265-275] using bovine serum albumin as standard.

Histology: Liver and kidney tissues were cut into small pieces and fixed in Bouin's solution. Following dehydration in an ascending series of ethanol tissue samples were cleared in xylene and embedded in paraffin and sliced in 5 µm sections. Slides were stained with Masson's triple dyes (Masson). All sections were examined under a light microscope (Olympus-CX 41).

Statistics: All data were expressed as mean ± standard deviation (SD). Data were analyzed by ANOVA test using "SPSS version 13.0 for Windows" statistical program and the individual comparisons of groups were obtained by using Scheffe`s multiple comparison procedure. p values of less than 0.05 and 0.001 were selected as the levels of significance

II. Results and discussion

A. Mortality

There were no mortalities in the methiocarb treatment groups.
No information reported for the other groups.

B. Clinical signs

None of the rats treated with methiocarb at 10 mg/kg bw for 5 consecutive day showed any signs of morbidity.
No information reported for the other groups.

C. Lipid peroxidation assay

The content of MDA in the rats treated with methiocarb was increased in liver by 1.35-fold (p<0.05) and by 1.5-fold in kidney (p<0.001) when compared to the control group. Pre-treatment with vitamin E and taurine resulted in a significant decrease in the content of hepatic (p<0.05) and renal (p<0.05, p<0.001, respectively) MDA-levels when compared to the methiocarb group.

Table 5.8.2/08- 1: Results of the lipid peroxidation assay – MDA levels (mean ± SD, n = 8)

Group	Control	MC	Vit. E	Vit. E + MC	T	T + MC
Organ	**MDA (nmol/mg protein)**					
Liver	3.86 ± 0.58	5.23 ± 0.66*	3.73 ± 0.46	4.08 ± 0.42 [$]	3.80 ± 0.44	4.16 ± 0.19 [$]
Kidney	5.26 ± 0.9	7.89 ± 0.94***	5.26 ± 0.49	5.69 ± 0.61 [$$$]	5.36 ± 0.24	6.07 ± 0.36 [$]

MC = methiocarb; Vit. E = vitamin E, T = taurine
* Significantly different from control, p < 0.05
*** Significantly different from control, p < 0.001
[$] Significantly different from methiocarb group, p < 0.05
[$$$] significantly different from methiocarb group, p < 0.001

D. GSH assay

The level of GSH in the rats treated with methiocarb was significantly decreased in liver (–12%, p<0.001) and kidney (–35%, p<0.001) when compared to the vehicle control group. However, pretreatment with vitamin E and taurine resulted in a significant increase in the level of hepatic (p<0.05) and renal (p<0.05) GSH in comparison with that of the methiocarb group.

Table 5.8.2/08- 2: Results of the GSH assay – GSH levels (mean ± SD, n = 8)

Group	Control	MC	Vit. E	Vit. E + MC	T	T + MC
Organ	**GSH (µmol/mg protein)**					
Liver	0.17 ± 0.01	0.15 ± 0.01***	0.18 ± 0.01	0.17 ± 0.01 [$]	0.17 ± 0.01	0.17 ± 0.01 [$]
Kidney	0.1 ± 0.014	0.08 ± 0.01***	0.1 ± 0.003	0.1 ± 0.003 [$]	0.1 ± 0.003	0.1 ± 0.01 [$]

MC = methiocarb; Vit. E = vitamin E, T = taurine
* Significantly different from control, p < 0.05; *** significantly different from control, p < 0.001
[$] Significantly different from methiocarb group, p < 0.05
[$$$] significantly different from methiocarb group, p < 0.001

E. Measurement of antioxidant enzymes

The activity of SOD and CAT was statistically significantly decreased in liver (–28% and –16%, both p < 0.001) and kidney (–19% and –21%, both p < 0.05) tissues of the methiocarb treatment group. However, pre-treatment with vitamin E and taurine resulted in a significant increase in the activity of SOD in liver (p<0.05) and kidney (p<0.05) in comparison with that of the methiocarb group.
The activity of GSH-Px in the methiocarb group was significantly increased in liver (36%, p<0.001) and kidney (29%, p<0.05) when compared to the vehicle control group. However, pre-treatment with vitamin E and taurine resulted in a significant decrease in the activity of GSH-Px in liver (p<0.05) and kidney (p<0.05) in comparison with that of the methiocarb group.
The activity of GSH-Rd in the methiocarb group was non-significantly increased in liver (8%) and kidney (6%) when compared to the control group. Pre-treatment with vitamin E and taurine showed no change in the activity of GSH-Rd in liver and kidney when compared to the methiocarb group.

Table 5.8.2/08- 3: Activities of SOD, CAT, GSH-Px and GSH-Rd (mean ± SD, n = 8)

Group	Control	MC	Vit. E	Vit. E + MC	T	T + MC
	SOD (U/mg protein)					
Liver	137 ± 16	98.31 ± 16***	138.7 ± 6.57	130.3 ± 7.1$	136.1 ± 9.8	123.4 ± 9.3$
Kidney	209.4 ± 19	166.1 ± 7.8***	199.6 ± 6.59	196.3 ± 9.7$	198.4 ± 7.7	189.7 ± 6.7$
	CAT (k/mg protein)					
Liver	555 ± 39.2	467 ± 53.3***	559 ± 18.7	549 ± 19.8$	556 ± 7.73	533 ± 11.0$
Kidney	548 ± 94.8	431 ± 42.1*	552 ± 10.3	539 ±13.8$	545 ± 12.8	527 ± 31.4$
	GSH-Px (U/mg protein)					
Liver	15.36 ± 2.4	20.89 ± 3.7***	15.11 ± 0.64	15.66 ± 0.8$	14.62 ± 1.1	16.12 ± 2.2$
Kidney	19.53 ± 1.9	25.15 ± 3.3*	19.64 ± 1.7	20.19 ± 0.99$	19.78 ± 0.6	20.98 ± 1.1$
	GSH-Rd (U/mg protein)					
Liver	6.11 ± 0.68	6.60 ± 0.93	6.42 ± 1.2	6.44 ± 0.36	6.27 ± 0.39	6.81 ± 0.40
Kidney	8.41 ± 0.56	8.88 ± 0.52	8.16 ± 0.37	8.23 ± 0.23$	7.96 ± 0.65	8.11 ± 0.47

MC = methiocarb; Vit. E = vitamin E, T = taurine
* Significantly different from control, $p < 0.05$
*** Significantly different from control, $p < 0.001$
$ Significantly different from methiocarb group, $p < 0.05$
k rate constant

D. Histopathology

Liver
Some histological changes that were not further described were noticed in liver tissues of control groups given especially only vitamin E or taurine. In the liver tissue after 5-day methiocarb treatment, moderate degenerative changes such as vacuolization and hyperemia, accumulation of collagen fibres in connective tissue around veins of some animals, few picnotic nuclei and mild sinusoidal dilatations were observed. While these degenerative changes were decreased except for mild vacuolization and hyperemia by vitamin E and except for mild hyperemia by taurine in 5-day methiocarb treated groups. The most improvement was noticed in peripheral area of central veins in the liver tissues.

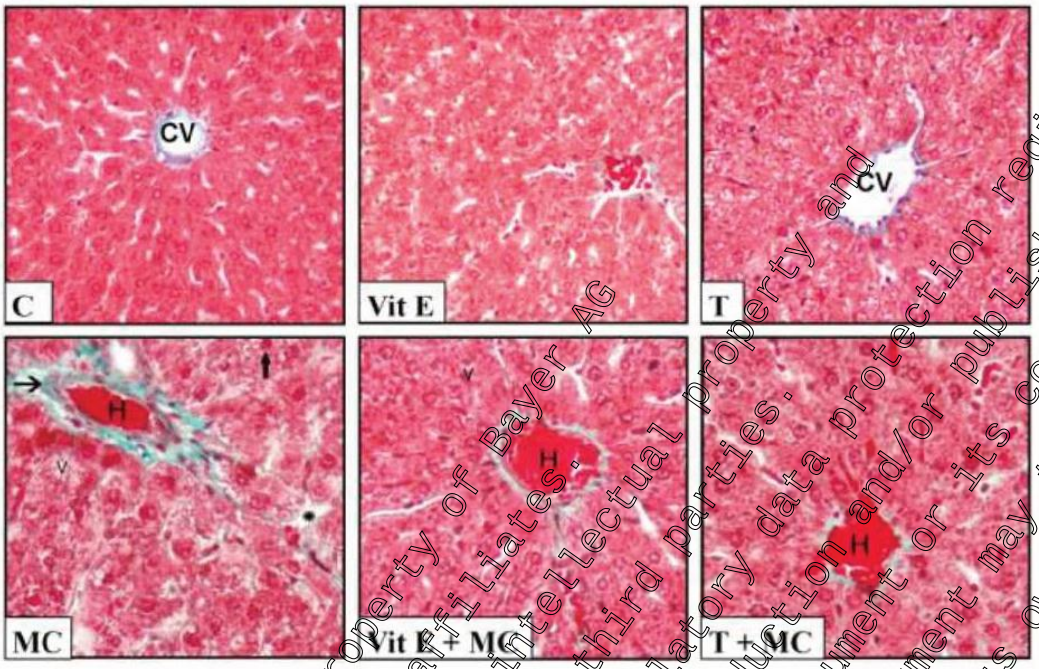


Figure 5.8.2/08- 1: The effects of vitamin E and taurine on histological changes in livers of methiocarb-treated rats.

CV: Central vein, V: vacuolization, H: hyperemia, picnotic nuclei (²), sinusoidal dilatation (✸), collagen accumulation in connective tissue (→).
C: Control, Vit E: Vitamin E-treated group, T: Taurine-treated group, MC: Methiocarb-treated group, VitE + MC: Vitamin E and Methiocarb-treated group, T + MC: Taurine and Methiocarb-treated group.
Masson stain, X 270.

Kidney

Partly histological changes were observed in the kidney tissue of the control animals. Methiocarb treatment caused marked alterations such as widening between parietal and visceral leaves of bowman capsules, shortening at the brush border, cytoplasmic debris and desquamated nuclei in the widened lumens of proximal tubules, accumulation of collagen fibers in necrotic areas, a mild hyperemia and few mononuclear cell infiltration. On the other hand, treatment of vitamin E and taurine partially decreased kidney damage in methiocarb-administered rats.

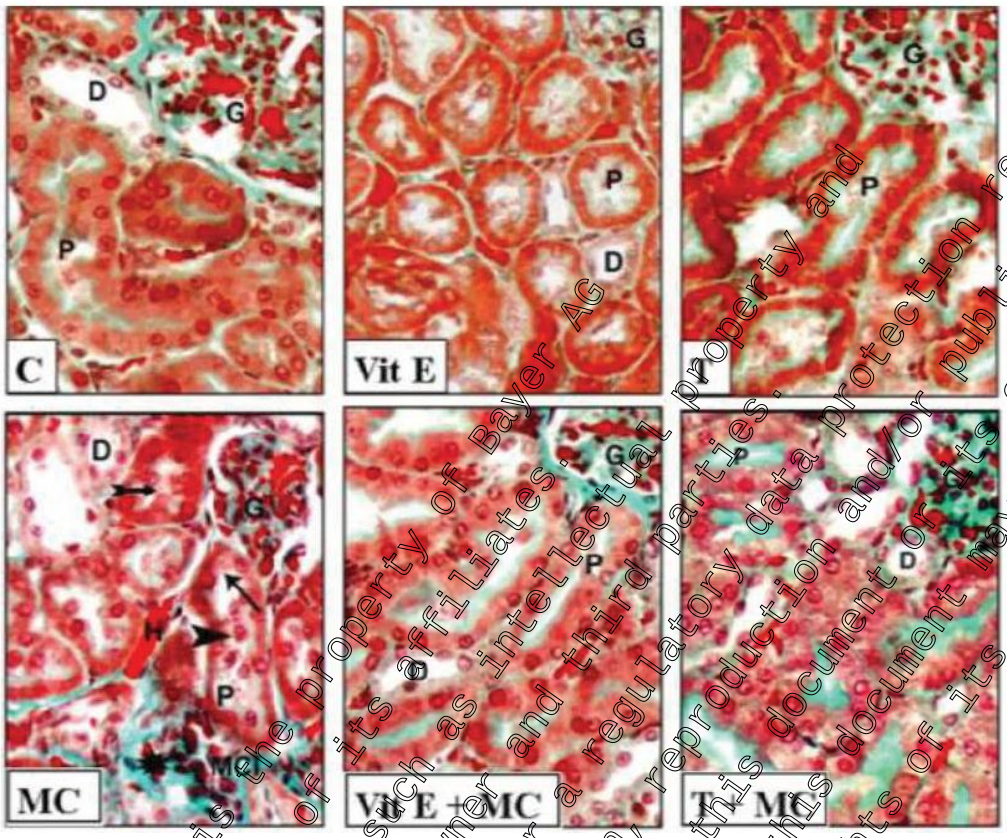


Figure 5.8.2/08- 2: The effects of vitamin E and taurine on histological changes in the kidney of methiocarb-treated rats.

The shortening at the brush border (a), desquamated nuclei (➤) in the proximal tubules (P) cytoplasmic debris (➥) in the proximal tubules (P), accumulation of collagen fibers in necrotic areas (✸), hyperemia (H) and few mononuclear cell infiltration (MCI) can be seen in the kidney of Methiocarb-treated rats. C: Control, Vit E: Vitamin E-treated group, T: Taurine-treated group, MC: Methiocarb-treated group, VitE + MC: Vitamin E and Methiocarb-treated group, T + MC: Taurine and Methiocarb-treated group, Masson stain, X270.

III. Conclusion

The authors concluded that oxidative stress may play a key role in methiocarb-induced liver and kidney toxicity as demonstrated by induction of lipid peroxidation, altered antioxidant status of cells and histological damages in liver and kidney. Furthermore, vitamin E and taurine may partially modulate the toxic responses resulting from 5-day exposure of methiocarb

Bayer conclusion:

This non-GLP study is considered to be not reliable (see reliability assessment below). The authors conclusions on the different assays for the assessment of oxidative damage based solely on statistical significances, without taking into account the biological relevance of the observed changes. For example the authors describe a decrease of GSH-levels of up to –12% after 5-day exposures to methiocarb when compared to control values. However, it is known that enhanced lipid peroxidation is only seen when the hepatic GSH-content decreases to about 20% of the initial / or control concentration. A change in antioxidant defense enzyme activity not necessarily results in organ damage. Only if the capacity of the antioxidant defense system is exhausted organ damage can occur. Regarding the histopathological findings, it has to be considered that the animals were not exsanguinated. Thus, the observed hyperemia and sinusoidal dilatation of the liver and kidney are probably artefacts. It is not reported if the animals were fasted before sacrifice. Non-fasting prior to

sacrifice could result in vacuolation. However, based on the presented pictures of the histopathology examinations, vacuolisation is not obvious. Furthermore, the authors considered the liver fibrosis as treatment-related. However, such a finding would not be observed after only 5 days of treatment.
It has to be noted that the applied staining method (i.e. Masson stain) is a special stain to distinguish cells from connective tissues, and is not suitable for the evaluation of cytoarchitectural changes (e.g. necrosis). Regarding the described kidney changes, like the alteration of the brush border, it should be noted that those changes are only observable with electron microscopy.
In addition, it was not stated in how many of the animals per group the findings were observed. Since also no positive and negative historical control data are provided it is not possible to draw a conclusion from the reported results.
Overall, it is concluded that the results of this study, besides the fact of its lack of reliability, have no impact on the risk assessment of methiocarb.

The reliability evaluation is given below.

Klimisch evaluation

Reliability of study:	**Not reliable (Klimisch code 3)**
Comment on reliability:	Non-GLP study with methodical and reporting deficiencies: • only one dose of methiocarb tested • only one sex tested • application volume for methiocarb test groups not provided • the conclusion for the enzyme assays based solely on statistical evaluation, the biological relevance of the measured values is not taken into account or not provided • exact number of animals in individual groups with histopathological findings not reported. • no historical control data reported
Comment on reliability Histopathology:	• animals were sacrificed (non-fasted) by cervical dislocation • animals were not exsanguinated • applied staining method not suitable for the assessment of cytoarchitectural changes
Relevance of study:	**Non relevant due to lack of reliability.**

Report:	KCA 5.8.2/11, [redacted]; [redacted]; [redacted]; [redacted]; [redacted], 2013; M-530999-01-1
Title:	Acute effects of methiocarb on oxidative damage and the protective effects of vitamin E and taurine in the liver and kidney of Wistar rats.
Report No.:	M-530999-01-1
Document No.:	M-530999-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

1. Test material: Methiocarb

Synonyms:	Mesurol
Source:	Bayer, [redacted], Turkey
Description:	Not reported
Lot/Batch no:	Not reported
Purity:	99.8%
Stability of test compound:	expiry date not reported
Additional chemicals:	Vitamin E (Evigen, 300 mg DL-a-tocopherol acetate) purchased from [redacted] ([redacted], Turkey)
	Taurine and all other chemicals were obtained from [redacted] ([redacted], Missouri, USA) and [redacted] ([redacted], Germany)
2. Vehicle:	For methicarb treatment: corn oil; for Vitamin E: olive oil for taurine: distilled water
3. Test animals:	
Species:	Rat
Strain:	Wistar albino
Age:	12-13 weeks
Sex:	Male
Weight at dosing:	160 ± 20 g
Source:	Not reported
Acclimatisation period:	Almost 1 week
Diet:	Standard dry pellet diet, *ad libitum*
Water:	water, *ad libitum*
Housing:	In polypropylene cages; number per cage not reported

B. Study design and methods

1. Animal assignment and treatment:

Dose:	Methiocarb: 28 x 2 mg/kg bw/d Vitamin E: 100 mg/kg bw/d Taurine: 50 mg/kg bw/d
Application volume:	0.5 mL/rat (corresponding to about 0.3 mL/100g bw)
Application route:	Applications were done in the morning (between 9 and 10 a.m.) to non-fasted rats Control & Methiocarb: oral gavage Vitamin E and Taurine: i.p.

