Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification: pethoxamid (ISO); 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenylprop-1-enyl)acetamide

EC Number:

CAS Number: **106700-29-2**

Index Number: **616-145-00-3**

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CONTENTS

1 PHYSICAL HAZARDS

Studies on the physical chemical properties of pethoxamid have been previously reviewed and are included in the EU draft Renewal Asssessment Report Volume 3 -B.2 (AS) (August 2016).

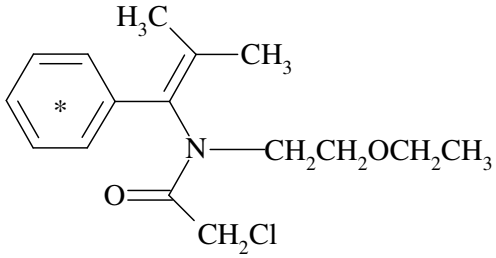
2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

2.1 Anonymous, 2000

Reference: Pethoxamid: Metabolism in the rat
Author(s), year: Anonymous, 2000
Report/Doc. number: 60 PXA (TOX2001-275) / TON '007/974328
Guideline(s): EPA FIFRA 85-1 ≈ 94/79/EEC ≈ JMAFF 59 Nohsan No. 4200; considered equivalent to OECD 417 (1984)
GLP: Yes
Deviations: Weight variation of animals exceeds 20%
Acceptability: Yes

Material and Methods:

Test material:



*[phenyl-U-14C]pethoxamid:

Specific activity: 1.11 GBq/mmol, Batch no.: CFQ9694, Radiochemical purity: >98 %.

Test animals: Sprague- Dawley rats (CRL:CD® BR), Charles River, Margate, UK.

In a pilot experiment no detectable radioactivity was found in the expired air traps, so these were not included in the main study. Furthermore, based on this pilot study, dose levels for the main study and sacrifice times for the tissue distribution experiments were selected.

Excretion studies:
Three groups of 5 male and 5 female rats were given a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw or 300 mg/kg bw, or fourteen consecutive daily oral doses of ^{14}C-pethoxamid at 8 mg/kg bw. Cooled urine was collected at 6, 12, and 24 hours and at 24-hour intervals up to 96 hours after dosing. Faeces were collected with 24-hour intervals up to 96 hours after final dosing. In the multiple dosing study, additional urine and faeces were collected at 24-hour intervals throughout dosing. Blood was sampled immediately prior to sacrifice. Upon sacrifice the residual carcass and organs were taken for analysis.

Biliary excretion was measured in another group of 5 male and 5 female rats after receiving a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw. Bile was collected and deep frozen at 1, 2, 4, 6, 8, 12, 24 and 48 hours after dosing. Urine was collected at 12, 24 and 48 hours after dosing and faeces were collected at 24 hour intervals until sacrifice at 48 hours after dosing. Residual carcass was taken for analysis.

Blood/plasma kinetics:
Groups of 5 male and 5 female rats received a single oral dose of 8 mg/kg bw or 300 mg/kg bw of ^{14}C-pethoxamid. At 4, 12, 48, 96, 120 and 168 hours after dosing blood samples were taken.

Tissue distribution studies:
Groups of 12 male and 12 female rats received a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw or 300 mg/kg bw. Groups of animals (3/sex) were sacrificed at 12, 48, 120 and 168 hours after dosing. Upon sacrifice the residual carcass and the organs were taken for analysis.

Whole-body autoradiography:
Four male and four female rats received a single oral dose of ^{14}C-pethoxamid at 8 mg/kg bw. Pairs of animals (1/sex) were sacrificed at 12, 48, 120 and 168 hours after dosing and used for autoradiography.

Analysis:
Radioactivity was measured using LSC. Tissue concentrations were determined by either combustion or solubilisation of replicate subsamples. Whole-blood concentrations were determined by direct combustion and radioassay. Urine, cage washes, plasma and bile concentrations were determined by direct radioassay of aliquots. Faeces were solvent extracted and aliquots of the extract and combustion of the residues were undertaken to determine residue levels. Subsequent tissue extracts, faeces extracts, bile and urine were analysed either by HPLC and TLC to assess the nature and proportion of any metabolites.

Results:

Excretion balance:
After a single oral dose of 8 mg/kg bw of ^{14}C-pethoxamid, the urinary excretion accounted for means of 35.0% and 31.4% in male and female rats, respectively, during 0-96 hours and 39.4% and 36.8%, respectively, after the high dose level of 300 mg/kg bw (Table 2.1.1-1). The majority of the remaining radioactivity was excreted via the faeces. The excretion pattern for both single dose levels, 8 and 300 mg/kg bw, was very similar. The majority of the urinary excretion took place in the first 24 hours, while the excretion in the faeces was mainly within 48 hours. After 96 hours the total recovery (urine, cagewash, faeces, carcass and tissue) was 95.9 (female) and 96.9% (male). After administration of 8 mg/kg bw/d for 14 days the excretion pattern was similar to that seen after a single dose.

Concentrations of radioactivity were measured in tissues at 96 hours (Table 2.1.1-6). The highest concentrations were found in the livers, lungs and spleens for all dosing regimes. Concentrations in the tissues from rats receiving 14 doses (8 mg/kg bw/d) were 3-16 fold higher than at the same time after a single dose (8 mg/kg bw).

Biliary excretion:
Rats with cannulated bile ducts excreted 73.2% of the radioactivity in females, and 75.6% in males through the bile (Table 2.1.1-2), with the majority occurring between 0 and 4 hours after dosing (37.3% for males and 48.1% for females). Recovery after 48 hours was >90%.

Pharmacokinetic parameters:
After a single low or high dose, the concentration of radioactivity in whole-blood and plasma was higher in males than in females, with maximum radioactivity 12 hours after dosing. At both dose levels the decline in radioactivity was monoexponential. The terminal half-lives of both whole-blood and plasma did not vary significantly between males and females or between the doses administered. However, the $t_{1/2}$ from whole-blood was much longer than from plasma (Table 2.1.1-3).

Tissue distribution:
Upon a single dose, the tissue distribution was similar between males and females. However, the values were slightly higher in males than in females. Highest concentrations in all tissues were 12 hours after dosing. The highest radioactivity occurred in whole-blood, plasma, liver and kidneys after single low or high dose

administration. After high dose administration, the lung also contained high radioactivity (Table 2.1.1-5). Mean tissue concentrations of radioactivity occurring 96 hours after a single oral dose (8 and 300 mg/kg bw), or 96 hours after the last dosing at 8 mg/kg bw for 14 days are shown in Table 2.1.1-6. The tissue concentrations of radioactivity in rats after 14 daily doses were 3-16 fold higher than after a single dose.

Whole-body autoradiography:
Tissue concentrations determined by luminography were generally in good agreement with those obtained by liquid scintillation counting. Differences occurred, may be attributed to the use of single animals, for luminography.

Metabolic pathway:
In urine up to eleven metabolites (all < 10% of the applied dose) were identified up to 48 hours after dosing. The single low and high dose and the 14 consecutive daily low doses showed a similar profile. In bile up to 24 hours, 10 metabolites were detected after administration of a single low level dose, of which polar metabolites accounted for 39.8% and 25.5% of the applied dose in males and females, respectively. In faeces extracts, up to 13 components were detected, generally accounting for <5% of the applied dose up to 48 hours (Table 2.1.1-7).

Pethoxamid is metabolised by glutathione-S-transferase to give a number of methylthio- metabolites, which are further oxidised to sulphonyl derivatives. This pathway is well known for herbicides which contain a chloroacetamide group. Metabolism also occurred by cleavage of the N-(2-ethoxyethyl) group and by oxidation of the methyl groups attached to the ethylenic bond. The sulphonic acid metabolite of pethoxamid (MET-42) was identified in faeces (eluted in F2-F4 region (Table 2.1.1-7) and accounted for maxima of 5.3% and 4.9% of the dose in females at 8 mg/kg bw and 300 mg/kg bw dose level, respectively. No metabolites derived from oxidation of the benzene ring were detected at all, in contrast to other well known chloroacetamide herbicides. A proposed metabolic pathway is shown in Figure B.6.1-1.