Treatment schedule:	Control group (I):0.5 mL corn oil/animal for 1 day Methiocarb group (II): 25 mg/kg bw for 1 day Vitamin E group (III): 100 mg/kg bw/d in olive oil (0.5 mL/rat) for 21 days; i.p. Vitamin E plus methiocarb group (IV): pre-treatment with 100 mg/kg bw/d Vit. E for 20 days (i.p), then methiocarb at 25 mg/kg bw along with Vitamin E for 1 day Taurine group (V): 50 mg/kg bw/d in distilled water (0.5 mL/rat) for 21 days; i.p. Taurine plus methiocarb group (VI): pre-treatment with 50 mg/kg bw/d for 20 days (i.p.), then methiocarb at 25 mg/kg bw/d along with taurine for 1 day
Group size:	8
Observations:	Clinical signs, histology (liver, kidney), lipid peroxidation assay, GSH-assay, measurement of antioxidant enzymes, protein assay
Tissue preparation for assays:	24 h after the last treatment rats were sacrificed by cervical dislocation. Liver and kidney samples were dissected out and washed immediately with ice-cold physiological saline (0.9% NaCl) and one part of the both tissues immediately stored at –80°C until analysis. The other parts of the both tissues were taken from the rats for histological analysis. The tissues were homogenized in 0.9% NaCl using an Ultra Turrax tissue homogenizer to make up the 10% tissue homogenate (w/v) and then centrifuged at 10000 × g at 4°C for 20 min to obtain cytosolic fraction. Tissue homogenates (10%) were used to determine levels of GSH and lipid peroxidation. Cytosolic fractions were used to determine activities of antioxidant enzymes.
Lipid Peroxidation Assay:	Quantitative measurement of lipid peroxidation was performed in liver and kidney homogenates (10%) according to the method of Buege and Aust (1976) [Method Enzymol, 52:302-310] based on the formation of thiobarbituric acid reactive substances (TBARS) and expressed as the extent of malondialdehyde (MDA) production. The amount of TBARS was determined in tissue homogenates by using phase HPLC with UV diode-array detection at 532 nm according to the modification of the HPLC method of Draper and Hadley (1990) [Method Enzymol, 186:421-431.]. The levels of TBARS were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as *nmol MDA per mg of protein*.

GSH assay: Levels of glutathion (GSH) were determined in the liver and kidney homogenates (10%) according to the method of Beutler (1975) [Red cell metabolism. A Manual of Biochemical Methods. Second ed. New York, Grune and Stratton, 71-73] by using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reagent. DTNB was reduced by free -SH groups of GSH to form 5-mercapto-2-nitrobenzoate and its absorbance were measured by spectrophotometric means at 412 nm. Results were expressed as *µmol GSH per mg of protein* using standard calibration curve.

Measurement of antioxidant enzymes: Activities of antioxidant enzymes (Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSH-Px), Glutathione reductase (GSH-Rd)) were determined in the cytosolic fractions of liver and kidney homogenates.

SOD assay: Superoxide dismutase (SOD) activity was determined according to the method of Sun et al. (1988) [Clin Chem, 34:497-500.] based on the inhibition of nitroblue tetrazolium (NBT) reduction by using the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the supernatants of 10 000g of ethanol/chloroform (5/3, v/v) extracts of tissue homogenates (10%). One unit of SOD was defined as the enzyme amount causes 50% inhibition in NBT reduction rate. Specific activity was expressed as unit *SOD per mg of protein* using standard calibration curve.

CAT assay: Catalase (CAT) activity was assayed in the cytosolic fractions of tissue homogenates by the decomposition of hydrogen peroxide according to the method of Aebi (1984) [Method Enzymol, 105:121-126]. This reaction follows a first-order kinetic given by equation $k = (2.3/t)\ (\log A0/A1)$. Specific activity was expressed as *k per mg of protein* using standard calibration curve.

GSH-Px assay: Glutathione peroxidase (GSH-Px) activity was determined in the cytosolic fraction of tissue homogenates according to the method of Pleban et al. (1982) [Clin Chem, 28:311-316] based on the decrease in the absorbance of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm.

Specific activity was expressed as unit GSH-Px per mg of protein using standard calibration curve. One unit of GSH-Px is defined as the amount of enzyme that oxidizes *1 µmol of NADPH per minute*.

GSH-Rd assay: Glutathione reductase (GSH-Rd) activity was performed in cytosolic fraction of tissue homogenates by monitoring the oxidation of NADPH in the presence of oxidized glutathione according to the method of Beutler (1969) [J Clin Invest, 48:1957-1966.]. Specific activity was expressed as mU GSH-Rd per mg protein using standard calibration curve. One unit of GSH-Rd is defined as the amount of enzyme that oxidizes *1 µmol of NADPH per minute*.

Protein assay: Contents of proteins were measured in the liver and kidney homogenates (10%) and cytosolic fractions of liver and kidney homogenates according to the method of Lowry et al. (1951) [J Biol Chem, 193:265-275] using bovine serum albumin (BSA) as standard.

Histology: Liver and kidney tissues were cut into small pieces and fixed in Bouin's solution. Following dehydration in an ascending series of ethanol, tissue samples were cleared in xylene and embedded in paraffin and sliced in 5 µm sections. Slides were stained with Masson's triple dyes (Masson). All sections were examined under a light microscope (Olympus-CX 41).

Statistics: All data were expressed as mean ± standard deviation (SD). Data were analyzed by ANOVA test using "SPSS version 13.0 for Windows", statistical program and the individual comparisons of groups were obtained by using Scheffe`s multiple comparison procedure. p values of less than 0.05 and 0.001 were selected as the levels of significance

II. Results and discussion

A. Mortality

There were no mortalities in the methiocarb treatment groups.
No information reported for the other groups.

B. Clinical signs

None of the rats showed any signs of morbidity after repeated oral treatment with 25 mg/kg bw/day methiocarb.
No information reported for the other groups.

C. Lipid peroxidation assay

The content of MDA in the rats treated with methiocarb was statistically significantly increased 2.33-fold in liver ($p<0.001$) and 1.54-fold in kidney ($p<0.001$) when compared to the vehicle control group.
Pre-treatment with vitamin E and taurine resulted in a statistically significant decrease in the content of hepatic ($p<0.001$) and renal ($p<0.001$, $p<0.05$, respectively) MDA when compared to the group treated with methiocarb alone.
Treatment of rats with vitamin E and taurine alone did not affect the levels of MDA when compared to the vehicle control groups.

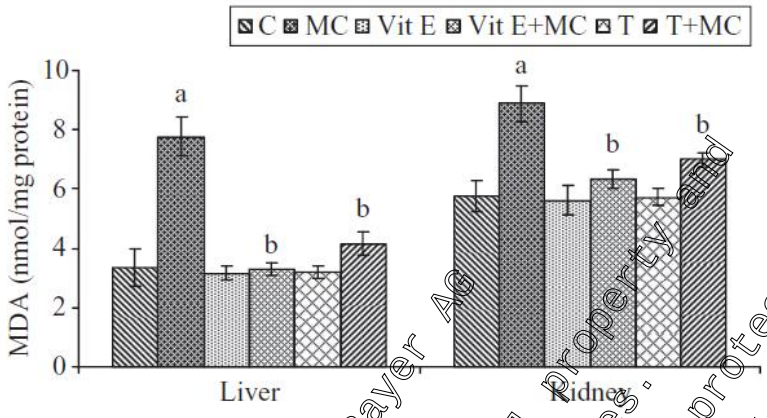


Figure 5.8.2/09- 1: Results of the lipid peroxidation assay – Effects of vitamin E and taurine on MDA content (nmol/mg protein) in liver and kidney of methiocarb treated rats. MDA levels (mean ± SD, n = 8)

a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.001$; one-way ANOVA).
MDA = malondialdehyde; MC = methiocarb; Vit E = Vitamin E, T = taurine

D. GSH assay

The level of GSH in the rats treated with methiocarb was significantly increased in liver (+25%, $p < 0.001$) and kidney (+33%, $p < 0.001$) when compared with the vehicle control group. However, pretreatment with vitamin E and taurine resulted in a significant decrease in the level of hepatic ($p < 0.05$) and renal ($p < 0.05$) GSH in comparison with that of the methiocarb group.
Treatment of rats with vitamin E and taurine alone did not affect the levels of GSH when compared to the vehicle control groups.

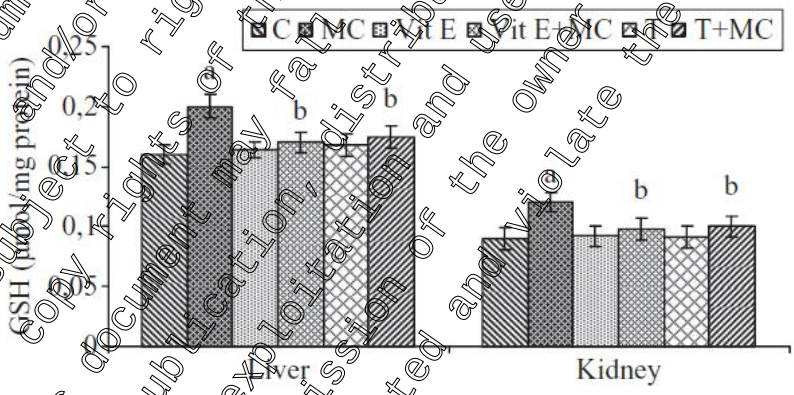


Figure 5.8.2/09- 2: Results of the GSH assay – Effects of vitamin E and taurine on GSH levels (µmol/mg protein) in methiocarb treated rat liver and kidney. GSH levels (mean ± SD, n = 8)

a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.05$) (one way ANOVA).
GSH = glutathione; MC = methiocarb; Vit E = Vitamin E, T = taurine

E. Measurement of antioxidant enzymes

SOD assay

The activity of SOD was statistically significantly increased in liver (+35%, $p < 0.001$) and kidney (+26%, $p < 0.001$) tissues of the methiocarb treatment group when compared to the vehicle control. However, pre-treatment with vitamin E and taurine resulted in a statistically significant decrease in the activity of SOD in liver ($p<0.05$) and kidney ($p<0.05$) in comparison with that of the methiocarb group.

Treatment of rats with vitamin E and taurine alone did not affect the levels of SOD activity when compared to the vehicle control groups.

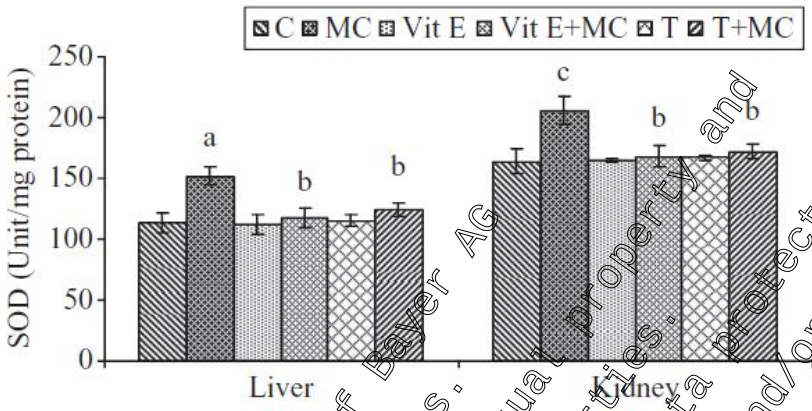


Figure 5.8.2/09- 3: Results of the SOD assay – The effects of vitamin E and taurine on SOD activity (U/mg protein) in methiocarb treated rat liver and kidney. (Values expressed as mean ± SD, n = 8)

a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.05$) (one way ANOVA).
c Significantly different from control group ($p < 0.05$, one way ANOVA).
SOD = superoxide dismutase; MC = methiocarb; Vit E = Vitamin E; T = taurine

CAT assay

The activity of CAT was statistically significantly increased in liver (+22%, $p < 0.001$) and kidney (+19%, $p < 0.05$) of the methiocarb treatment group when compared to the vehicle control. However, pre-treatment with vitamin E and taurine resulted in a statistically significant decrease in the activity of CAT in liver ($p < 0.001$) and kidney ($p < 0.05$) in comparison with that of the methiocarb group. Treatment of rats with vitamin E and taurine alone did not affect the levels of CAT when compared to the vehicle control groups.

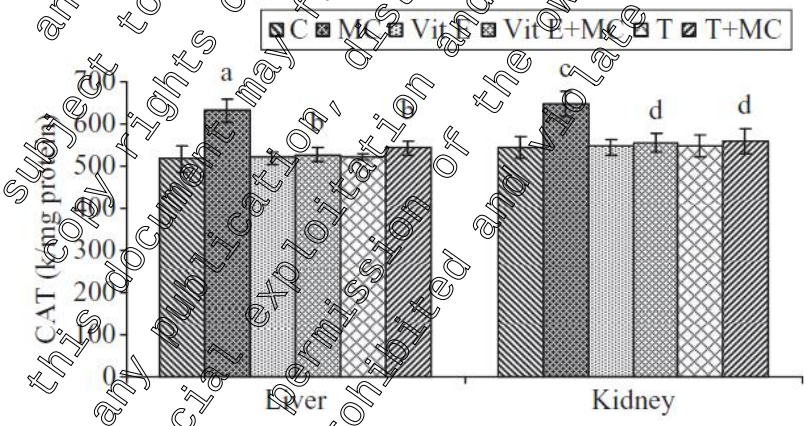


Figure 5.8.2/09- 4: Results of the CAT assay –Effects of vitamin E and taurine on CAT levels (k/mg protein) in methiocarb treated rat liver and kidney. (Values expressed as mean ± SD, n = 8)

a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.001$).
c Significantly different from control group ($p < 0.05$).
d Significantly different from methiocarb group ($p < 0.05$; one way ANOVA).
CAT = catalase; MC = methiocarb, Vit E = Vitamin E, T = taurine

GSH-Px assay

The activity of GSH-Px in the methiocarb group was significantly increased in liver (+31%, p<0.001) and kidney (+30%, p<0.05) when compared to the vehicle control group. However, pre-treatment with vitamin E and taurine resulted in a significant decrease in the activity of GSH-Px in liver (p<0.001) and kidney (p<0.05) in comparison with that of the methiocarb group.
Treatment of rats with vitamin E and taurine alone did not affect GSH-Px levels when compared to the vehicle control groups.

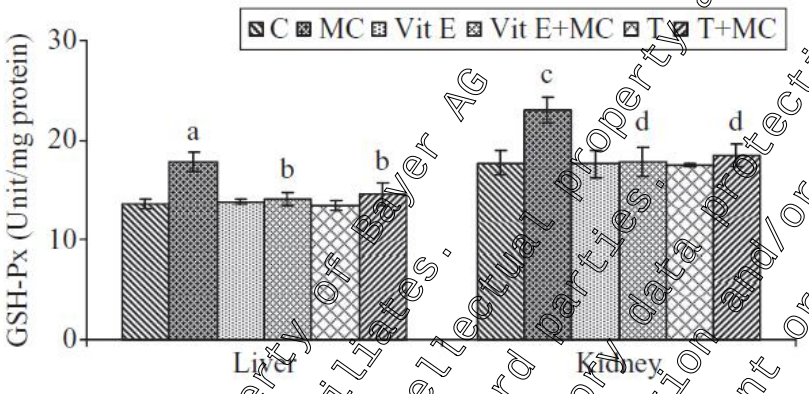


Figure 5.8.2/09- 5: Results of the GSH-Px assay – The effects of vitamin E and taurine on GSH-Px levels (U/mg protein) in liver and kidneys of methiocarb treated rats. (Values expressed as mean ± SD, n = 8)

a Significantly different from control group ($p < 0.001$).
b Significantly different from methiocarb group ($p < 0.001$).
c Significantly different from control group ($p < 0.05$).
d Significantly different from methiocarb group ($p < 0.05$; one way ANOVA).
GSH.Px = glutathione peroxidase; MC = methiocarb; Vit E = Vitamin E, T = taurine

GSH-Rd assay

The activity of GSH-Rd in the methiocarb group remained unchanged in liver and kidney when compared to the control group. Pre-treatment with vitamin E and taurine showed no change in the activity of GSH-Rd in liver and kidney when compared to the methiocarb group.
Treatment of rats with vitamin E and taurine alone did not affect GSH-Rd levels when compared to the vehicle control groups.

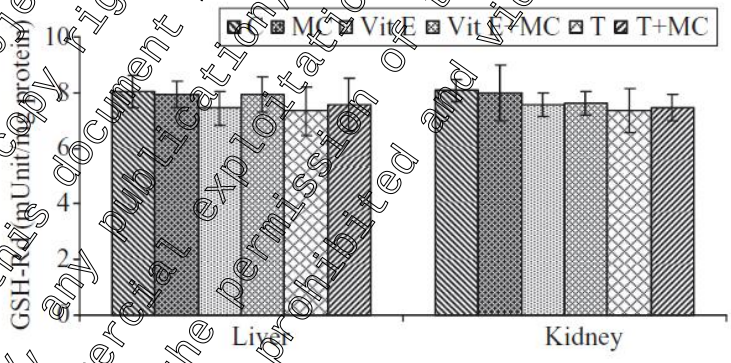


Figure 5.8.2/09- 6: Results of the GSH-Rd assay – The effects of vitamin E and taurine on GSH-Rd levels (mU/mg protein) in liver and kidneys of methiocarb treated rats. (Values expressed as mean ± SD, n = 8)

GSH-Rd = Glutathione reductase; MC = methiocarb; Vit E = Vitamin E, T = taurine

D. Histopathology

Liver

There were no histopathological findings observed in the livers of control animals. Treatment with vitamin E and taurine caused a partly histological change that was not further described. The administration of methiocarb produced mild degenerative changes such as a moderate hyperemia and mild sinusoidal dilatations, vacuolization and accumulation of collagen fibers around veins in the liver tissue. In the presence of vitamin E (Figure 5.8.2/09- 7(b)) and taurine (Figure 5.8.2/09- 7(c)), mild degeneration in the liver tissue was still visible.

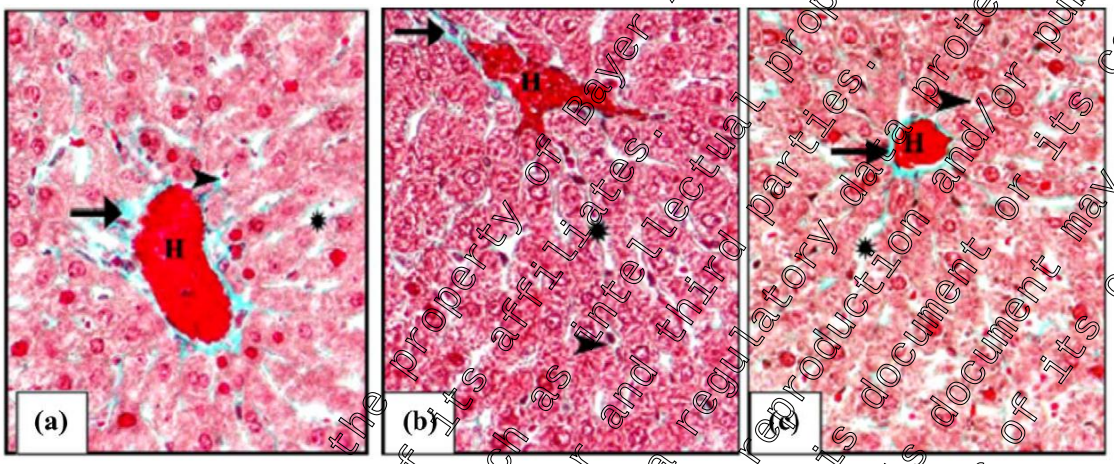


Figure 5.8.2/09- 7: The effects of vitamin E and taurine on histological changes in the liver of methiocarb treated rats. Liver tissues of rats in methiocarb group (a), in Vit E + methiocarb group (b) and in Taurine + methiocarb group (c).