Table 2.1.1-1: Mean excretion and retention after single low and high dose and repeated (14-daily) low dose administration of pethoxamid

Dose level	**8 mg/kg bw**		**300 mg/kg bw**		**8 mg/kg bw/d**	
Sample	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Urine	35.0	31.4	39.4	36.8	34.4	31.1
Cagewash	0.3	0.3	0.4	0.4	1.2	1.9
Faeces	57.6	62.7	53.2	58.0	60.5	62.4
Carcass	1.8	1.2	1.6	1.1	0.7	0.6
Total recovery	94.5	95.6	94.6	96.3	96.9	95.9

Results expressed as % administered dose, 96 hours after dosing

Table 2.1.1-2: Mean excretion and retention in bile-duct cannulated rats after single low dose administration of pethoxamid

Dose level	**8 mg/kg bw**	
Sample	**Male**	**Female**
Urine	6.6	10.6
Cagewash	0.1	0.1
Faeces	8.5	6.1
Bile	75.6	73.2
Carcass	1.5	1.7
Total recovery	92.4	91.5

Results expressed as % administered dose, up to 48 hours after dosing

Table 2.1.1-3: Pharmacokinetic parameters of pethoxamid in plasma and whole-blood following a single low or high oral administration of ^{14}C-pethoxamid

	Plasma				Whole-blood			
Dose level	**8 mg/kg bw**		**300 mg/kg bw**		**8 mg/kg bw**		**300 mg/kg bw**	
Parameter	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
$t_{1/2}$ (hours)	43.7	46.7	41.0	45.2	145.7	122.5	149.2	148.2
AUC_{168}	55.9	49.3	2297.8	1840.9	286.8	175.3	14753.8	8779.0

Half life values calculated between 12 and 168 hours, except for male (whole-blood) 8 mg/kg bw, calculated between 48 and 168 hours.

Table 2.1.1-4: Mean tissue concentrations of radioactivity following a single oral dose of ^{14}C-pethoxamid at a nominal level of 8 mg/kg bw

Sacrifice time	**12 hours**		**48 hours**		**120 hours**		**168 hours**	
Animal numbers	**73M - 75M**	**76F - 78F**	**79M - 81M**	**82F - 84F**	**85M - 87M**	**88F - 90F**	**91M - 93M**	**94F - 96F**
Adrenal glands	0.385	0.305	0.130	0.125	0.064	0.069	0.099	0.053
Bone	0.059	0.069	nd	0.029	0.014	nd	0.015	nd
Brain	0.126	0.105	0.055	0.040	0.063	0.032	0.050	0.028
Carcass	0.890	1.316	0.209	0.285	0.105	0.095	0.066	0.058
GIT	62.577	42.909	3.555	3.786	0.306	0.396	0.172	0.143
Heart	0.438	0.329	0.232	0.168	0.168	0.082	0.126	0.070
Kidney	1.360	1.175	0.379	0.388	0.223	0.158	0.176	0.112
Liver	2.882	2.056	0.766	0.678	0.398	0.348	0.305	0.184
Lung	0.563	0.478	0.324	0.317	0.255	0.162	0.195	0.113
Muscle (skeletal)	0.233	0.187	0.082	0.066	0.060	0.029	0.043	0.023
Ovary	ns	0.367	ns	0.141	ns	0.052	ns	0.039
Plasma	0.795	0.773	0.329	0.381	nd	nd	0.067	0.051
Spleen	0.540	0.399	0.288	0.278	0.251	0.162	0.201	0.114
Testis	0.207	ns	0.067	ns	0.032	ns	0.021	ns
Thyroid	0.584	0.305	0.243	0.161	0.137	0.082	0.077	nd
Whole-blood	2.276	1.591	1.810	1.417	1.664	0.984	1.384	0.805
Fat (abdominal)	0.222	0.224	0.056	0.054	0.037	0.018	0.026	0.011

Results are expressed as µg pethoxamid equivalents/g; ns: no sample; nd: not detected

Table 2.1.1-5: Mean tissue concentrations of radioactivity following a single oral dose of ^{14}C-pethoxamid at a nominal level of 300 mg/kg bw