Vacuolization (➤), hyperemia (H), sinusoidal dilatation (✸), accumulation of collagen fibers around veins (➔). Masson stain. Original magnification x270

Kidney

The kidneys of control animals have a normal histological appearance except of a partly histological change in the kidney tissues of some animals.

After acute methiocarb exposure widening between parietal and visceral leaves of Bowman's capsules, shortening at the brush border, cytoplasmic debris and desquamated nuclei in the widened lumens of proximal tubules were observed. The moderate kidney damage was decrease after acute exposure of methiocarb in the presence of vitamin E (Figure 5.8.2/09- 8(e)) and taurine (Figure 5.8.2/09- 8(f)).

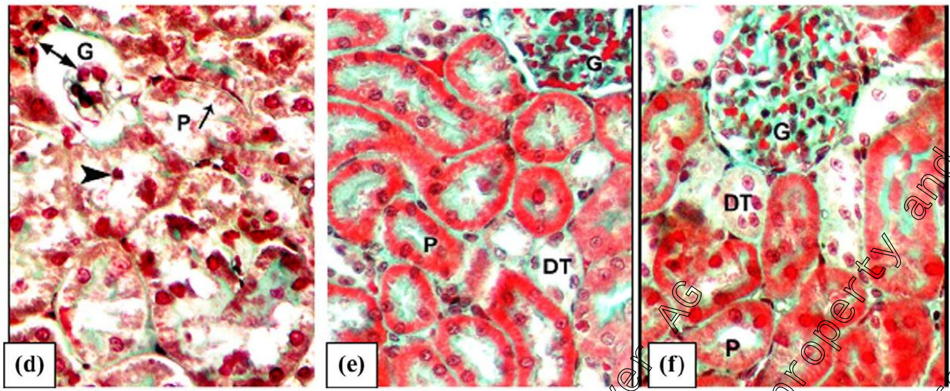


Figure 5.8.2/09- 8: The effects of vitamin E and taurine on histological changes in kidney of methiocarb treated rats. Kidney tissues of rats in Methiocarb group (d), in Vit E + Methiocarb group (e) and in Taurine + Methiocarb group (f).

Widening between parietal and visceral leaves of Bowman capsules (↔), the shortening at the brush border (→), and desquamated nuclei (➤) in the proximal tubules (P), glomerulus (G) and distal tubule (DT) can be seen in kidney tissue of methiocarb treated rats. Masson stain. Original magnification x270.

III. Conclusion

The authors concluded that acute methiocarb exposure caused lipid peroxidation and significant changes on antioxidant defence system, as well as histological changes in liver and kidney. The biochemical parameters (enzyme activities) are changed more easily than the histological appearance. Moreover, they proposed that the results of the study showed that pre-treatment of vitamin E and taurine may attenuate methiocarb-induced oxidative damage by decreasing lipid peroxidation and altering antioxidant defence system in liver and kidney of rats. However, vitamin E and taurine might reduce histological changes related with oxidative stress in the kidney more significantly than in the liver of methiocarb-treated rats.

Bayer conclusion:

This non-GLP study is considered to be not reliable (see reliability assessment below). The authors conclusions on the different assays for the assessment of oxidative damage based solely on statistical significances, without taking into account the biological relevance of the observed changes. It has to be considered that a change in antioxidant defense enzyme activity not necessarily results in organ damage. Only if the capacity of the antioxidant defense system is exhausted organ damage can occur.

Regarding the histopathological findings, it has to be considered that the animals were not exsanguinated. Thus, the observed hyperemia and sinusoidal dilatation of the liver are probably artefacts. In addition, the animals were not fasted before sacrifice. Non-fasting prior to sacrifice could result in vacuolation. However, based on the presented pictures of the histopathology examinations, vacuolisation is not obvious. Furthermore, the reported finding of accumulation of collagen fibers around liver veins is questionable, since such a finding would not be observed after a single treatment. It important to note that the applied staining method (i.e. Masson stain) is a special stain to distinguish cells from connective tissues, and is not suitable for the evaluation of cytoarchitectural changes (e.g. degenerative changes). Regarding the described kidney changes, like the alteration of the brush border, it should be bear in mind that those changes are only observable with electron microscopy and not with light microscopy.

In addition, it was not stated in how many of the animals per group the findings were observed. Since also no positive and negative historical control data are provided it is not possible to evaluate / draw a conclusion from the reported results.

Overall, it is concluded that the results of this study, besides the fact of its lack of reliability, have no impact on the risk assessment of methiocarb.

The reliability evaluation is given below.

Klimisch evaluation

Reliability of study:	Reliable with restrictions (Klimisch code 3)
Comment on reliability:	Non-GLP study with methodical and reporting deficiencies: • only one dose of methiocarb tested • application to non-fasted rats • only one sex tested • results presented only as illustrations, • the results for the enzyme assays are not presented in tabulated form (i.e. no means and no individual number of affected animals/group reported). • the conclusion for the enzyme assays based solely on statistical evaluation, the biological relevance of the measured values is not taken into account • exact number of animals in individual groups with histopathological findings not reported, • no historical control data provided
Comment on reliability Histopathology:	• animals were sacrificed (non-fasted) by cervical dislocation • animals were not exsanguinated • applied staining method not suitable for the assessment of cytoarchitectural changes
Relevance of study:	Non relevant due to lack of reliability.

Report: KCA 5.8.2/12 [redacted]; 2011; M-459624-01-1

Title: In vitro screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR) and PPAR and quantitative analysis of in vivo induction pathway

Report No.: M-459624-01-1

Document No.: M-459624-01-1

Guideline(s): not applicable

Guideline deviation(s): not applicable

GLP/GEP: **no**

I. Materials and methods

A. Materials

1. Test material: Methiocarb (among 200 other tested pesticides; only the assays conducted with Methiocarb are summarised in this document.)

Source: Not specified (several sources were listed for the 200 pesticides)

Description: Not reported

Lot/Batch no: Not reported

Purity: Not specified (in the range of 95-100%)

2. Vehicle/negative and positive control:

Vehicle:	DMSO (max. 0.1% in assay)
Positive controls:	PPARα WY-14643 (= pirinixic acid) (10^{-8} - 10^{-5} M) PPARγ Pioglitazone (10^{-8} - 10^{-5} M)

3. Test system:

Species/strain:	Monkey kidney CV-1 cells transfected with a mouse PPARα and γ-receptor/LUC-reporter construct
Source:	Created in course of this study, see details below
Details on the test system:	
Cell line:	CV-1 monkey kidney cells were obtained from Dainippon Pharmaceutical Co. Ltd. The cells were routinely cultured in phenol red-free MEM supplemented with 10% FBS and antibiotics at 37 °C in an atmosphere of 5% CO_2/95% air under saturating humidity, and passaged every week by trypsinization with 0.25% trypsin/0.02% EDTA.
Plasmids:	Expression plasmids, pCMX-mPPARα, pCMX-mPPARγ and pCMX-mRXRα, and the PPRE-containing reporter plasmid, PPRE3-TK-LUC, were gifts from Dr. R. M. Evans (Salk Institute, La Jolla, CA, USA). The construction of these plasmids has already been described by Umesono et al. (1991) and Kliewer et al. (1992). The internal control plasmid pCMVβ-Gal was purchased from Clontech (Palo Alto, CA, USA).
Generation of the reporter gene assay:	Host CV-1 cells were plated in 48-well microtiter plates (Corning Costar Corporation, NY, USA) at a density of 50,000 cells per well in phenol red-free MEM supplemented with 10% CD-FBS (complete medium) 1 day before transfection. To detect mPPARα or mPPARγ activity, host cells were transfected with 24 ng of either pCMX-mPPARα or pCMX-mPPARγ, 96 ng PPRE3-TK-LUC and 20 ng pCMVβ-Gal per well using the FuGene6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) following the manufacturer's instructions.

B. Study design and methods

1. Treatment:

Concentration tested:	Not explicitly stated (≤10^{-5} M)
Replicates:	3
Observations:	Luciferase activity (normalized for β-galactosidase activity)

PPARα and PPARγ reporter gene assay:	After a 3-h period of transfection, various concentrations of pesticides, positive control compounds or 0.1% DMSO (vehicle control) in complete medium were administered to the cells. To avoid cell toxicity by the pesticides, assays were performed for pesticides at concentrations of less than 10^{-5} M. After an incubation period of 24 h, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (100 µL/well; Promega, Madison, WI, USA).
Luciferase assay:	The firefly luciferase activity was measured with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany) in a reaction tube with a 10 µl aliquot of cell lysate using the Luciferase Assay System (Promega) following the manufacturers instructions. The β-galactosidase activity was also measured in the cell lysate using the fluorescence method previously reported (Takeuchi et al., 2005). The luciferase activity was normalized based on the β-galactosidase activity for each treatment. Results are expressed as means ± SD from at least three independent experiments. To estimate the potency of receptor-agonistic activity of the compounds tested, the concentration of the compound exhibiting the response equal to 20% of the maximal response of 1×10^{-5} M WY-14643 or 1×10^{-5} M pioglitazone for PPARα or PPARγ, respectively, was evaluated from a dose-response curve of the luminescence intensity, and expressed as the 20% relative effective concentration (REC20).
Statistics:	All data were expressed as mean ± standard deviation (SD). As there was no biologically relevant modulation of luciferase activity for either PPARα or PPARγ, the data were not statistically analysed.

II. Results and discussion

A. Luciferase assay

According to the text, methiocarb did not induce relative PPRE-Luc reporter gene activation of more than 20% of the WY-14643- or piglitazone-induced maximal activity (10^{-5} M), the threshold for a positive agonistic response in this study. According to the provided figure (Figure 2 in the publication), methiocarb did not induce any relevant relative PPRE-Luc reporter gene activation for either PPARα or PPARγ.

III. Conclusion

The authors conclude that methiocarb does not activate PPARα or PPARγ in the PPRE-Luc reporter assay.

Bayer conclusion:
The non-GLP in vitro assay was used as a screening tool for the evaluation of agonistic activity for PPARα and PPARγ of pesticides including methiocarb. Due to the reporting deficiencies this study is regarded as supplemental information. Results do not change existing endpoints or have an effect on the risk assessment.

The reliability evaluation is given below

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study with methodical and reporting deficiencies • source and purity were not specified • tested concentrations were not specified • passage of the CV-1 cells not stated • no antagonisation experiments • no endogenous genes were studied
Relevance of study:	**Supplemental information that does not change existing endpoints or leads to a more conservative risk assessment.**

Report: KCA 5.8.2/13 [redacted]; 2011; M-459484-01-1
Title: In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron.
Report No.: M-459484-01-1
Document No.: M-459484-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: **no**

I. Materials and methods

A. Materials

1. Test material: Methiocarb
(among 200 other tested pesticides; only the assays conducted with Methiocarb are summarised in this document.)
Source: Not specified (several sources were listed for the 200 pesticides)
Description: Not reported
Lot/Batch no: Not reported
Purity: Not specified (in the range of 95-100%)

2. Vehicle/negative and positive control:
Vehicle: DMSO (max. 0.1% in assay)
Positive controls: 10^{-10} M TCDD (as specified in Figure 2 of the publication)

3. Test system:
Species/strain: DR-EcoScreen cells, i.e. mouse hepatoma Hepa1c1c7 cells stably transfected with a reporter plasmid containing seven copies of dioxin-response element (DRE)

Source:	Created in course of this study, see details below.
Details on the test system:	
Cell line:	Mouse hepatoma Hepa1c1c7 cells were obtained from the American Type Culture Collection.
Plasmids:	The expression plasmid, *pIND-GCDR7,* was generated in course of this study and was based on the genome of the C57BL/6 strain mouse. The new reporter plasmid for the Aryl Hydrocarbon Receptor (AhR)-mediated transcriptional assay using pIND-MCS-LUC, based on the ecdysoninducible mammalian expression vector pIND/Hygro, harboring hygromycin resistant gene (Invitrogen), the construction method of pIND-MCS-LUC being previously described (Kojima et al.,2003). After each recombinant DNA treatment, the arrangement of the insert was verified by DNA sequencing. Details are presented in the publication.
Generation of the reporter gene assay:	About 16 h prior to the transfection, Hepa1c1c7 cells were seeded at a confluency of 30% in a 6-well plate in 2 mL of culture medium (α-MEM with 5%FBS) per well. Transfection was carried out with FuGENE®6 Transfection Reagent according to the instruction of the manufacturer. Briefly, 1µg of pIND-GCDR7 and 3 µL of FuGENE®6 Transfection Reagent were added to each well. After 24 h incubation, the cells in each well were trypsinized, resuspended in the culture medium containing 150 µg mL^{-1} of hygromycin, and divided equally to plate in two 100 mm petri dishes. The culture medium was replaced every four days until colonies became available for isolation (for about two weeks). After cells were exposed to 20 pM of TCDD for 24 h, luciferin (final concentration: 20 nM) was added into a 100 mm petri dish. After further incubation for 5 min, luminescence image was scanned through a photon detecting charge-coupled device (CCD) camera (Night OWL, Perkin-Elmer), which took 10 min per dish. Each luciferase positive clone was isolated using a cloning ring and individually cultured in a well of a 24-well plate. After proliferation, each clone was trypsinized, resuspended, seeded a 1/10 volume into a 100 mm petri dish, and further cultured. With repeated these steps, the clone having the highest luciferase activity and the greatest fold of induction ratio to background luciferase activity was selected, and named as DR-EcoScreen.

Culture conditions:	The DR-EcoScreen cell line was maintained in α-MEM supplemented with 5% FBS, antibiotics and glutamine at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity, and passaged twice every week by trypsinization with 0.25% trypsin/0.02% EDTA For screening assay, cells were trypsinized and suspended at a density of 1.0×10^5 cells mL^{-1} in α-MEM containing 5% of CD-FBS.

B. Study design and methods

1. Treatment:

Concentration tested:	Not explicitly stated (1 x 10^{-7} to 1 x 10^{-5} M)
Replicates:	3 independent experiments performed in duplicate
Observations:	Luciferase activity (relative to 10^{-10} M TCDD), cytotoxicity
Luciferase assay:	Ninety µL of the cell suspension was seeded in a well of a 96-well white bottom plate (final density: 9000 cells $well^{-1}$). After 24 h cultivation at 37 °C, 10 µL of various concentrations of chemicals that were dissolved in 1% DMSO was added to each well (final concentration of DMSO was 0.1%). Following further 24 h of cultivation, 100 µL of Steady-GloTM reagent was added to each well. After the plate was shaken at room temperature for five min, the luminescence was measured with a microplate-luminometer (Wallac 1420 ARVOTM SX, Perkin-Elmer).
Cytotoxicity test:	DR-EcoScreen cells were plated and cultured in the same way as the luciferase reporter gene assay, and added alamar blue dye instead of Steady-Glo™ reagent. The cytotoxicity was measured in accordance with the manufacturer's protocol of alamarBlue™ (AbD Serotec, Kidlington, UK) (O'Brien et al., 2000). This is a colorimetric assay to measure the metabolic activity of living cells based on the conversion of alamar blue dye (resorufin, blue) into resorufin (red). Absorbance was read at a test wavelength of 575 nm and a reference wavelength of 610 nm with a microplate-spectrophotometer, MPRA4i (TOSOH Co., Ltd., Osaka, Japan).
Aryl Hydrocarbon Recetor (AhR) agonistic activity:	To estimate the potency of receptor-agonistic activity of the pesticide tested, its concentrations exhibiting the response equal to 50% and 20% of the maximal response of 10^{-10} M TCDD for AhR were evaluated from a dose-response curve of the luminescence intensity, and expressed as 50% and 20% relative effective concentration, REC50 and REC20, respectively. When the activity of the test compound was higher than REC20 within the concentration tested (~10^{-7} - 10^{-5} M), the pesticide was judged to be positive for activity.
Statistics:	All data were expressed as mean ± standard deviation (SD) of three experiments performed in duplicate.

II. Results and discussion

A. Luciferase assay

Methiocarb did not induce any relevant relative luciferase activity. Thus, methiocarb had no AhR agonistic activity in this luciferase reporter gene assay in DR-EcoScreen cells.

III. Conclusion

According to the results methiocarb does not induce any relevant AhR agonistic activity.

Bayer conclusion:
This non-GLP study was conducted according to scientific principles.
The in vitro luciferase assay was used as a screening tool for the evaluation of AhR agonistic activity of pesticides including methiocarb. Due to the reporting deficiencies this study is regarded as supplemental information. Results do not change existing endpoints or have an effect on the risk assessment.

The reliability evaluation is given below

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study, conducted according to scientific principles with reporting deficiencies. • Methiocarb not obtained from BCS (impurity and purity not reported). • tested concentrations were not exactly specified • passage of the Hepa1c1c7 cells not stated • no antagonisation experiments
Relevance of study:	**Supplemental information that does does not lead to a more conservative risk assessment**

Publications cited in this publication:

[redacted] 2003. Effects of a diphenyl ether-type herbicide, chlornitrofen, and its amino derivative on androgen and estrogen receptor activities. Environ. Health Persp. 111, 497–502

Report: KCA 5.8.2/14 [redacted]; 2011; M-459845-01-1
Title: Comparative study of human and mouse pregnane X receptor agonistic activity in 200 pesticides using in vitro reporter gene assays.
Report No.: M-459845-01-1
Document No: M-459845-01-1
Guideline(s): not applicable
Guideline deviation(s): not applicable
GLP/GEP: no

A. Materials

1. Test material:	Methiocarb
Further test substances:	In total 200 pesticides (29 organochlorines, 11 diphenyl ethers, 56 organophosphorus pesticides, 12 pyrethroids, 22 carbamates, 12 acid amides, 7 triazines, 7 ureas, and 44 others)
Lot/Batch no:	Not reported
Purity:	≥ 97 %
Source:	Cannot be concluded from publication
Stability of test compound:	expiry date: not reported
2. Vehicle:	DMSO
3. Test system:	
Cells:	COS-7 simian kidney cells
Cell source:	American Type Culture Collection
Expression plasmids:	pSG5-hPXR and pSG5-mPXR (both encoding the full-length receptor protein) (Dep. of Molecular Biology, University of Texas, Southwestern Medical Center, Dallas, TX, USA).
Reporter plasmid:	pXREM-3A4-Luc (High Throughput Biology, Discovery Research, GlaxoSmithKline, Research Triangle Park, NC, USA).
Internal control plasmid:	pCMVß-Gal (Clontech, Palo Alto, CA, USA)

B. Study design and methods

1. treatment:	
Transfection of plasmids to cells:	Cells were seeded at a concentration of 8400 cells/well (96-well microtiter plates) in DMEM containing 10 % charcoal-dextran treated fetal bovine serum (CD-FBS) 1 day before transfection. Cells were transfected with 12 ng pSG5-hPXR or pSG5-mPXR, 48 ng pXREM-3A4-Luc, and 12 ng pCMVß-Gal per well using FuGENE®6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) by incubation for 3 h.
Test substance incubation:	Incubation with various concentrations of test substances or vehicle control in complete medium. After 24 h, cells were rinsed with phosphate-buffered saline and lysed with passive lysis buffer (50 µg/well; Promega, Madison, WI, USA). Culture conditions are not reported.
Test substance concentrations:	Screening assay for cytotoxicity: 1 x 10^{-7} – 1 x 10^{-5} M Subsequently, the concentration ranges were modified to avoid cytotoxicity of each individual compound. Final test concentration for methiocarb: ≤ 1 x 10^{-5} M (range not further specified)
Positive controls:	For human pregnane X receptor (hPXR): Rifampicin (RIF) For mouse pregnane X receptor (mPXR): pregnenolone 16-α-carbonitrile (PCN) (both from [redacted])
Negative (solvent) control:	0.1 %DMSO

Specificity controls:	To confirm that luciferase inductions of test chemicals are PXR-dependent: COS-7 cells transfected with 48 ng pXREM-3A4-Luc, and 12 ng pCMVß-Gal (without the PXR expression plasmid).
Luciferase activity assay:	Firefly luciferase activity was measured in a 5-µl aliquot of the cell lysate in one reaction tube with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany), using the Luciferase Assay System (Promega), according to the manufacturer's instructions. The luciferase activity was normalized against the ß-galactosidase activity for each treatment. Results are expressed as mean ± SD from at least three indipendent experiments prformed in triplicate.
ß-Galactosidase activity assay:	To check the cytotoxicity of the test chemicals against transcriptional activity using a fluorescence method (not described in detail).
Evaluation of PXR agonistic activities:	To estimate the potency of the receptor agonistic activity of the tested compounds, the luminescence intensity of the assay was presented as a dose–response curve. The obtained concentration of the compound equal to 20 % of the maximal response of positive control (RIF or PCN) from the dose–response curve of the luminescence intensity and was expressed as an REC_{20} value (20 % relative effective concentration). When the agonistic activity of the test compound was higher than the REC_{20} value for the concentration tested (~1 × 10⁻⁴ M), the pesticides was judged to be positive for agonistic activity against hPXR or mPXR. Each REC_{20} value represents the mean of three independent experiments.

II. Results and discussion

Of the 200 pesticides tested, 106 and 93 activated hPXR and mPXR, respectively, and a total of 111 had hPXR and/or mPXR agonistic activity with greater or lesser inter-species differences.
Methiocarb is not listed as tested positive in the human and/or mouse PXR transactivation assay.

III. Conclusion

Methiocarb is not mentioned among the carbamate-type pesticides showing hPXR and/or mPXR agonistic activities. Methiocarb showed no pregnane X receptor (PXR) agonistic activity under the experimental conditions described.

Bayer conclusion:
The non-GLP in vitro assays revealed no methiocarb specific results. Since methiocarb was not listed in the result table with positive tested chemicals, it is assumed that methiocarb was not an activator of hPXR and / or mPXR. Due to the reporting deficiencies and the absence of methiocarb-specific results

this study is regarded as supplemental information. Results do not change existing endpoints or have an effect on the risk assessment.

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP *in vitro* assays, conducted according to scientific principles but with reporting deficiencies: • source for methiocarb not specified • tested concentration range were not specified • passage of the COS-7 cells not stated • applied test substance volumes not reported • no results for methiocarb presented.
Relevance of study:	**Supplemental information. Results do not change existing endpoints or lead to a more conservative risk assessment.**

CA 5.8.3 Endocrine disrupting properties

No specific studies on endocrine disruption were conducted with methiocarb. However, a review of the whole data base on methiocarb was conducted to identify possible effects of methiocarb on endocrine organs, tissues or parameters. Methiocarb treatment led to no findings linked to the thyroid as well as to findings linked to steroidogenesis. There are also no indications for effects on the pancreas (including blood glucose, urinalysis data or histopathology), pituitary (organ weight, histopathology), thymus (histopathology), adrenals (based on blood cholesterol levels, organ weight and histopathology) or parathyroid (based on blood calcium levels and histopathology) in rat, mouse or dog. There were also no effects on reproductive organs (male and female) observed in any of the studies in rats, mice and dogs. In addition, there was no carcinogenic potential, and no reproductive effects with an endocrine background observed for methiocarb.

In addition, three publications emerged from public literature between 2009 and 2011, which describe potential endocrine effects of methiocarb in different test systems *in vivo* and *in vitro*. Due to methodological and reporting deficiencies the publications were considered to be reliable with restrictions.
One publication described a non-dose-related increase of the testicular ERα protein expression, while no expression of ERβ was observed. The results based on Western-blot analysis of testicular tissues obtained after repeated i.p. applications of low doses methiocarb to mice. Since there were no correlated effects on sperm concentration or sperm quality the results are considered as supplemental information that does not change existing endpoints or lead to a more conservative risk assessment.
The other two publications described anti-androgenic activities of methiocarb *in vitro*. Due to the fact that the tested concentrations in these *in vitro* assays are much higher than *in vivo* concentrations that can be reached in blood at dose levels that cause (toxic) effects, the results are of no relevance for the human risk assessment of methiocarb. This is supported by the fact that known potent androgen receptor antagonists like hydroxyflutamide or 17β-estradiol, have at least 10 times lower IC_{25} concentrations than the IC_{25} observed for methiocarb.

The summaries of the publications, as well as the reliability and relevance assessments are provided below.

Publications

Report:	KCA 5.8.3/01 [redacted]; 2009; M-495809-01-1
Title:	Increased expression of the testicular estrogen receptor alpha in adult mice exposed to low doses of methiocarb.
Report No.:	M-495809-01-1
Document No.:	M-495809-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

1. Test material: Methiocarb

Source:	[redacted], MO, USA
Description:	Not reported
Lot/Batch no:	Not reported
Purity:	Not reported
Stability of test compound:	expiry date: not reported

2. Vehicle: Corn oil

3. Test animals:

Species:	Mice
Strain:	DBA/2 Slc
Age:	18 weeks at start of treatment
Sex:	Male
Weight at dosing:	Not reported
Source:	SLC Inc., Tokyo, japan
Acclimatisation period:	At least 2 month
Diet:	AIN-76A rodent feed (Research Diets, [redacted], NJ, USA), *ad libitum*
Water:	water, *ad libitum*
Housing:	Not reported

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0-0.03-0.3-1.0-3.0 µg/kg bw/day
Application route:	i.p.
Application volume:	0.1 mL
Treatment schedule:	20 consecutive days
Group size:	6-7 mice
Observations:	Body weights, organ weights (testis, epididymis, Cowper's gland, kidneys, liver, thymus), effects on spermatogenesis, sperm motility, expression of testicular ERα (by western blot analysis)

Tissue sampling for western blot:	On day 20 of treatment, animals were euthanized by cervical dislocation. Collected tissue samples were submerged in RNAse-free water pre-treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma) and then snap frozen in liquid nitrogen gas. All tissue samples for western blot were stored at −80 °C.
Western blot analysis:	Tissue samples were homogenized with cold protein extraction solution (Pro-prep; Intron Biotechnology, Sungnam, Korea) using a PRO 200 homogenizer (PRO Scientific Inc., Oxford, CT, USA). To obtain protein, homogenates were treated as per the manufacturers′ instruction. Quantification of protein was measured using the Bradford protocol using BSA as a reference standard. A 25 µg aliquot of protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electro transferred onto nitrocellulose membranes. The equal loading of proteins was confirmed by probing the same membrane with a monoclonal antibody against GAPDH (AB Frontier, Seoul, Korea) at 1:10 000. Membranes were stripped and incubated overnight with polyclonal antibodies against SC-542 ERα (1:1000), SC-8974 ERβ (1:1000) and SC-14245 aromatase (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, membranes blots were incubated at room temperature for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody at a 1:3000 dilution (Santa Cruz Biotechnology) before visualization using enhanced chemiluminescence (AB Frontier, Seoul, Korea) and detected using a Fujifilm LAS3000 Intelligent Dark Box Imager (Fuji Photo Film, Tokyo, Japan). Relative binding (%) was calculated based on protein densities of immunoprecipitates measured with MultiGauge V 3.0 (Fuji Film Life Science, Stamford, CT, USA).
Sperm analysis (computer-assisted)	The cauda distal epididymides were punctured in a 60 mm organ culture dish (Orange Scientific Inc., Braine-I'Alleud, Belgium) containing 1.0 ml of pre-warmed Dulbecco's modified Eagle medium with Ham's F-12 nutrient mixture (DMEM/F-12; Welgene, Seoul, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Welgene). Sperm concentration and motion analyses were conducted within 30 min after puncture. Quantitative parameters of sperm motility were measured by computer-assisted sperm analysis (CASA) using the integrated visual optical system (IVOS) motility analyzer (Hamilton-Thorne Research Inc., Beverly, MA, USA), software version 10.7.

Statistics:

Data analysis was performed using SAS version 8.0 (SAS Institute, Cary, NC, USA). All data were tested for normality by the Shapiro–Wilks test at the 5% level of significance. Non-normal data were log-transformed and retested. Nonparametric one-way analysis of variance (ANOVA) using the NPAR1WAY procedure from SAS was performed for non-normal data. The Kruskal–Wallis test was used to compare between control and tested groups. Data passing the normality test were analyzed with a repeated measures ANOVA using the PROC general linear models procedure of SAS.
To compare between control and treated groups, Dunnett's and Student's t-tests were performed. The level of significance was set at $P < 0.05$ and reported in the Results

II. Results and discussion

A. Body weights
There were no treatment-related effects on body weights.

B. Organ weights
There were no significant differences in absolute and relative testis and epididymis weights in methiocarb-treated groups when compared with controls.
In the 1.0 µg methiocarb/kg bw/d treated group, kidney weights were significantly increased, compared with the control group (59%) ($p < 0.05$). Relative (i.e. adjusted) organ weights of kidney and liver were significantly increased in the 1.0 µg methiocarb/kg bw/d treated group in comparison to the controls.
In the 3.0 µg methiocarb/kg bw/d group, absolute and relative kidney and liver weights were significantly increased.

Table 5.8.3/01- 1: Body and absolute organ weights (mean ± SE)

Group [µg/kg bw/d]	Terminal bw [g]	Paired testis	Paired epididymis	Paired Cowper´s gland
	Absolute organ weights [g]			
0	27.95 ± 0.83	0.2099 ± 0.0097	0.0801 ± 0.0047	0.1218 ± 0.0192
0.03	29.52 ± 1.56	0.2043 ± 0.0090	0.0779 ± 0.0042	0.1130 ± 0.0087
0.30	28.10 ± 1.58	0.2183 ± 0.0102	0.0785 ± 0.0036	0.1050 ± 0.0118
1.00	26.90 ± 0.40	0.2201 ± 0.0111	0.0751 ± 0.0012	0.1425 ± 0.0193
3.00	29.30 ± 0.53	0.2113 ± 0.0060	0.0759 ± 0.0043	0.1348 ± 0.0093
Group [µg/kg bw/d]	**Terminal bw [g]**	**Paired kidneys**	**Liver**	**Thymus gland**
	Absolute organ weights [g]			
	27.95 ± 0.83	0.4791 ± 0.0124	1.3389 ± 0.0745	0.0230 ± 0.0024
0.03	29.52 ± 1.56	0.5047 ± 0.0775	1.3436 ± 0.1470	0.0268 ± 0.0082
0.30	28.10 ± 1.58	0.6156 ± 0.0489	1.5998 ± 0.1260	0.0456 ± 0.0086
1.00	26.90 ± 0.40	0.7619 ± 0.0342*	1.6647 ± 0.0817	0.0417 ± 0.0065
3.00	29.30 ± 0.53	0.7358 ± 0.0353*	1.7596 ± 0.0924*	0.0412 ± 0.0083

* Significantly different from control, $p < 0.05$

C. Expression of estrogen receptor α in testes

There was a significant increase in the protein levels of testicular ERα in 0.3, 1.0 and 3.0 µg methiocarb/kg body weight-treated groups, after adjustment relative to the protein level of endogenous GAPDH. The highest expression was observed in the 1.0 µg treatment group by approximately a 1.8-fold increase compared with the control. The authors implied from these results that increasing dose of methiocarb exhibits a biphasic effect on testicular ERα expression (see Figure 5.8.3/01- 1below). In contrast, methiocarb treatment did not significantly change the protein levels of testicular aromatase in the mice testes.

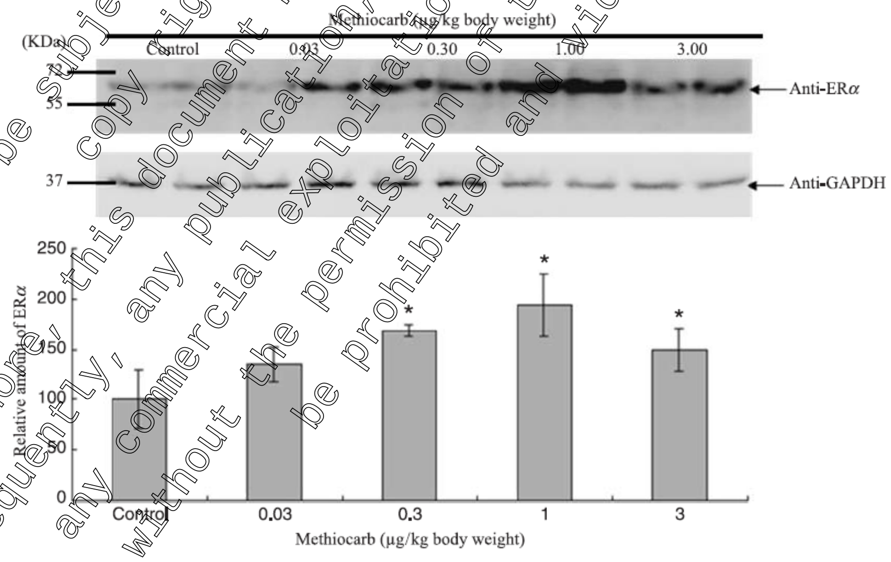


Figure 5.8.3/01- 1: Quantitative analysis of the testicular ERα protein expression presented as a bar graph. Four independent testicular lysates of the mice exposed with or without methiocarb (0, 0.03, 0.30, 1.00, and 3.00 µg methiocarb/kg bw) were prepared, and western blot analyses were performed with polyclonal antibodies against anti-ERα.

ERα protein expression was quantified by the normalization with the levels of GAPDH expression. Quantitative data (mean ± SD) were presented as relative amounts of ERα in which the levels of control are considered as 100%.

*Significantly different from the control ($P < 0.05$).

D. Effects of methiocarb on epidermal sperm concentration and quality

Methiocarb exposure did not alter epididymal sperm concentration including numbers of motile sperms and progressive sperms in adult male mice.

There was an increased trend in linearity in all test substance groups when compared to control (0.2, 5.3, 5.5 and 6.1%). However, the differences were not statistically significant.

Table 5.8.3/01- 2: Epididymal sperm concentration and sperm quality (mean ± SE)

	Methiocarb dose (µg/kg bw/day)				
	0	**0.03**	**0.3**	**1.0**	**3.0**
No. of animals	7	6	6	6	7
Sperm concentration (10^6 sperm/mL)	24.88±2.83	26.83±3.85	26.72±1.73	23.43±2.80	26.01±1.85
Motile sperm (10^6 sperm/mL)	11.37±2.69	15.00±3.06	14.70±2.64	14.95±3.06	13.22±1.96
Progressive (10^6 sperm/mL)	2.69±0.79	4.02 ± 1.45	2.70 ± 0.59	3.77 ± 1.20	3.01 ± 0.53
Smoothed path velocity (µm/s)	54.77 ± 5.95	54.55 ± 5.55	53.31 ± 4.90	47.88 ± 3.98	62.67 ± 3.87
Track velocity (µm/s)	43.45 ± 5.10	42.19 ± 4.17	40.03 ± 3.71	36.99 ± 2.67	49.82 ± 3.35
Straight line velocity (µm/s)	89.29 ± 8.82	94.98 ± 9.08	93.04 ± 7.95	84.58 ± 8.19	106.33 ± 6.34
Amplitude of lateral head displacement (µm)	4.66 ± 0.54	6.26 ± 0.71	4.59 ± 0.53	3.79 ± 0.32	4.57 ± 0.17
Beat cross frequency (Hz)	22.23 ± 1.64	24.66 ± 2.30	24.08 ± 2.20	19.79 ± 1.41	21.14 ± 1.40
Straightness (%)[A]	78.21 ± 2.47	79.08 ± 2.06	75.42 ± 2.81	79.50 ± 2.36	78.93 ± 1.73
Linearity (%)[B]	44.82 ± 1.26	44.91 ± 1.16	47.18 ± 2.25	47.27 ± 1.50	47.57 ± 2.30
Elongation (%)[C]	92.77 ± 1.92	92.92 ± 2.07	91.45 ± 1.44	92.25 ± 1.81	91.14 ± 1.72

A = straightness (ratio of VSL:VAP), linearity (ratio of VSL:VCL). Elongation = head shape (ratio of minor to major axis of sperm head).

III. Conclusion

Intraperitoneal treatments of male mice with methiocarb doses of 0.3, 1.0 and 3.0 µg/kg bw/day for 20 consecutive days resulted in a significant increase of testicular ERα protein levels, without affecting sperm concentration and quality.

The authors concluded that methiocarb is probably an estrogenic compound.

Bayer conclusion:

This non-GLP mouse study was conducted with low doses of methiocarb that were applied by a non-relevant exposure route (i.e. i.p.) for humans.