Sacrifice time	**12 hours**		**48 hours**		**120 hours**		**168 hours**	
Animal numbers	**133M - 135M**	**136F - 138F**	**139M - 141M**	**142F - 144F**	**145M - 147M**	**148F - 150F**	**151M - 153M**	**154F - 156F**
Adrenal glands	16.269	24.408	4.785	4.625	3.948	3.392	3.558	3.132
Bone	4.173	3.556	1.170	0.756	0.632	0.555	0.596	nd
Brain	6.266	9.306	2.588	1.572	1.819	1.713	1.750	1.300
Carcass	17.434	35.006	7.020	7.025	3.929	5.046	2.842	3.421
GIT	1632.5	1926.4	65.075	65.380	5.340	7.376	2.680	3.087
Heart	16.375	23.025	8.377	4.893	5.950	4.406	5.318	3.724
Kidney	53.574	52.933	12.869	11.338	8.672	7.616	5.825	5.795
Liver	74.446	87.842	15.679	12.670	9.736	8.025	6.516	5.854
Lung	22.841	30.391	12.429	10.292	8.960	7.997	7.131	7.233
Muscle (skeletal)	9.099	12.478	3.215	1.881	2.342	1.547	1.865	1.315
Ovary	ns	19.657	ns	4.340	ns	3.009	ns	1.645
Plasma	25.836	33.614	12.127	10.304	4.199	4.749	2.032	2.606
Spleen	19.876	22.322	11.697	8.646	8.643	7.350	10.113	6.877
Testis	8.111	ns	2.097	ns	1.085	ns	0.717	ns
Thyroid	15.258	19.416	7.486	6.178	5.679	4.159	4.841	3.907
Whole-blood	74.576	76.418	70.582	51.676	56.611	46.254	49.628	43.666
Fat (abdominal)	12.445	24.053	1.809	1.985	1.155	1.272	0.671	0.869

Results are expressed as µg pethoxamid equivalents/g; ns: no sample; nd: not detected

Table 2.1.1-6: Mean tissue concentrations at 96 hours after single low or high dose and repeated low dose administration of TKC-94

Dose level	8 mg/kg bw		300 mg/kg bw		8 mg/kg bw/d	
Tissue	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Adrenal gland	0.093	0.074	8.156	4.209	1.126	0.797
Bone	0.023	nd	1.071	0.929	0.142	0.103
Brain	0.051	0.026	3.170	2.053	0.599	0.302
GIT	0.465	0.503	6.564	5.183	0.830	0.743
Heart	0.153	0.087	10.044	4.640	1.352	1.024
Kidney	0.229	0.183	12.139	7.706	1.976	1.295
Liver	0.425	0.312	11.447	7.311	2.629	1.681
Lung	0.251	0.173	15.482	9.868	2.609	1.975
Muscle (skeletal)	0.069	0.033	4.039	1.672	0.458	0.230
Ovary	ns	0.069	ns	2.977	ns	0.500
Plasma	0.168	0.178	8.998	5.680	0.520	0.520
Spleen	0.316	0.222	15.653	7.570	2.825	1.878
Testis	0.037	ns	2.064	ns	0.187	ns
Thyroid	0.112	0.071	15.401	13.692	1.430	1.126
Whole-blood	1.468	0.945	96.029	53.432	15.951	10.400
Fat (abdominal)	0.042	0.027	1.965	1.957	0.197	0.140