In the Western blot analysis a non-dose-related increase of the testicular ERα protein expression was seen. Expression of ERβ was not observed. No correlated effects of sperm concentration and quality

were observed. Therefore, the results do not change existing endpoints and do not change the risk assessment.

The reliability evaluation is given below.

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study, conducted according to scientific principles with reporting deficiencies. • Methiocarb not obtained from BCS (impurity and purity not reported). • no details of tissue homogenisation reported • no details for homogenate treatment given • Unclear if pooled samples or individual samples were analyzed by Western blot quantification.
Relevance of study:	**Supplemental information which does not change existing endpoints and does not lead to a more conservative risk assessment.**

Report:	KCA 5.8.3/02 [redacted]; [redacted]; [redacted]; [redacted]; 2013; M-474483-01-1
Title:	Widely used pesticides with previously unknown endocrine activity revealed as in vitro antiandrogens
Report No.:	M-474483-01-1
Document No.:	M-474483-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

1. Test material:	Methiocarb
Source:	[redacted], UK
Description:	Not reported
Lot/Batch no:	Not reported
Purity:	>98.7%
Source:	[redacted]; [redacted], UK
Stability of test compound:	expiry date: not reported
2. Vehicle:	none
3. Test system:	
Cells:	MDA-Kb2 cells
Cell description:	Human breast cancer cells stably transfected with firefly luciferase reporter gene that is driven by an androgen-response element containing promotor.
Source:	Not reported

B. Study design and methods

1. treatment:

MDA-kb2 assay:	Cells were seeded at a concentration of 1 × 10^5 cells/mL in phenol red–free Leibowitz-15 medium containing 10% (charcoal-stripped) fetal calf serum in white luminometer plates and allowed to attach for 24 hours. Cells were then exposed to eight serial dilutions of selected pesticides with or without dihydrotestosterone (DHT) (0.25 nM). After 24 hr, luciferase activity was determined with SteadyGlo assay reagent (Promega UK Ltd., Southampton, Hampshire, UK) and measured in a plate reader (FLUOstar Optima, BMG Labtech GmbH, Offenburg, Germany).
Test substance concentrations:	Range-finder: 0.64 nM – 50 µM (5x dilutions) Subsequently, the concentration ranges were modified to reflect the potency and toxicity of each individual compound Test substances were co-exposed to DHT (0.25 nM)
Positive controls:	Positive control: DHT serial dilutions (0.002–10 nM)
Negative (solvent) control:	ethanol
Internal quality control for anti-androgenic activity:	Initially flutamide (0.013–8 µM) (serial dilutions) Flutamide was replaced by procymidone (0.005–3.2 µM) serial dilutions
Replicates	All concentrations were tested in duplicate over two plates, and each pesticide was measured at least twice in separate experiments
Evaluation:	For comparative purposes, luminescence was normalized to DHT alone at co-exposure concentration (maximum response: 100%) and solvent-only (ethanol) controls (minimum response: 0%) Because cytotoxic effects could not be distinguished from anti-androgenic effects in the co-exposed treatments, any readings of the pesticide statistically significantly below the mean ethanol control level (0%) were considered toxic to MDA-kb2 cells and the corresponding co-exposure data were not classified as anti-androgenic. Sixty percent of the pesticides were repeat tested using the same product but with new stock solutions and by a different experimenter Results were expressed as anti-androgen IC_{20} (= inhibitory concentration that inhibits the androgenic activity of DHT by 20%), as well as androgen EC_{20} (= concentration that causes a 20% effect)
YAS assay:	Method not described in detail. For details reference was made to *Sohoni and Sumpter 1998 (Several environmental oestrogens are also anti-androgens. J Endocrinol 158(3):327–339).* Briefly, stimulation of the transfected AR causes a color change in the media, which is measured by absorbance at 540 nm. Plates were also measured at 620 nm to measure cell

	growth (turbidity) to check for any cytotoxic effects that may have occurred. Serial dilutions of test substances were co-incubated with DHT (6.4 nM). Incubation time was 53 hr at 28°C. Where turbidity readings were significantly depressed, toxicity was indicated and the effect could not be considered antiandrogenic; therefore, these dilutions were removed from analysis.
Test substance concentrations:	Serial dilutions, varied according to potency observed in MDA-kb2 assay but was between 0.016 and 750 µM for all test compounds.
Positive controls:	DHT (6.4 nM), serial dilutions (0.0026-100 nM)
Negative (solvent) control:	ethanol
Internal quality control for anti-androgenic activity:	Initially flutamide (0.19–100 µM) (serial dilutions)
Replicates:	All concentrations were tested in duplicate over two plates, and each pesticide was measured at least twice in separate experiments
Evaluation:	Results were expressed as antiandrogen IC_{20} (= inhibitory concentration that inhibits the androgenic activity of DHT by 20%)
Cytotoxicity assessment:	In both assays cytotoxicity was determined as EC_{20} (concentration that produces a 20% effect)
Statistics:	To analyze antiandrogenic action, raw luminescence readings were normalized on a plate-by-plate basis to the means of the positive DHT controls (n = 8) and the solvent controls (n = 8) (Ermler et al. 2010). We pooled all data from the same test compound and conducted statistical concentration–response regression analyses using the best-fit approach (Scholze et al. 2001). Specifically, a variety of nonlinear regression models were fitted independently to the same data set, and the best-fitting model was selected using a statistical goodness-of-fit criterion. Concentration–response data from different researchers were first analyzed one by one using regression models, and differences in regression analyses due to data from different researchers were judged as statistically significant when the 95% CIs of the regression curves did not overlap. Such statistical differences between researchers were not observed, and thus data were pooled for final analysis. Luminescence readings from pesticides tested in the absence of DHT were divided by the mean of the solvent controls from the same plate and analyzed for negative and positive trends (suggestive of cytotoxic or androgenic action,

respectively) by nonparametric contrast tests (Neuhaeuser et al. 2000). Data considered to be statistically significant at $p < 0.05$ were analyzed using the best-fit approach as described above. All statistical analysis was performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA). From the best-fitting model, we derived inhibitory concentrations for antiandrogenicity and effect concentrations for cytotoxicity.

II. Results and discussion

A. MDA-kb2 assay

In the MDA-kb2-assay methiocarb showed anti-androgenic activity. The determined IC_{20} was 6.82 µM. In contrast the most potent anti-androgen determined in this assay was fenitrothion with an IC_{20} of 0.098 µM. The least potent anti-androgen pyrimethanil had an IC_{20} of 27.2 µM.

Table 5.8.3/02- 1: Results for methiocarb in the MDA-kb2-assay

Compound	Antiandrogen IC_{20} (µM)	Cytotoxic EC_{20} (µM)	Androgen EC_{20} without DHT (µM)
Methiocarb	6.82	>46	negative
Fenithrothion*	0.098	>50	4.9
Pyrimethanil*	27.2	>125	27.8
Procymidone**	0.163	>50	negative

* For comparison the most potent (fenithrothion) and the least potent (pyrimethanil) substance tested in the MDA-kb2-assay are presented.

** Internal quality control for antiandrogenic activity

In addition, to the determined IC_{20} value the response curve over the tested concentration was determined. The results for methiocarb and some other substances (grouped according to sum of maximum EU residue, top 10 fruit/cereal list, JMPR estimated intake, fruit/vegetable EFSA report) are depicted in Figure 5.8.3/02- 1below. As can be seen in the figure, the NOEC of methiocarb for anti-androgenic activity in the MDA-kb2-assay is about 2 µM, the IC_{50} is greater than 10 µM.

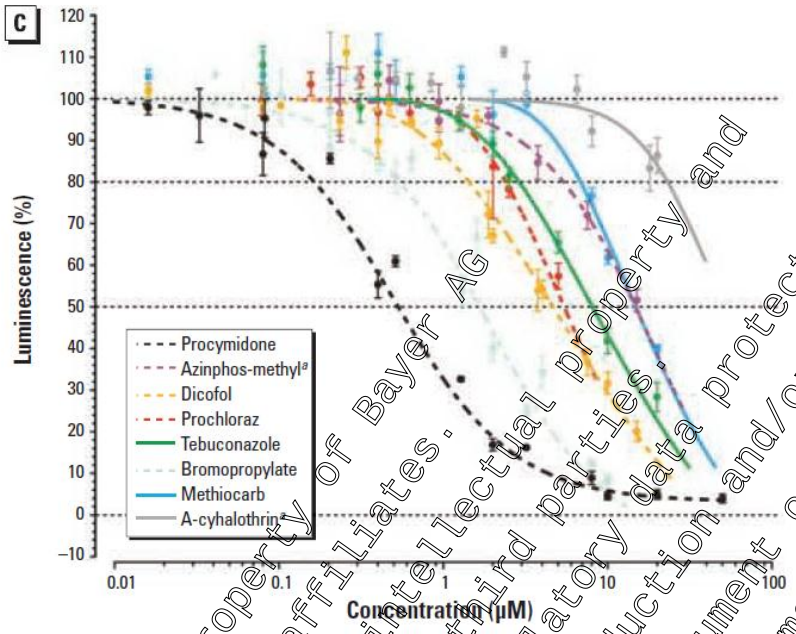


Figure 5.8.3/02- 1: **Results of the MDA-kb2 assay showing regression curves for anti-androgenic pesticides**

Values for luminescence were normalized to those of controls. Compounds are grouped by exposure scoring (based on sum of maximum EU residue, top 10 fruit/cereal list, JMPR estimated intake, fruit/vegetable EFSA report); procymidone is shown as a point of reference. Regression lines end at the toxic threshold. Dashed lines indicate pesticides with lapsed registration, and solid lines indicate pesticides with current registration.
[a]Newly described anti-androgens

B. YAS assay
Pesticides that were newly described as highly active antiandrogens in the MDA-kb2 assay and 4 pesticides for which the QSAR prediction differed from the experimental result (including 1 out of the model domain) were subjected to further testing using the YAS (n = 14).
Methiocarb was neither highly active, nor was the QSAR prediction different from the MDA-kb2-result. Therefore, methiocarb was not tested in the YAS assay.

C. Cytotoxicity
The cytotoxicity evaluation revealed that the methiocarb concentration that produces cytotoxicity in this assay is about 6.7 times (derived from the ratio: EC_{20} for cytotoxicity/IC_{20} for antiandrogenic activity) higher than the concentration needed to produce antiandrogenic activity. The results are summarised in Table 5.8.3/02- 1.

III. Conclusion

The authors concluded, that methiocarb has antiandrogenic activity in the MDA-kb2-assay *in vitro*.

Bayer conclusion:
This non-GLP study was conducted according to scientific principles. The described anti-androgenic effects of methiocarb in the MDA-kb2-assay were observed in vitro at methiocarb concentrations of greater than 2 µM (corresponding to the determined NOEC in this assay) with an observed IC_{20} of 6.82 µM.

It has to be considered that the total maximum plasma concentration of methiocarb observed after treatment of rats with 0.91 mg/kg bw by (actual dose) oral gavage was 0.0513 µM (based on a maximum dose-normalized concentration CN_{Max} of 0.012 kg bw/kg blood ([redacted] 2009; M-348264-02-1; see also KCA 5.1.1/05)). The corresponding calculated total plasma concentration of the acute oral LD_{50}-dose of 19 mg/kg bw is 1.07 µM (for details of the calculation see box below). Taken this into consideration the total maximum plasma concentrations of rats at an acute lethal oral dose of 19 mg/kg bw (i.e. LD_{50}) is about a factor 1.9 lower than the observed NOEC of the in vitro assay, and about 6.4 times lower than the IC_{20}. Furthermore, it has to be considered that due to protein-binding in blood the unbound fraction of methiocarb is even lower than the total concentration. This consideration leads to a further increase of the factors between the tested concentrations and the methiocarb in vivo concentrations.

Therefore, it is concluded that the results of this study, have no impact on the exposure situation in vivo.

Regarding the described AR-agonistic activity of some of the tested compounds it has to be considered that the used cell line expresses in addition to the androgen receptor (AR) also endogenous glucocorticoid receptor (GR). However, co-exposure with a known antiandrogen (e.g. hydroxyflutamide, OHF) was conducted to distinguish between AR- and GR-agonistic activities.

The reliability evaluation is given below.

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study, conducted according to scientific principles with reporting and methodological deficiencies. • Methiocarb not obtained from BCS (impurity and purity not reported), • Number of cell passages not reported • No differentiation between AR- and GR-agonistic activities. In addition, the tested concentrations in this *in vitro* assay are not comparable to *in vivo* concentrations that can be reached in blood at dose levels that cause (toxic) effects.
Relevance of study:	**Due to the large differences between the determined NOEC and IC_{20} and the maximum total plasma concentrations of methiocarb determined *in vivo* the results of this *in vitro* MDA-kb2-assay are of no relevance for the human risk assessment.**

From [redacted], 2009; M-348264-02-1; see also KCA 5.1.1/05:

CN_{Max} = 0.012 kg bw/kg blood ≙ 0.012 mg/kg blood

With a dose (D) of 19 mg/kg bw the resulting c_{Max} is

$c_{Max} = CN_{Max} * D$

c_{Max} = 0.228 mg/kg blood

Taken into account a blood density of 1.06 kg/L the resulting c_{Max} is

c_{Max} = 0.242 mg/L

with a molecular weight of methiocarb of 225.3 g/mol the total maximum concentration of methiocarb in blood is:

c_{Max} = 1.07 µM

Abbreviations

CN = dose-normalized concentration

Report:	KCA 5.8.3/03 [redacted],; [redacted]; [redacted]; [redacted]; 2011; M-477708-01-1
Title:	Screening of 397 Chemicals and Development of a Quantitative Structure-Activity Relationship Model for Androgen Receptor Antagonism.
Report No.:	M-477708-01-1
Document No.:	M-477708-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Only results for MTC reported

I. Materials and methods

A. Materials

1. Test material: Methiocarb

Synonym: Mercaptodimethur

Source: Not reported

Other test substances: In total 397 chemicals (natural and synthetic hormones, pesticides, PCBs, PAHs, brominated flame retardants, heterocyclic amines, plasticizers and plast additives, food and cosmetic additives, antioxidants, plant compounds and others) were tested.

Description: Nor reported

Lot/Batch no: Not reported

Purity: Not reported

Source: Not reported

Stability of test compound: expiry date: not reported

2. Vehicle: Ethanol, or DMSO or dimethylformamid (DMF)
none of these solvents had an effects in this assay.

3. Test system:

Cells: CHO-K1

Cell description: Not reported

Source: [redacted], USA

Maintaining medium: DMEM/F12 (Gibco, UK), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B and 10 % FBS

Culture conditions: 37°C in a humidified atmosphere of 5%CO_2/air

B. Study design and methods

1. treatment:

AR transactivation assay (luciferase reporter assay):

The cells were seeded in white 96 well plates (PerkinElmer Life Sciences, Packard) at a density of 7000 cells/well in DMEM/F12 containing 10% charcoal-treated fetal bovine serum (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) and incubated at 37 °C in a humidified atmosphere of 5% CO_2/air. After 24 h, cells were transfected for 5 h with a total of 75 ng cDNA/well consisting of the expression vector pSVAR0 (human AR) and the MMTV-LUC reporter

	plasmid (gifts from Dr. Albert Brinkmann, Erasmus University, Rotterdam) in a ratio of 1:100 using 300 nL of the transfection reagent FuGene (Roche Diagnostics A/S, Hvidovre, Denmark). The ratio of DNA (µg) to Fugene (µL) was kept at 0.25. The test substances were tested in the presence of 0.1 nM R1881. After incubation for 20 h, cells were lysed by adding 20 µL well lysis buffer (25 mM triphosphate, pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM DTT, and 8 mM $MgCl_2$) followed by shaking at room temperature for 10 min.
Measurement of luciferase activity:	The luciferase activity was measured directly using a BioOrbit Galaxy luminometer by automatic injection of 40 µL of substrate containing 1 mM luciferin (Amersham Int., Buckinghamshire, United Kingdom) and 1 mM ATP (Boehringer Mannheim, Germany) in lysis buffer, and the chemiluminescence generated from each well was measured over a 1 s interval after an incubation time of 2 s. We determined cytotoxicity in parallel by transfecting cells with a plasmid (pSVAR13) encoding for a constitutively active AR, which lacks the ligand-binding domain (a gift from Dr. Albert Brinkmann, Erasmus University, Rotterdam). These experiments were designed exactly as was the AR reporter gene assay except that the ratio between pSVAR13 and MMTV-LUC was 2:100.
Cytotoxicity assessment:	Cytotoxicity was determined in parallel to the luciferase measurement by transfecting cells with a plasmid (pSVAR13) encoding for a constitutively active AR, which lacks the ligand-binding domain (a gift from Dr. Albert Brinkmann, Erasmus University, Rotterdam). These experiments were designed exactly as was the AR reporter gene assay except that the ratio between pSVAR13 and MMTV-LUC was 2:100.
Test substance concentrations:	1-3-10-30 µM
Positive controls:	R1881 (= methyltrienolone): 0.1 nM (NEN, Bosten, USA)
Negative (solvent) control:	Not specified for methiocarb
Internal quality control for anti-androgenic activity:	None
Replicates	Each test substance concentration tested in triplicate.
Evaluation:	All data were related to the response of 0.1 nM R1881, which was set to 100%.

Concentration-response analyses were performed, and the IC_{25}, that is, the concentration of test compound showing a 25% inhibition of the activity induced by 0.1 nM R1881, was calculated for each compound.
The criteria for determination of "a positive" was that a 25% inhibition of the 0.1 nM R1881-induced response should be reached at non-cytotoxic concentrations ≤10 µM.
Based on the obtained IC_{25} values the chemicals tested positive for AR-antagonism that were not cytotoxic were divided into potency classes as follows:

1 $IC_{25} \leq 0.1$ µM;
2 0.1 µM $< IC_{25} \leq 0.3$ µM;
3 0.3 µM $< IC_{25} \leq 1$ µM;
4 1 µM $< IC_{25} \leq 3$ µM;
5 3 µM $< IC_{25} \leq 10$ µM.

Statistics: Only for the QSAR model part.

II. Results and discussion

A. in vitro AR-assay

The presented data for the in vitro AR-transactivation assay consisted in total of 397 chemicals. However, only 295 of the chemicals were tested in the laboratory of the authors. Results from further 102 chemicals were obtained from an external laboratory. The results of the 295 chemicals tested internally are presented in the following. The chemicals were divided into the following chemical classes: natural hormones (seven chemicals, 71% positive), synthetic hormones and drugs (30 chemicals, 53% positive), pesticides (68 chemicals, 56% positive), PCBs (39 chemicals, 74% positive), PAHs (35 chemicals, 54% positive), brominated flame retardants (six chemicals, 83% positive), roast mutagens (nine chemicals, no positives), plasticizers and plast additives (13 chemicals, 31% positive), food additives and cosmetics (16 chemicals, 25% positive), antioxidants (10 chemicals, 20% positive), plant compounds (six chemicals, no positives), and a miscellaneous group of chemicals (56 chemicals, 23% positive). The numbers in parentheses refer to the total number of tested chemicals in each class and the percentage of positive compounds. In total, 45.7% of the chemicals were positive AR antagonists according to the defined criteria, 48.5% of the chemicals were negative, and 5.8% of all chemicals were cytotoxic.
A total of 4.4% of all tested chemicals were relatively potent with an $IC_{25} \leq 0.3$ µM (belonging to potency groups 1 and 2).

The results for methiocarb in comparison with some more or less potent chemicals are provided in the following table. It has to be noted that the anti-androgenic IC_{25} was only provided as range and not as a specific value for all the compounds.

Table 5.8.3/03- 1: Results for methiocarb in the in vitro AR-assay

Compound*	Tested concentrations (µM)	Anti-androgen IC_{25} range (µM)	Cytotoxic (yes/no)	Potency class**
4-androsten-3,17-dione	1-3-10-30	> 10	No	**neg**
Methiocarb	1-3-10-30	>3 ≤10	No	**5**
17α-estradiol	0.1-0.3-1-3	>0.1 ≤0.3	No	**2**
17β-estradiol	0.1-0.3-1-3	>1 ≤3	No	**4**
progesterone	0.1-0.3-1-3	>0.1 ≤0.3	No	**2**
hydroxyflutamide	0.01-0.03-0.1-0.3	≤0.10	No	**1**

* For comparison the very potent (hydroxyflutamide), medium potent (progesterone, estradiol) and negative (4-androsten-3,17-dione) substances tested in the *in vitro* AR-assay are presented.

** Positive potency classes:
1 $IC_{25} \leq 0.1$ µM; 2 $0.1 \mu M < IC_{25} \leq 0.3 \mu M$; 3 $0.3 \mu M < IC_{25} \leq 1 \mu M$;
4 $1 \mu M < IC_{25} \leq 3 \mu M$; 5 $3 \mu M < IC_{25} \leq 10 \mu M$.

It was stated that the selection of chemicals for the training set was biased, as most known AR antagonizing chemicals were included in the test.

III. Conclusion

Based on the presented results methiocarb can be considered to have weak AR-antagonizing activity in this AR-assay *in vitro*.

Bayer conclusion:
The publication describes the development of a QSAR model. For the development in total 397 chemicals of a broad range of chemical classes were screened in vitro for androgen-receptor (AR) antagonism. Since the QSAR model development is not relevant for the evaluation of methiocarb technical, only the results of the in vitro AR assay are considered here.
Due to the fact that the results were used for development and validation of a QSAR model, the assay results were only given as ranges which were then transferred into potency classes.
Based on the measured IC_{25} range of >3 and ≤ 10 µM, methiocarb can be considered to have weak AR-antagonizing activity according to the defined criteria.
However, comparing this concentration range to a maximum possible plasma concentration of 1.07 µM (please refer to the calculation provided with the assessment of KCA 5.8.1/18, M-495823-01-1 presented above) that can be reached in vivo after a lethal oral dose of methiocarb (i.e. 19 mg/kg bw/day = LD_{50}) the observed weak antagonistic effect is biologically not relevant for humans.
In addition, when compared to potent AR antagonist like hydroxyflutamide or 17β-estradiol with 100- and 10-times lower IC_{25} ranges, the weak AR-antagonizing effect of methiocarb in vitro is of no relevance the exposure situation in vivo.

The reliability assessment is given below.

Klimisch evaluation

Reliability of study:	**Reliable with restrictions (Klimisch code 2)**
Comment on reliability:	Non-GLP study, conducted according to scientific principles with reporting and methodological deficiencies. • Methiocarb not obtained from BCS (impurity and purity not reported). • No internal quality control for AR-antagonistic activity included
Relevance of study:	**Due to the (large) differences between the determined IC_{25} values and the maximum total plasma concentration that can be reached *in vivo* after a lethal dose of methiocarb the result of the *in vitro* AR assay is of no relevance for the human risk assessment of methiocarb.**

CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Medical surveillance data contain confidential business information. Please refer to Document JCA 5.9.1.

CA 5.9.2 Data collected on humans

There is only one single report in literature about a forensic examination of an elderly lady, who committed suicide with methiocarb. Concentrations in heart blood were 4 µg/ml, and 6,100 µg/g stomach content.

Report:	[redacted] 6; [redacted]; [redacted]; [redacted]; [redacted] ;2009; M-505200-01-1
Title:	Suicidal poisoning with mercaptodimethur -morphological findings and toxicological analysis
Report No.:	M-505200-01-1
Document No.:	M-505200-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

CA 5.9.3 Direct observations

See CA 5.9.2.

CA 5.9.4 Epidemiological studies

No epidemiological studies have been reported or published up to now.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

Methiocarb and its metabolites can by analysed, but in regard of the speed of manifestation and decrease of toxicity signs this does not make sense. Even acetylcholinesterase measurements take too long.

Methiocarb (=MIPC) is a carbamate insecticide.

Signs and Symptoms of Poisoning:

Carbamate insecticides inhibit esterases in the organism, the key enzyme inhibited and accounting for signs and symptoms is the acetylcholinesterase (AChE). Thus the mechanism of action is similar to organophosphate insecticides, but carbamates do not bind irreversibly to AChE, and their effect is much shorter.

The inhibition of AChE leads to the accumulation of the neurotransmitter acetylcholine in the central and peripheral nervous system both at the nicotinic and muscarinic receptors, resulting in an endogenous acetylcholine intoxication with the following signs and symptoms:

Organ	Nicotinic	Muscarinic
Eye		Miosis (pinpoint pupils), lacrimation (watering of the eyes), vision problems
Mouth		Salivation
Heart/circulation		hypotension, bradycardia
Lung		Bronchial secretion, bronchospasm
Gastrointestinal tract		Nausea, vomiting, diarrhea
Skin		Sweating
Muscles	Fibrillation, tics, myoclonus, paralysis of respiratory muscles, peripheral respiratory failure	
Central nervous system	Somnolence, coma, central respiratory depression and failure, hypothermia, convulsions	

There are indications that symptoms in – small – children may be different to those in adults with a preponderance of CNS depression and less specific symptoms.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

First Aid:

- -Remove patient from exposure/terminate exposure under self-protection (e.g. long gloves)
- -Thorough skin decontamination with copious amounts of water and slightly alkaline soap/detergent
- -Flushing of the eyes with lukewarm water for 15 minutes

- -Whilst induction of vomiting may be useful for the active ingredient in case of ingestion, it is strictly forbidden, if a formulation containing solvents has been swallowed. Induction of vomiting should only be considered if a significant amount has been swallowed (more than a mouthful), if the ingestion was less than one hour ago, and if the patient is fully conscious. Induced vomiting can remove maximum 50% of the ingested substance.

Treatment:

In regard of the high toxicity treatment should be started as quickly as possible.

- -In case of ingestion a gastric lavage within the first hour after ingestion and after intubation only with consecutive application of activated charcoal and sodium sulphate should be performed, if a significant amount has been swallowed.

- -Before treatment is started, either clear symptoms of carbamate insecticide poisoning as described above should be present or a reduction of cholinesterase activity to below 30% of normal should be present.

- -The following antidote is generally accepted: atropine (see below).

- -Additionally a benzodiazepine (e.g. diazepam) should be given in case of seizures/convulsions according to standard regimens.

- **-Atropine:**

  Atropine will counteract only the muscarinic symptoms.

  2 regimens for initial atropine treatment are currently suggested, in both cases the cessation of the cholinergic symptoms salivation, bronchial secretion, sweating and bradycardia indicates sufficient atropinization. The skin should be dry, the lungs should be clear on auscultation and the heart rate should be in a range of 80 to 100/minute.
  Overdoses of atropine have to be strictly avoided, as these can promote heart rhythm disturbances (torsades des pointes).

  Adults:

  Regimen 1:
  2-10 mg atropine i.v., followed every 15 minutes by 2 mg atropine i.v. until cessation of the symptoms as above.

  Regimen 2:
  2 mg atropine i.v., 5 minutes wait, if symptoms persist or reappear
  4 mg atropine i.v., 5 minutes wait, if symptoms persist or reappear
  8 mg atropine i.v., 5 minutes wait, if symptoms persist or reappear
  16 mg atropine i.v., 5 minutes wait, if symptoms persist or reappear
  32 mg atropine i.v.

  No higher doses of atropine should be given nor are necessary.
  It is mandatory to allow 5 minutes after each dose for atropine to become fully effective. The next higher dose must not be given earlier and only if the above symptoms are persisting.

  Regimen 2 currently is advisable.

Further atropine treatment should be done by continuous application of 1 – 2 mg/hour. Atropine treatment can be stopped, when the plasma cholinesterase level has returned to above 30% of normal.

Children:

For children the dosage has to be more careful due to a higher sensitivity of children to atropine. The initial dose should be 0.1 mg/kg body weight, then careful repletion or increase depending on the reversal of symptoms as described above.
Both giving too much and too little atropine should strictly be avoided, the dosage should be adjusted to the signs of esterase-inhibitor poisoning.

Note: Oxime therapy has not been convincingly shown to have additional value in the treatment of carbamate poisoning. On the other hand – with the one possible exception of carbaryl – it has been shown not to have negative effects. Thus in cholinesterase-inhibitor poisoning with an unknown substance, oxime therapy is recommended.

CA 5.9.7 Expected effects of poisoning

No data on survived poisonings are available, but carbamates are not expected to cause delayed or persistent effects of poisoning.

Overall conclusions

Acute toxicity

Methiocarb was very toxic after acute oral and inhalation exposure rats, but displayed a low toxicity after dermal exposure to rats and rabbits. The symptoms of intoxication in mammals were cholinergic effects typical of carbamates and included trembling, cramps, muscular fasciculations, salivation, diarrhoea, vomiting and ataxia. The symptoms at sublethal doses diminished rapidly.

Methiocarb did not cause skin or eye irritation in rabbits, and was not sensitising to the skin of the guinea pig in tests by the Buehler method and by the Magnusson and Kligmann method.

An *in vitro* 3T3 NRU phototoxicity test did not give any indication for a phototoxic potential of methiocarb.

Furthermore, methiocarb showed no phototoxic potential in an *in vitro* 3T3 NRU phototoxicity test.

Short-term toxicity

Short-term dietary toxicity studies were conducted in rats and dogs. Dermal route and inhalation route studies were performed in rabbits and rats respectively.

It has to be noted that most of the short-term toxicity studies with methiocarb were pre-GLP and not according to current guidelines. However, the 13-week rat study and the 3-month dog study are according to GLP and according to currently valid guidelines. These two studies cover all the required parameters for the assessment of short-term toxicity.

The target effects are cholinesterase inhibition and reduction of body weight gain.

In rats, biologically significant reduction in cholinesterase in the submaxillary glands and plasma were observed at 10 ppm and above in a 16 week study. There were also changes of erythrocyte and brain cholinesterase, but these were not considered toxicologically significant. Sub-chronic dietary exposure caused body weight reductions that were associated with sporadic findings in clinical chemistry and changes in erythrocyte parameters of uncertain toxicological significance at 900 ppm. Biologically significant cholinesterase inhibition was not observed at dose levels of ≤ 900 ppm (67.6 mg/kg bw/day in males or 90.7 mg/kg bw/day in females) in this study. The NOAEL was 100 ppm (equivalent to 7.34 mg/kg bw/day in males and 10 mg/kg bw/day in females). The observation of low cholinesterase inhibition was noted to be inconsistent with findings in other dietary studies in rats. However, it has to be considered that rats were not fasted prior to blood sampling for cholinesterase assays.

Sub-acute inhalation exposure study to rats caused significant reduction in plasma and brain cholinesterase activities at dose levels of ≥ 23 mg/m³.

In dogs, cholinesterase inhibition, clinical signs and reduction of body weights were observed. In a 90-day dietary study in dogs erythrocyte and retinal cholinesterase was reduced, while there was no effect on brain cholinesterase inhibition. Clinical signs consisted of an increased incidence of vomiting. There was no evidence of functional impairment in a functional observation battery of tests. In a two year feeding study beagle dogs displayed impaired feed consumption, vomiting and clinical signs including mild weakness in the hind limbs, trembling and slightly reduced alertness suggestive of significant cholinesterase effects.

In rabbits, dermal exposure to methiocarb resulted in reduced food consumption which were associated with reductions in plasma cholinesterase activity at dose levels > 150 mg/kg bw/day. In a further study, the only observed effects at the 500 mg/kg bw/day dose in New Zealand White rabbits were reduction in body weight, reduction in food consumption and mild behavioural changes. Reductions in plasma cholinesterase at the 500 mg/kg bw/day dose level were very slight and not of biological significance.

The lowest relevant NOAEL for short term toxicity is 1.33 mg/kg bw/day based on cholinergic signs, reduction in body weight gain, and erythrocyte cholinesterase inhibition and retinal cholinesterase inhibition observed in the 90-day dog study.

Genotoxicity testing
Methiocarb was tested in in vitro and in vivo genotoxicity studies. Methiocarb was not mutagenic in studies in vitro including the bacteria/mammalian microsome assay (Ames test) for point mutations in histidine auxotrophic strains of Salmonella typhimurium; the HGPRT assay for forward mutations in cultured Chinese hamster ovary) (CHO) cells; the Pol test in Escherichia coli for the potential to induce chromosomal damage; the unscheduled DNA synthesis assay rat primary hepatocytes; and the Sister chromatid exchange (SCE) assay in CHO cells.
Methiocarb was clastogenic at high concentrations in the in vitro chromosomal aberration assay in CHO cells. However, the positive result was not confirmed in a micronucleus test in vivo that was re-evaluated taken into account a factor of two for counting the micronucleated PCEs. This was done to compensate that only 1000 PCEs were evaluated in the assay.
Furthermore, there was no evidence of genotoxicity in other in vitro and in vivo studies sensitive to potential clastogenic compounds. Carcinogenicity was not observed in chronic studies in the rat and mouse. In addition, methiocarb was not mutagenic in the in vivo mouse dominant lethal test for germ cell chromosomal and dominant gene mutations.

Overall, based on the evaluation of the results of all *in vitro* and *in vivo* genotoxicity studies methiocarb is considered to have no genotoxic / mutagenic potential.

Long-term toxicity
Long-term studies have been conducted in the rat and mouse.

In rats the main effect was a significant reduction of body weights at the high dose of 600 ppm (3.3 / 4.98 mg/kg bw/day (males / females)). The NOAEL was 200 ppm (9.3 / 13.89 mg/kg bw/day (males / females). Plasma, erythrocyte and brain cholinesterase activities were not significantly depressed at the tested dose levels.

In mice the main effect was cholinesterase inhibition as well as changes in alanine aminotransferase activity. The NOAEL in the 24-month feeding study in mice was 67 ppm (19.8 mg/kg bw/day) based on changes in alanine aminotransferase and transient but biologically relevant reductions of plasma and brain cholinesterase activities at 200 ppm (57 mg/kg bw/day).

In both studies in rats and mice there were no treatment-related increases in incidences of benign and malignant tumours.

Overall methiocarb was not carcinogenic in mice and rats.

Reproductive toxicity
No structural malformations were observed in any of the reproductive and developmental toxicity studies on methiocarb. In the different generational studies on methiocarb findings indicative of reproductive / developmental toxicity, i.e. increased prenatal loss, reduced litter size, reduced viability, and a reduced lactation index, were observed. However, these findings were not consistently observed in all studies, generations and at comparable dose levels.

Prenatal loss
Only marginally increased incidences of prenatal loss close to the upper higher limit of the historical control data were observed at 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder as well as at 500 ppm or 89 mg/kg bw/day in the 2nd generation of the second two-generation study. All other values in the dose range finder as well as in all generations of the first two-generation study as well as in the 1st generation of the second two-generation study with doses up to 83.9 mg/kg bw/day

were in the range of the historical control data. Since this borderline finding was not consistent over generations and doses, it is not considered to fulfil the requirements for a clear treatment related effect.

Litter size

Mean litter size was also marginally reduced at 150 and 500 ppm or 15.4 and 52.1 mg/kg bw/day in the 1st generation of the first two-generation study as well as at 500 ppm or 89 mg/kg bw/day in the 2nd generation of second two-generation study. All three values were close to the lower limit of the historical control values indicating maximally a borderline effect. Furthermore, no effect on litter size was observed in the 2nd generation (F2 and F2b) of the first two-generation study as well as in the 1st generation of the second two-generation study up to the high dose of 500 ppm. Apart from these three borderline cases a slight reduction in litter size (below the lower limit of the historical controls) was only seen at 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder. However, the fact that no effect was seen at the higher dose of 500 ppm or 83.9 mg/kg bw/day in the first generation of the second two-generation study shows that also a possible marginal effect on litter size is not consistent over doses and generations and, as such, no clear treatment related effect.

Viability

A marginal reduction of pup viability on lactation day 4 (LD 4) was observed at the high dose of 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder. However, the value was at the lower limit of the historical control data (i.e. 87.4%). In the 2nd two-generation study there was no effect on pup viability at all up to and including to the high dose of 500 ppm or 89 mg/kg bw/day. Based on these data it is improbable that there is a treatment related effect of methiocarb on pup viability. In the 1st two-generation study there was also no effect on pup viability in the F1 and F2 pups. In F2b pups, however, reduced viability indices were noted for all dose groups including controls. Although there seemed to be a very slight dose relationship, it is not clear, if in this single case an additional influence, i.e. a (subclinical) infection of the pups or an unspecific reduction in nursing capability in the F1 animals, contributed to this finding. It is noticeable that pups in these groups displayed more frequently severe clinical signs indicative of a poor general condition. They were thin and had a food pasted mouth/nose and empty stomach (no milkspot). Taken all these facts together, an effect of methiocarb alone on pup viability on lactation day 4 is not probable due to the missing consistency over doses and generations.

Lactation index

The lactation index was reduced at the high dose of 900 ppm or 76.5 mg/kg bw/day in the one-generation dose range finder with a value of 71.43% below the lower limit of the respective historical control data (for the 1st generation: 85.1 - 100%, for the 2nd generation: 51.7 -100%). The parameter was also statistically significantly reduced in the 1st generation of the 2nd two-generation study at the high dose of 500 ppm or 83.9 mg/kg bw/day with a value of 53.1 %. All other values in both studies including the lactation index of the 2nd generation of the second two-generation study at 500 ppm or 89 mg/kg bw/day of 75.93% were in the range of the respective historical control data.