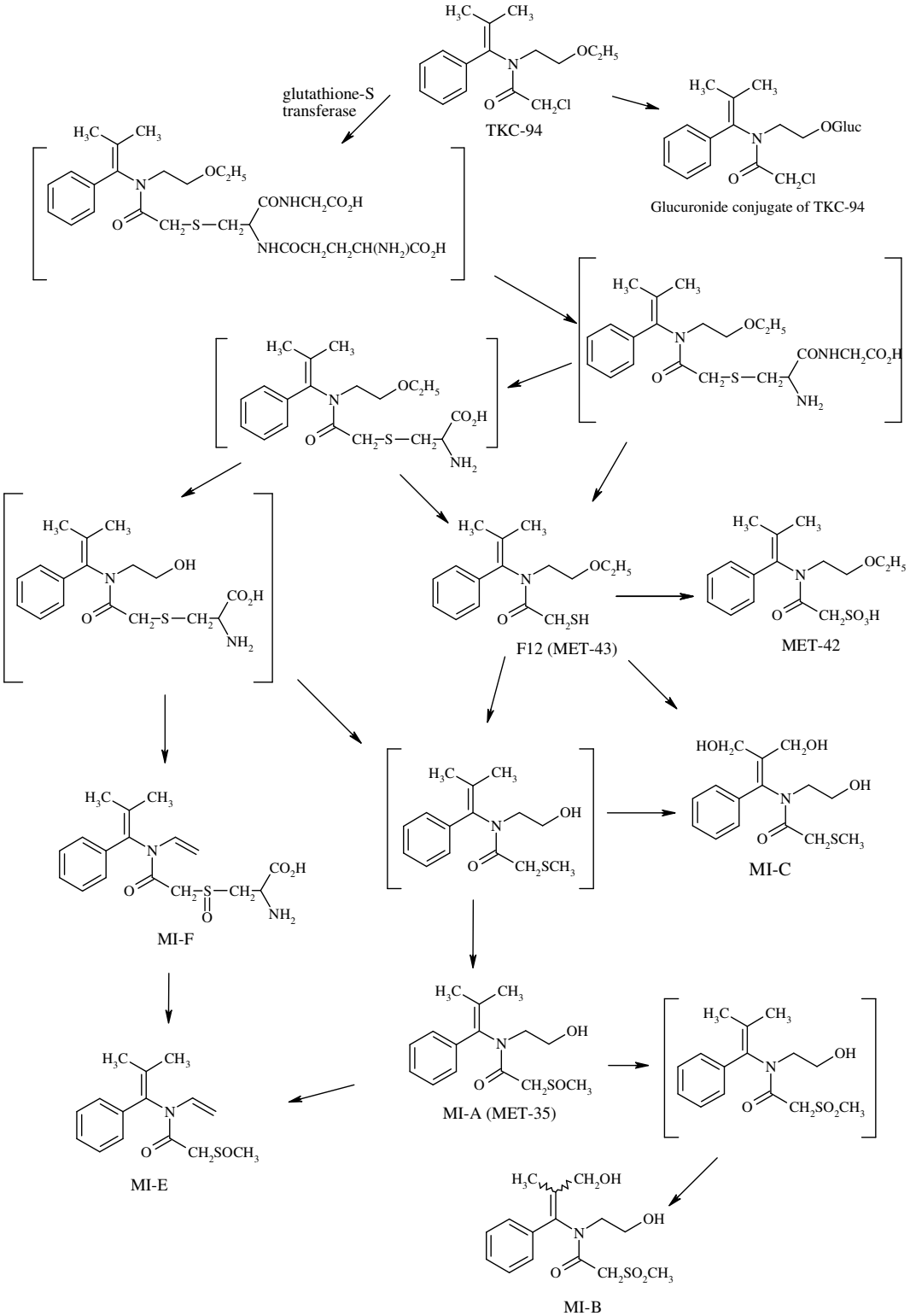
Results expressed as µg test compound equivalents/g; ns: no sample; nd: not detected

Table 2.1.1-7: Radioactive components expressed as % of the administered dose in urine, faeces and bile after oral administration of ^{14}C-pethoxamid to rats[1]

Component	8 mg/kg bw		300 mg/kg bw		8 mg/ kg bw/d	
	Male	Female	Male	Female	Male	Female
Urine						
U1	0.7	1.0	1.4	0.7	0.1	0.5
U2	1.4	2.1	nd	1.5	0.4	0.4
U3	1.9	1.0	4.1	2.2	0.5	0.2
U4	nd	1.7	nd	2.0	0.3	0.3
MI-F	2.7	5.0	5.2	4.5	0.5	0.6
MI-E	1.4	2.8	1.2	4.5	0.2	1.0
MI-D	1.9	3.7	3.3	5.7	0.6	0.5
U8	0.8	nd	2.3	1.4	nd	nd
MI-C	9.2	1.7	6.7	2.2	0.1	0.2
MI-B	5.4	2.6	5.8	2.9	1.3	0.2
MI-A	2.6	2.3	3.7	4.2	0.7	0.5
Pethoxamid	2.0	1.5	0.8	1.1	0.5	0.2
Others[2]	3.9	3.9	3.8	2.7	0.1	0.4
Faeces[3]						
F1	0.7	2.2	3.7	5.3	2.7	2.6
F2	3.6	2.7	1.3	2.3	nd	2.3
F3	2.5	4.0	1.2	1.6	nd	0.3
F4	1.2	3.0	1.3	2.5	0.3	nd
F5	0.5	4.9	nd	nd	1.4	0.3
F6	nd	2.4	nd	nd	0.5	0.2
F7	0.8	2.1	0.3	6.4	0.5	<0.1
F8	5.5	1.4	0.3	1.3	0.3	<0.1
F9	3.5	0.7	5.9	nd	0.1	0.1
F10	2.2	0.2	2.0	0.9	0.2	0.4
F11	0.9	1.5	3.2	nd	0.2	nd
F12	1.0	2.0	3.7	1.9	0.6	0.6
F13	0.9	1.4	2.4	nd	nd	nd
Pethoxamid	1.5	0.4	6.8	11.0	nd	nd
Others[1]	6.9	5.7	7.8	10.2	1.0	1.2
Unextracted	22.3	25.3	10.8	11.4	nd	nd
Bile						
B1	6.0	8.7				
B2	8.6	4.2				
B3	19.2	4.7				
B4	6.0	7.9				
MI-F	6.1	11.8				
MI-E	0.8	5.8				
MI-D	16.6	4.6				
MI-C	1.9	2.0				
MI-B	1.2	1.1				
MI-A	0.5	0.9				
Pethoxamid	0.9	5.2				
Others [2]	7.0	15.4				