In the 1st generation of the first two-generation study the lactation index was in the range of the historical control values. However, starting with the F2 pups the lactation index was overall low in F2 and F2b pups in the first two-generation study. Values below the lower limit of the historical controls (51.7 – 100%) were noted in F2 pups at 50 and 500 ppm (with 46.23 and 42.7%) as well as in all doses including controls in F2b pups (46.23 – 37.3 – 34.04 – 28.68 % at 0 – 50 – 150 – 150 ppm (0 – 6.0 – 18.6 – 61.3 mg/kg bw/day)). Pups in these groups displayed more frequently severe clinical signs indicative of a poor general condition (thin pups, pups with a food pasted mouth/nose and empty stomach (no milkspot)). With also the control groups showing low viability (F2b) and lactation indices in the F2 and F2b pups, it is possible that other influences like for example a (subclinical) infection of the young pups or an unspecific reduction in nursing capability in the F1 animals may have contributed to pup deaths. Since a clear treatment related effect cannot be derived from these data, the lactation indices of the 2nd generation of the first two-generation study should not be taken as the basis for the evaluation of a compound related effect.

Overall, there seemed to be a limited effect on the lactation index at the high maternally toxic dose of 83.9 mg/kg bw/day in the 1st generation of the second two-generation study. However, again, this finding was not consistent between doses and generations (i.e., there was no effect at 89 mg/kg bw/day in the 2nd generation of the second two-generation study).

The overall NOAEL for parental toxicity, reproduction and offspring toxicity was 50 ppm (equivalent to 4.3/5.5 mg/kg bw in males or females).

In conclusion, neither of the possible effects on prenatal loss, litter size, pup viability on lactation day 4 or the lactation index was consistently observed over doses and generations. Therefore, a specific effect of methiocarb on these parameters can be ruled out. What could be possible is that methiocarb contributes to pup death as a secondary, non specific consequence of maternal toxicity.

Developmental toxicity studies were conducted in rats and rabbits.
In a developmental toxicity study in FB30 strain rats, the NOAEL for maternal toxicity was 3 mg/kg bw/day based on reduction of maternal body weights and for fetotoxicity and teratogenicity at 10 mg/kg bw/day the highest test dose.

In a subsequent developmental toxicity study in Wistar rats the maternal NOAEL was 0.5 mg/kg bw/day based on cholinergic signs, muscle fasciculations at 1.5 mg/kg bw/day. The NOAEL for developmental toxicity in rats was 5.0 mg/kg bw/day based on the absence of foetotoxicity or teratogenicity at the highest dose tested.

In a developmental toxicity study in New Zealand White rabbits, the NOAEL for maternal toxicity was 3 mg/kg bw/day based on observed clinical signs at the top dose level and for fetotoxicity and teratogenicity at 10 mg/kg bw/day the highest test dose.

In a dermal route developmental toxicity study in Chinchilla rabbits, the NOAEL was 50 mg/kg bw/day for maternal and foetal toxicity based on reduction in food consumption and body weight gain in dams and birth weight in foetuses.
In this study there was an increased incidence of delayed ossification and non-ossification of the number 5 sternebrae noted (litter basis) that was considered treatment-related. However, a comparison with historical control data on a fetal basis (report 2nd addendum page 7) demonstrated that the incidence of non-ossification, as well as delayed ossification is well within the historical control range. Therefore, the finding is considered to be incidental. Overall, there was no teratogenicity at the highest test dose (250 mg/kg bw/day).
Overall, the NOAELs in the rat were 0.5 mg/kg bw for maternal toxicity and 5 mg/kg bw for developmental toxicity, while in the rabbit they were 3 mg/kg for maternal toxicity and 10 mg/kg bw for developmental toxicity.

Methiocarb is not a primary reproductive toxin.

Neurotoxicity studies
No specific acute and repeated dose neurotoxicity studies are available for methiocarb.

However, methiocarb is a carbamate and as such an inhibitor of acetylcholinesterase, which acts on the nervous system of insects and vertebrates as well. Carbamates reversibly inhibit the enzyme acetylcholinesterase (AChE), which is responsible to hydrolyze the neurotransmitter acetylcholine (ACh) at the postsynaptic membrane. Inhibition of AChE leads to accumulation of ACh in synapses and neuromuscular junctions, resulting in muscarinic and nicotinic symptoms. At the muscarinic nerve endings this excites increased tear flow and salivation, increased bronchial secretion, bronchospasms

(leading to dyspnoea), increased gastric and intestinal secretion, contractions and spasms (leading to colic, vomiting, diarrhoea), myosis of the eye and a decrease in blood pressure caused by bradycardia and a decreased tonus of the blood vessels. At the vegetative ganglia and at the neuromuscular plate excess of ACh causes nicotinergic effects like stiffness of muscles in face and neck, tremor, muscle fasciculations, tonic-clonic cramps. Furthermore, after crossing of the blood-brain barrier, also central effects like seizures, respiratory depression and CNS depression occur.
The typical clinical signs, mortality at higher doses, as well as acetylcholinesterase inhibition were observed in almost all of the available acute and repeated dose toxicity studies with methiocarb. The measurements of AChE inhibitions in rat (gavage) studies revealed peak effects at 20 min for plasma and RBC AChE inhibition, and at 2 h post-dosing for brain AChE inhibition. Recovery to control levels was within 6 to 24 h after dosing.
In dog (capsule) studies of AChE inhibition peaked 1 hour after application, and resolved within 5 hours after application.

However, detailed assessments of neurotoxicity were performed in two sub-chronic toxicity studies in rats and dogs. These included neurobehavioral investigations (i.e. functional observational battery (FOB) investigations including observations in the home cage, during handling, in the open field, and manipulative tests in rats, and neurological examinations consisting of the assessment of mental status/behaviour, gait characteristics, postural status and reactions, and spinal/cranial reflex tests in the dog. These investigations, except that there was **no automatic** assessment of motor activity in the rat study, and no *in situ* fixation for histopathology, and no histopathological examination of some PNS tissues (dorsal root ganglia, dorsal and ventral root fibres proximal tibial nerve (at knee), tibial nerve calf muscle branches, skeletal muscle (calf muscle)), fully cover the investigations of acute and sub-acute neurotoxicity screening studies according to OECD guideline 424.

In the 13-week dietary study in Wistar rats there were no treatment-related mortalities and no clinical signs of toxicity observed at any dose level up to and including 900 ppm (67.2 / 96.7 mg/kg bw/day (males / females). Body weight gain was decreased in females from 300 ppm and above and in males at 900 ppm (up to 21% and 29% for males and females, respectively). General behaviour, posture and respiration were normal at any dose level. Functional observational battery (FOB) tests provided no indications for treatment-related effects in both sexes up to the highest dose tested (i.e. 900 ppm). AChE activity measurements revealed a slight, not biologically significant reduction of brain AChE activity (–9%) in high dose females at the end of the treatment period.
Grip strength measurements performed at the termination of the study showed in male rats relative changes in forelimb grip strength of –10%, –23%, and –14% (increasing dose levels) and for hind limbs of –4%, –25%, and –14%. In females relative changes in forelimb strength were –9%, –15, –29%, and for hind limbs –1%, –3%, –17%. At the end of the recovery period the relative changes were –4% in males for forelimbs and hind limbs, and in females –2% for forelimbs and 0% for hind limbs. These not clearly dose-related effects occurred at dose levels of ≥ 300 ppm which have to be regarded as close to or already above the maximum tolerated dose (MTD). Therefore, the observed changes in grip strength were not regarded as specific neurotoxic effects. Overall, except for the slight AChE inhibition in brain in the high-dose group, no specific dose-related neurotoxic effects were observed in both sexes up to 900 ppm.

In the 90-day dietary study in beagle dogs with doses of 0, 10, 50, and 250 ppm increased incidence of vomiting was observed at the highest dose group, while no other relevant dose-related clinical signs were observed. Also in the high-dose group food consumption and body weight gain was reduced. Plasma and RBC cholinesterase activities were statistically and biologically relevant decreased at the high dose, while there was no decrease of the brain AChE. Neurological examination of mental status / behaviour, gait characteristics, postural status and reactions, and spinal/cranial reflex tests conducted on all animals by a veterinarian at pre-treatment and just prior to termination revealed no treatment-related neurological defects.

In addition, there are also acute and sub-acute studies in rat and dog available which were conducted for the determination of AChE inhibition.
After acute exposure rats exhibited typical signs of AChE inhibition (trembling, cramps) at methiocarb doses of ≥ 10 mg/kg bw. The symptoms appeared within 5 min to 10 min after application, and were resolved 2 h later. The maximum dose-related levels of cholinesterase inhibition were recorded 20 minutes after application of ≤25 mg/kg bw methiocarb, and 20 minutes to 2 hours after application of 50 mg/kg bw. Brain AChE inhibition peaked after 2 hours at dose levels of 10 and 20 mg/kg bw methiocarb. Thereafter, brain AChE activity increased again.
In the sub-acute experiments rats exhibited biologically significantly decreased AChE 20-30 min after gavage application of 2 or 10 mg/kg bw. Brain AChE was also inhibited at 10 mg/kg bw two hours after application.
In dogs 29-day capsule treatment with methiocarb at 0.05 and 0.5 mg/kg bw/day caused signs of toxicity and significant AChE inhibition in plasma and RBC at 0.5 mg/kg bw/day. Maximum depression of AChE was observed 1 to 3 hours after application, with full recovery within 6 hours.

Taken all this together the main neurotoxic effect of methiocarb is AChE inhibition of plasma, RBC and brain. Clinical signs indicative of neurotoxicity are cholinergic signs like tremors, salivation, lacrimation, decreased locomotor- and motoractivity, decreased grip strength muscular fasciculations were observed after acute and repeated exposure, but there was no cumulative effect on the AChE activity observed. In addition, in none of the studies with methiocarb there were any histopathological findings indicative of neurotoxicity observed.
Except for the inhibition of AChE and correlated symptoms, there are no further signs indicative of neurotoxicity observed in any of the toxicity studies. The subchronic rat and dog studies cover all neurobehavioral investigations that are necessary for neurotoxicity screening. It is concluded that the neurotoxic potential of methiocarb is adequately assessed in the available toxicity studies.

Methiocarb did not show any evidence of a potential to cause **delayed neurotoxicity** in strains of domestic hens. The acute oral LD_{50} in White Leghorn hens was 380 mg/kg bw. Pre-treatment with atropine sulphate provided protection against the neurotoxic effects and hens survived repeated dosing at the 380 mg/kg bw dose level.

To assess that there are no further neurotoxic signs to be expected after methiocarb exposure, publically available data of 9 further N-methylcarbamates (carbofuran, oxamyl, methomyl, pirimicarb, thiodicarb, carbaryl, formetaneate, aldicarb and propoxur) were assessed. All these N-methyl carbamates showed the same or comparable effects like methiocarb. They inhibited AChE in plasma, RBC and brain. The peak of the AChE inhibition was shortly after application (i.e. 15-30 min post dosing). Full recovery was in general within 24 hours. Also due to the AChE inhibition, reduced locomotor, and motoractivity was observed in parallel to the AChE inhibition. Available FOB evaluations revealed besides the mentioned effects also decreases of fore and hind limb strength, reduced extensor trust, tail and toe pinch response, and increased hind limb splay. Like for methiocarb, there were also no histopathological neurotoxicity findings observed for these N-methylcarbamates. Furthermore, none of these carbamates induced delayed polyneuropathy in hens.

Overall, except for the designated effect of methiocarb to inhibit AChE activity, with related cholinergic signs, there is no evidence for treatment-related neurotoxic effects. Since the AChE inhibition is only transient (i.e. reversible), and not accumulative, it can be concluded that methiocarb exhibits no potential to permanently impair the central or peripheral nervous system (i.e. methiocarb does not induce significant functional changes in the central or peripheral nervous system, signs of CNS depression, effects on the senses (sight, hearing, smell), and histopathological damage to the brain). In addition, methiocarb did not induce delayed polyneuropathy.

Toxicity studies on metabolites

During the previous EU review, the toxicological properties of several animal, plant and/or soil/groundwater metabolites (methiocarb sulfoxide (M01), methiocarb sulfone (M02), methiocarb phenol (M03), Methiocarb sulfoxide phenol (M04), Methiocarb sulfone phenol (M05), N-hydroxymethyl methiocarb (M06), N-hydroxymethyl methiocarb sulfone (M07) and N-hydroxymethyl sulfoxide (M08)) had already been evaluated based on studies on acute oral toxicity in rats. For methiocarb sulfoxide (M01) sub-acute studies in rats and dogs were also evaluated.

In addition, new studies on methiocarb phenol (M03), Methiocarb sulfoxide phenol (M04), Methiocarb sulfone phenol (M05),) on acute oral toxicity, and mutagenicity *in vitro* (bacterial reverse mutation assay (Ames test)) were conducted.

The results of all available studies on the above mentioned metabolites are provided in the following paragraphs.

Methiocarb sulfoxide (M01)

The animal, plant and soil/groundwater metabolite methiocarb sulfoxide is more toxic than the parent compound and is also a more potent cholinesterase inhibitor.

Methiocarb sulfone (M02)

The plant, animal and soil metabolite methiocarb sulfone (M02) is of less acute toxicity than the parent compound methiocarb.

Methiocarb phenol (M03)

The animal, plant and soil/groundwater metabolite methiocarb phenol (M03 has a lower acute oral toxicity than the parent compound methiocarb. There were no mortalities, clinical signs or symptoms of AChE inhibition observed at an acute dose of 2000 mg/kg bw. Furthermore, methiocarb phenol (M03) was negative for point mutations in the bacterial reverse mutation test.

In addition, one publication emerged from public literature from 2011 that described estrogenic activity in two *in vitro* test systems. The publication based on non-GLP studies, which due to methodically deficiencies and large differences between the determined effect concentrations and maximum plasma concentrations that can be obtained after non-lethal doses *in vivo* are considered to be not relevant.

Methiocarb sulfoxide phenol (M04)

The rat, plant, animal, soil and groundwater metabolite methiocarb sulfoxide phenol (M04) has a lower acute oral toxicity than the parent compound methiocarb. After an acute oral dose of 2000 mg/kg bw, no mortality, clinical signs or symptoms of AChE inhibition were observed. Furthermore, methiocarb sulfoxide phenol (M04) gave a clear negative result for point mutations in the bacterial reverse mutation test.

Methiocarb sulfone phenol (M05)

The rat, plant, animal, soil and groundwater metabolite methiocarb sulfone phenol (M05) has a lower acute oral toxicity than the parent compound methiocarb. In addition, methiocarb sulfone phenol (M05) was negative for point mutations in the bacterial reverse mutation test.

N-hydroxymethyl methiocarb (M06), N-hydroxymethyl methiocarb sulfone (M07) and N-hydroxymethyl sulfoxide (M08)

For the three metabolites N-hydroxymethyl methiocarb (M06), N-hydroxymethyl methiocarb sulfone (M07) and N-hydroxymethyl sulfoxide (M08) acute oral LD_{50} values of > 112, >112 and >160 mg/kg bw compared to 33 and 47 mg/kg bw in males and females for methiocarb technical were observed.

Supplementary studies on the active substance

Supplementary studies for methiocarb were done for specific examinations on cholinesterase inhibition, antidote effects and potentiation.

Studies on cholinesterase inhibition

The mode of action of methiocarb is inhibition of the AChE. Therefore, measurements of AChE inhibition in plasma, RBC and brain were conducted in most of the repeated dose toxicity studies. A specific study for AChE inhibition after acute and repeated 28-day exposure to methiocarb was also conducted.

In the acute study typical symptoms of AChE inhibition (trembling, cramps) were observed at ≥ 10 mg/kg bw. The symptoms appeared within 5 min to 10 min after application, and were reserved 2 h later. The maximum dose-related levels of cholinesterase inhibition were recorded 20 minutes after the application in the dose groups of 25 mg/kg bw and below, and 20 minutes to 2 hours after application in the highest dose group. Two hours after the application, a marked increase in enzyme activity was already noted again in all dose groups except the highest one. In the lower dose groups AChE was only slightly, and not biologically significant (<20%) reduced. The brain AChE inhibition peaked after 2 hours at dose levels of 10 and 20 mg/kg bw methiocarb. Thereafter, brain AChE activity increased again.

In the sub-acute experiment rats (28-day gavage) plasma and RBC AChE was biologically significantly decreased at the highest tested dose of 10 mg/kg bw 20 min after application. On after the final application AChE was still slightly but not biologically relevant reduced (i.e. inhibition was <20%). Brain AChE was also inhibited at 10 mg/kg bw two hours after application. In other dose groups brain AChE was slightly reduced (not biologically significant). Cumulative cholinesterase inhibition was not observed during the 4 week treatment.

In another sub-acute study (4-week, gavage) a significant reduction of plasma AChE was observed during the first three weeks. At 2 mg/kg bw/day a significant reduction of plasma AChE was observed during the first three weeks. RBC AChE was reduced only during the first week. There was a decreasing effect with increasing duration of treatment observed for the plasma AChE. Recovery to normal activity was within four hours after treatment.

In a 29-day study dogs received daily applications of methiocarb at 0.05 and 0.5 mg/kg bw/day by capsules. Signs of toxicity and significant AChE inhibition in plasma and RBC were observed at 0.5 mg/kg bw/day. Maximum depression of AChE was observed 1 to 3 hours after application, with full recovery within 6 hours.

Antidote studies

In investigations of antidotal effects in rats, the acute oral LD_{50} of methiocarb was 67 mg/kg bw (without antidote); 367.5 mg/kg bw (with atropine), 158 mg/kg bw (with PAM), 225 mg/kg bw (with BH6), 497.5 mg/kg bw (atropine + PAM) and 512.5 mg/kg bw (atropine + BH6). Atropine was shown to provide significant antidotal protection against the cholinergic effects of methiocarb. PAM and BH6 did not provide significant antidotal effects. Atropine sulphate also provided greater antidotal effect than TEAC. The acute oral LD_{50} of methiocarb was 104.5 mg/kg bw (without antidote); 415 mg/kg bw (with TEAC); 641 (with atropine sulphate); and 580 (with atropine sulphate + TEAC).

Potentiation studies

In studies for potential potentiation effects of methiocarb with other anticholinesterase insecticides, there was no evidence for synergistic effects.

Publications

In addition, seven articles were published between 2009 and 2013. In four publications the potential of methiocarb to cause oxidative damage in rats after acute and repeated *in vivo* exposure was evaluated. The other three publications reported investigations on binding activities to three receptors (i.e. PPAR, AhR and PXR).

Three of the four publications that investigated the potential of methiocarb to cause oxidative damage had different deficiencies and are considered to be not reliable and therefore not relevant.

The fourth publication on this topic had also some deficiencies and was assessed to be reliable with restrictions. This publication provides supplemental information on potential oxidative stress induced by methiocarb treatment, but has no influence on existing endpoints and the risk assessment for methiocarb.
The three publications providing information on *in vitro* activation / binding activities of methiocarb to three different receptors based all on non-GLP studies that were conducted according to scientific principles. Due to methodological and reporting deficiencies there were assessed to be reliable with restrictions. They provide supplemental information that has no influence on existing endpoints and do not lead to a more conservative risk assessment.

Endocrine disrupting properties

No specific studies on endocrine disruption were conducted with methiocarb. However, a review of the whole data base on methiocarb was conducted to identify possible effects of methiocarb on endocrine organs, tissues or parameters. There were no indications of an endocrine disrupting potential in any of the submitted toxicity studies of methiocarb. Specifically there were no effects on endocrine organs, as well as no indications for a carcinogenic and reproductive toxic potential observed for methiocarb.

In addition, three publications emerged from public literature between 2009 and 2011, which describe potential endocrine effects of methiocarb in different test systems *in vivo* and *in vitro*. Due to methodological and reporting deficiencies the publications were considered to be reliable with restrictions.
One publication described a non-dose-related increase of the testicular ERα protein expression based on Western-blot analysis of testicular tissues obtained after repeated i.p. applications of low doses methiocarb to mice. Since there were no correlated effects on sperm concentration or sperm quality the results are considered as supplemental information that does not change existing endpoints or lead to a more conservative risk assessment.
The other two publications described anti-androgenic activities of methiocarb *in vitro*. The tested concentrations in these *in vitro* assays are not comparable to *in vivo* concentrations that can be reached in blood at dose levels that cause (toxic) effects. Therefore, the results are of no relevance for the human risk assessment of methiocarb.

In the following table the relevant studies used for derivation of reference values are summarised.

Table 5.10- 1: Summary of relevant toxicity studies for deriving regulatory reference doses

Study Doses tested	Sex	NO(A)EL	LO(A)EL	Main findings observed at LO(A)EL	Reference
		(mg/kg bw/day)			
Rat 4-week, oral (gavage) 0-3-10 mg/kg bw/day	M F	3 3	10 10	↓ of plasma, erythrocyte and brain AChE activity; cholinergic symptoms, No accumulation of AChE inhibition	[redacted]., 1973 M-009378-01-1
Rat 4-week,oral (gavage) 0-0.5-2.0 mg/kg bw/day	F	0.5	2	Tremors (during the first days only), ↓ (> 25%) of plasma and erythrocyte AChE activity (during the first week only)	[redacted] 1981 M-009348-01-1
Dog 29-day, oral (capsule) 0-0.05-0.5 mg/kg bw/day	M F	0.05 0.05	0.5 0.5	Cholinergic signs, slight ↓ of erythrocyte AChE activity (2 h post application)	[redacted] 1981 M-009577-01-1

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
Rat 16-week, oral (diet) 0-5-10-50 ppm	M F	1 1 (10 ppm)	(50 ppm)	↓ of AChE activity in submaxillary glands with significant ↓ of plasma AChE activity (both sexes)	[redacted] 1962 M-016133-01-1
Rat, 13-week + 4 weeks recovery, oral (diet) 0-100-300-900 ppm 0-7.34/10.0-22.72/30.71-67.59-90.74 mg/kg bw/day (m/f)	M F	7.34 10.0 (100 ppm)	22.72 30.71 300 ppm	≥ 300 ppm: stat. sign. ↓ glucose (m), ↓ urine volume, ↑ absolute adrenal weights (f) due to lower bw	[redacted] C., 2001 M-088469-01-1
Dog, 3-month, oral (diet) 0-10-50-250 ppm 0-0.3/0.25-1.32/1.33-6.46/5.91 mg/kg bw/day	M F	1.32 1.33 (50 ppm)	6.46 5.91 (250 ppm)	250 ppm: vomiting, trend of ↓ food consumption, marked ↓ bw gain (non-significant) ↑ N-OEM (m), stat. sign. ↓ erythrocyte AChE activity	[redacted] 2000 M-030181-01-1
Dog, 2-year, oral (diet) 0-15 (5)**-60-240 ppm 0-0-2-2.2-8.6 mg/kg bw/day	M F	2.2 2.2 (60 ppm)	8.6 8.6 (240 ppm)	Cholinergic effects, vomiting, ↓ food consumption associated with ↓ plasma AChE activity	[redacted] 1980 M-010201-01-1
Rabbit 21-day dermal 0-60-150-375 mg/kg bw/day	M F	150	375	stat. sign. ↓ plasma AChE activity (m), ↓ food consumption No effects on erythrocyte and brain AChE activity	[redacted], 1988 M-009558-01-1
Rabbit 21-day, dermal 0-500 mg/kg bw/day	M F	500	500	↓ Food consumption, ↓ bw (f), stat. sign. ↓ plasma AChE activity (not biol. relevant) No effects on erythrocyte and brain AChE activity	[redacted], 1989 M-009552-01-1
Rat 3-week, inhalation 0-6-23-96 mg/m³ (6h/day, 5 days/week)	M F	6 mg/m³	23 mg/m³	stat. sign. ↓ plasma AChE activity (m), moderate ↓ plasma AChE activity (f), moderate ↓ brain AChE activity (m)	[redacted], 1983 M-009879-01-1
Dog, 2-year, oral (diet) 0-15 (5)**-60-240 ppm 0-0-2-2.2-8.6 mg/kg bw/day	M F	2.2 2.2 (60 ppm)	8.6 8.6 (240 ppm)	Cholinergic effects, vomiting, ↓ food consumption associated with ↓ plasma AChE activity	[redacted] 1980 M-010201-01-1

Study Doses tested	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL	Main findings observed at LO(A)EL	Reference
Rat 2-year, oral (diet) 0-67-200-600 ppm 0-3.27/4.98-9.3/13.9-29/42 mg/kg bw/day	M F	9.3 13.9 (200 ppm)	29 42 (600 ppm)	Slight stat. sign. ↓ bw, (−6.2%/−7.6% (m/f))	[redacted] 981 M-009809-02-1
Mouse 2-year, oral (diet) 0-67-200-600 ppm 0-14.67/19.8-42.8/57-131.9/173.3 mg/kg bw/day	M F	14.7 19.8 (67 ppm)	57 (200 ppm)	↑ ALT indicating liver toxicity	[redacted] 1983 M-008825-02-1
Rat multi-generation (diet) 0-30-100-300 ppm	M F	15 15 (300 ppm)	>15 >15 (300 ppm)	No significant maternal, fetal or reproductive toxicity at the highest dose tested	[redacted], 1970 M-010170-01-1
Rat, 1-generation (diet, dose-range finder) 0-100-300-900 ppm 0-6.1/8.1-18.5/22.4-52.4/76.5 mg/kg bw/day	M F	6.1 8.1 (100 ppm)	18.5 22.4 (300 ppm)	Maternal: retarded body weight gain, ↓ plasma AChE in week 4 Offspring: retarded body weight (up to −10%) and body weight gain reproduction: no effects	[redacted] 2002 M-035507-01-1
Rat 2-generation (diet) 0-50-150-500 ppm 0-4.3/5.5-12.5/15.[illegible]-41/52.1 mg/kg bw/day	M F	4.3 5.5 (50 ppm)	12.5 15.[illegible] (150 ppm)	Maternal: ↓ body weights during lactation (F1) offspring: ↓ litter size (F1) reproduction: ↓ lactation index (F2&F2b)	[redacted] 2002 M-036790-01-1
Rat 2-Generation (diet)	Dam Fetal	6.9 (50 ppm) 16.3/22.3 (150 ppm)	21.3 (150 ppm) 69.0/89.0 (500 ppm)	↓ RBC AChE offspring: ↓ body weight (F1&F2), clinical signs, ↓ lactation index (F1) ↓ plasma & RBC AChE slightly delayed vaginal opening and balano-preputial separation in F2 due to lower pup weights ↑ incidence of pups with poor condition (food pasted mouth/nose and empty stomach or intestine)	[redacted], 2002 M-064945-01-1

Study Doses tested	Sex	NO(A)EL	LO(A)EL (mg/kg bw/day)	Main findings observed at LO(A)EL	Reference
Rat, special sub-chronic (diet)	Dam Fetal	 (50 ppm)	 (150 ppm)	Offspring: ↓ plasma AChE in both sexes	[redacted] 2003 M-088195-01-1
Rat oral (gavage) developmental	Dam Fetal	3 10	10 > 10	Maternal: significant ↓ body weight	[redacted] 1971 M-009391-02-1
Rat oral (gavage) developmental	Dam Fetal	0.5 5	1.5 >5	Maternal: cholinergic signs and muscle fasciculation	[redacted] 2002 M-038693-01-1
Rabbit oral (gavage) developmental	Dam Fetal	3 10	10 > 10	Maternal: clinical signs (increased respiratory rate, loss of coordination, muscular tremors, marked transient ↓ body weight	[redacted] 1981 M-010210-01-1
Rabbit oral (gavage) developmental	Dam Fetal	50 50	250 250	Maternal: ↓ food consumption, ↓ body weight gain Offspring: ↓ birth weight no teratogenicity	[redacted], K., 1992 M-009985-03-1

M: male F: female ↑: increase(d) ↓: decrease(d) bw: body weight
Stat: statistically sign.: significant(ly) AChE: acetylcholine esterase
N-DEM: N-demethylase (m): males (f): females
*: New studies, i.e. studies previously not submitted /evaluated on EU level, are written black. Previously evaluated studies are written in light grey.
**: The lowest test dose was reduced from 15 to 5 ppm after 15 days due to the depression of plasma AChE activity at 15 ppm.

Calculation of the acceptable daily intake (ADI)

The critical studies for the setting of regulatory endpoints are summarised in Table 5.10- 1.

It is notable that the toxicity profile in the same species is frequently inconsistent. This is attributed to two critical aspects:

i) metabolism and toxicity profile
ii) methodology in the conduct of the studies.

Methiocarb is very rapidly absorbed and eliminated. Cholinesterase inhibition is for the most part transient and it may be described as a weak inhibitor. For this reason, the timing of cholinesterase measurements is critical. Consequently in the experimental procedures, the timings for blood sampling and feeding status in dietary status is also critical in determining the nature of the responses measured. This is recognized to be difficult to determine or control in routine dietary studies in which feed is provided ad libitum. In experiments in which gavage doses were administered followed by optimalised timings of cholinesterase measurements, dramatically low NOAELs were obtained comparable to some of the most potent organophosphate compounds. However, the application of such data requires caution. The pharmacokinetic profile, absorption profile of a neat dose in fasted animals is not comparable to that of dietary equivalent dose mixed with food as absorption of the active substance is slower with a clear shift to the right in optimal collection times and maximum concentration (Cmax) attainable. The proposed regulatory dose levels carefully considers this point. The most sensitive species appeared to be dogs > rats > mice. This could be related to feeding habits

of the different species. Dogs tend to consume a large meal at a time, even if food is provided ad libitum.

Based on the study data provided for methiocarb the dog is the most sensitive species and the most sensitive endpoint is AChE inhibition (see also Table 5.10- 1). As can be seen the dog is the most sensitive species. Comparison of available dietary and gavage/capsule studies in rats and dogs reveals that derived LOAEL and NOAELs after dietary exposure are in a similar range.

In dietary studies on methiocarb in rat and dog the lowest LOAELs are 5 and 5.9 mg/kg bw and NOAELs are 1 and 1.3 mg/kg bw, for rats and dogs, respectively. In the gavage/capsule studies differences seem to be more pronounced: the lowest LOAEL was 1.5 mg/kg bw in rat and 0.5 mg/kg bw in dog, while the lowest NOAEL was 0.5 mg/kg bw in rat and 0.05 mg/kg bw in dog. However, this very low NOAEL derived in the capsule study in dogs is rather a product of dose spacing than a real NOAEL. The tested dose levels in dogs were: 0-0.05-0.5 mg/kg bw/day (i.e. factor of 10 between doses), and the tested dose levels in the rat study were 0-0.5-2.0 mg/kg bw/day (i.e. factor of 3). Due to the borderline erythrocyte AChE inhibition measured 1 to 2 h after dosing with 0.5 mg/kg bw (mean value: –26%, biologically relevant reduction: >20%) in the dog study it can be assumed that the real NOAEL is higher than 0.05 mg/kg bw/day or only slightly below 0.5 mg/kg bw/day, respectively.

It has also to be considered that following uptake of methiocarb in the feed, the absorption from the gastro-intestinal tract is retarded (in comparison to bolus application by gavage or capsule) and together with the quick detoxification of this compound considerably lower AChE inhibition values are reached.

Since potential methiocarb exposure will be due to ingestion and not as a bolus but together with a high volume of food stuff, dietary studies regarded to be more relevant when selecting the most appropriate study and NOAEL for the establishment of the reference values. Thus, it is proposed to consider the results of feeding studies for this purpose.

An ADI of 0.013 mg/kg bw/day is proposed. This is derived from the NOAEL of 50 ppm (1.3 mg/kg bw/day) in the 90-day dietary study in dogs with investigation of neurological function and it allows for a 100-fold safety factor. The NOAEL is based on reduction in erythrocyte and retinal cholinesterase activities and increased incidence of vomiting at 250 ppm. The NOAEL in the older 2 year study was 60 ppm (2.2 mg/kg bw/day) and after comparison with the 90-day study an overall NOAEL in dogs based on the same cholinesterase endpoint was determined to be 1.3 mg/kg bw/day. There is greater confidence in an overall NOAEL of 1.3 mg/kg bw/day as a basis for risk assessment. It is observed that methiocarb sulphoxide, the primary metabolite of methiocarb was slightly more potent in the depression of cholinesterase activity in oral repeat dose studies in which the compound was administered in capsules. The slightly more conservative overall NOAEL in the new dog study is considered to address the potentially increased risk presented by exposure to methiocarb sulphoxide.

As explained above the use of the NOAEL in the 4-week dog capsule study of 0.05 mg/kg bw/day would produce an ADI of 0.0005 mg/kg bw/day which is 17000X greater than the dose survived by 8 dogs in the 2-year chronic toxicity study. In the 3-month dietary dog study with neurofunctional examination significant erythrocyte cholinesterase depression, vomiting and impaired body weight gain was observed at 250 ppm (5.9 –6.5 mg/kg bw/day) but no neurofunctional deficits were observed at 50 ppm (1.3 mg/kg bw/day). These findings would therefore appear to confirm that the capsule dose is not representative of the normal dietary exposure.

ADI = 0.013 mg/kg bw/day

Acceptable Operator Exposure Level (AOEL)

No distinction is required for a short-term or long-term AOEL for methiocarb as the cholinergic toxicity is rapidly reversible.

The exposure profile for operators clearly does not support a bolus oral dose exposure. Together with the considerations presented above the dietary studies are considered to be the most appropriate studies for setting the AOEL. Therefore an AOEL of 0.013 mg/kg bw/day is proposed. This is derived

from the NOAEL of 1.3 mg/kg bw/day in the 90-day dietary study in dogs and allows for a 100-fold safety factor. The NOAEL is based on reduction in erythrocyte and retinal cholinesterase activities and increased incidence of vomiting at 250 ppm. No correction for the extent of oral absorption is considered to be required as it is estimated to be > 80% of an oral dose.

AOEL = 0.013 mg/kg bw/day

Acute Reference Dose (ARfD)

An ARfD of 0.013 mg/kg bw/day is proposed. This is derived from the NOAEL of 1.3 mg/kg bw/day in the 90-day dietary study in dogs with neurotoxicity investigations and allows for a 100-fold safety factor. The NOAEL is based on reduction in erythrocyte and retinal cholinesterase activities and increased incidence of vomiting at 250 ppm. Although this is not a short-term study, it is considered to be the most reliable study in terms of cholinergic investigations.

ARfD = 0.013 mg/kg bw/day

Comparison of the study results with CLP criteria according to REGULATION (EC) No 1272/2008[7]

Based on the derived oral LD_{50} of 19 mg/kg bw/day, and the LC_{50} of 433 mg/m³ (for females) methiocarb is considered to be fatal after acute oral and inhalation exposure. No classification is triggered for acute dermal exposure, skin and eye irritation, as well as skin sensitisation.

There are also no significant toxic effects at non-lethal dose levels that were not already covered by the acute toxicity assessment. There is also no indication of transient effects like respiratory tract irritation (RTI) and narcotic effects (NE) after single exposure to methiocarb. Therefore, classification of methiocarb for STOT-SE is not warranted.

In the repeated dose toxicity studies with methiocarb there were no significant toxic effects observed at non-lethal dose levels. Thus, classification of methiocarb for STOT-RE is not warranted.

Based on the overall result of the *in vitro* and *in vivo* mutagenicity tests with methiocarb conducted in bacteria and somatic cells it is concluded that methiocarb does not possess a genotoxic / mutagenic potential. In addition, the dominant lethal test in mice was also negative, indicating that methiocarb has no potential to cause mutations in germ cells. Thus, according to the actual CLP criteria no classification is triggered for methiocarb regarding mutagenicity / genotoxicity.

In the combined chronic toxicity carcinogenicity studies conducted in rats and mice there were no treatment-related increases in the incidence of benign and malignant tumors. Thus, according to the CLP criteria, taking also into account that methiocarb possesses no genotoxic potential, classification of methiocarb for a carcinogenic potential is not warranted.

In none of the reproductive and developmental toxicity studies there were no structural malformations observed. In the different generational studies on methiocarb findings indicative of reproductive / developmental toxicity, i.e. increased prenatal loss, reduced litter size, reduced viability on lactation day four, and a reduced lactation index was observed. Neither of these possible effects was consistently observed over doses and generations. Therefore, a specific effect of methiocarb on these parameters can be ruled out. What could be possible is that methiocarb contributes to pup death as a secondary, non-specific consequence of maternal toxicity.

In developmental studies in rats and rabbits there were no developmental or teratogenic effects up to the highest dose tested. Thus, according to the CLP criteria classification of methiocarb for reproductive toxicity is not warranted.

Thus, the following classification/labelling is triggered for methiocarb:

Regulation (EC) No 1272/2008 (CLP):	Acute Toxicity Category 2 H300 (Fatal if swallowed) H330 (Fatal if inhaled)