[1] Sampling: Single administration: Urine and faeces 0-48 hours; bile 0-24 hours; 14 days administration: Urine 0-15 days, faeces 0-14 days; [2] Radioactivity not associated with specific components; [3] MET-42 (in F2-F4 region eluting): 8 mg/kg bw: 0.5% (males), 5.3% (females), 300 mg/kg bw: 1.5% (males), 4.9% (females); nd: not detected.

Figure 2.1.1-1: Proposed metabolic pathway of pethoxamid in the rat



3 HEALTH HAZARDS

Acute toxicity

3.1 Acute toxicity - oral route

3.1.1 Animal data

3.1.1.1 Anonymous, 1994a

Reference: Acute oral toxicity to the rat of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 63 PXA (TOX2001-280)/ TKS '15/932187/AC
Guideline(s): OECD 401 (1987)
GLP: Yes
Deviations: No
Acceptability: Yes

Materials and methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: Groups of 5 male and 5 female CD Sprague-Dawley rats, seven to ten weeks old, 204 to 248 g from Harlan Olac Ltd.,Bicester, Oxon, England.

Test method: The test material was administered as supplied to the rats by oral gavage at dose levels of 800, 1260 and 2000 mg/kg bw (the maximal application volume was 1.8 mL/kg bw). The observation period was 14 days post-exposure.

Results:

Table 3.1.1-1: Mortality

Sex	**Dose (mg/kg bw)**			**LD50 (95% C.I.) (mg/kg bw)**
	800	1260	2000	
Male	2/5	3/5	5/5	983 (623 to 1360)
Female	0/5	2/5	4/5	1472 (1039 to 2235)
Male and Female	2/10	5/10	9/10	1196 (878 to 1579)

Table 3.1.1-2: Incidence of clinical observations

Finding	**Dose level (mg/kg bw)**					
	800		1260		2000	
	male	female	male	female	male	female
Piloerection	5	5	5	5	5	5
Abnormal body carriage (hunched posture)	5	5	5	5	5	5
Abnormal gait (waddling)	5	5	5	5	5	5

Lethargy	5	5	5	5	5	5
Decreased respiratory rate	5	0	3	0	5	5
Partially closed eyelids	5	0	1	0	2	5
Pallor of the extremities	5	0	5	5	5	5
Soft or liquid faeces	5	5	5	3	5	4
Increased salivation	5	5	2	5	4	5
Unsteadiness	0	5	0	0	0	1
Body tremors	0	0	0	0	2	1
Dilation of pupils	0	0	0	0	5	4
Cold to the touch	0	0	0	0	1	0
Staining of the ano-genital region	0	0	2c	2b	1b	2c
Staining on cage litter tray	0	0	5a	0	0	1d
a: Yellow staining; b: Yellow/orange staining; c: Yellow/red staining; d: Red/brown staining						

Body weight: The rats that died showed bodyweight loss, and revealed changes to subcutaneous tissue, liver, spleen, kidneys, stomach, intestines and testes at macroscopic examination.
All surviving rats were recovered at day 6. Slightly low bodyweight gains were recorded on day 8 for two males dosed at 800 and 1260 mg/kg bw, and a slight bodyweight loss for one female at 2000 mg/kg bw.

Necropsy: At macroscopic examination no abnormalities were recorded for the surviving animals.

Conclusion: The acute oral median lethal dose (LD_{50}) and its 95% confidence limits in male rats was estimated to be 983 (623 to 1360) mg/kg bw. Therefore, according to the criteria in Council Directive 67/548/EEC, the test material is classified as harmful (Xn) and labelled with R22, "harmful if swallowed".

According to Annex VI of Regulation (EC) 1272/2008, ATP01 (Regulation (EC) 790/2009), pethoxamid is classified as **Acute Tox. Cat. 4 and labelled with H302, "Harmful if swallowed".**

3.1.2 Human data

No relevant studies.

3.1.3 Other data

No relevant studies.

3.2 Acute toxicity - dermal route

3.2.1 Animal data

3.2.1.1 Anonymous (1994b)

Reference: Acute dermal toxicity to the rat of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 64 PXA (TOX2001-277) / TKS '16/932186/AC

Guideline(s): OECD 402 (1981)
GLP: Yes
Deviations: No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: 5 male, 5 female CD Sprague-Dawley rats.

Test method: The test material was administered as supplied to the rats at 2000 mg/kg bw to the shaved skin of each animal (application volume 1.8 mL/kg). A gauze pad was then placed over the treated area and held in place with a non-irritative dressing for 24 hours. The observation period was 14 days post-exposure.

Findings:
Clinical findings: No mortalities were observed. No clinical signs were noted during the study. No irritation or other dermal changes were observed in any animal throughout the observation period.

Body weight: Slightly low bodyweight gains were recorded for all five males and two females on Day 8. In addition, at this time, two females showed either no change or a slight body weight loss. By Day 15, three males had achieved the anticipated body weight gains. Slightly low body weight gains were than evident in two males and three females. One female achieved the anticipated body weight gain throughout the study.

Necropsy: No abnormalities were noted at necroscopy.

Conclusion: The acute dermal median lethal dose (LD_{50}) in rats was found to be greater than 2000 mg/kg bw. Therefore according to the criteria in Council Directive 67/548/EEC classification is not required.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding acute dermal toxicity is not required.

3.2.2 Human data

No relevant studies.

3.2.3 Other data

No relevant studies.

3.3 Acute toxicity - inhalation route

3.3.1 Animal data

3.3.1.1 Anonymous (1994)

Reference: Acute inhalation toxicity to the rat of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 65 PXA (TOX2001-278) / TKS '14/932316
Guideline(s): OECD 403 (1981)

GLP: Yes
Deviations from OECD 403 (2009): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: Albino rats, (Sprague Dawley); one control group and one test group each of 5 male and 5 female rats, six and eight weeks old respectively, 200 g of weight, from Charles River UK Ltd, Manston Road, Margate,Kent, UK.

Test method: The rats were exposed to the test material or to clean dried air by continuous whole body exposure for 4 hours by inhalation. The test atmosphere contained a liquid droplet aerosol generated from the melted test substance. After the observation period of 14 days post exposure, the animals were macroscopically examined. The lungs were also examined microscopically.

Table 3.3.1-1: Exposure parameters

Parameter		**Value**
Chamber concentration (mg/L)	Chemical analysis	4.16
	Gravimetric analysis	4.38
Particle size (µm)	MMAD ± GSD	3.3 ± 2.14
	% respirable (<7 µm)	84.1
Chamber air temperature (°C)	Control group	24
	Test group	23
Relative humidity (%)	Control group	45
	Test group	96
MMAD: Mass median aerodynamic diameter, GSD: Standard geometric deviation		

Results:
Clinical observations: No mortalities were observed. Signs seen during exposure to the test substance included partial closing of the eyes, wetness around the eyes, snout and mouth and matted fur. During the observation period, signs seen in rats exposed to pethoxamid included residues of the test material on the fur, brown discharge from the eyes, wet fur and brown staining around the snout, jaws and on the underbody, and matted fur. Exaggerated respiratory movements were observed in 1 male rat. Recovery from the effects was evident from Day 6 in male rats and Day 8 in female rats. All rats were recovered on Day 11.

Body weight: Reduced body weight or decreased rate of body weight gain was observed in treated rats for one day. Subsequently, weight gain was similar to that of control rats.

Pathological examination: At necropsy, the lungs of one female rat exposed to pethoxamid had dark subpleural foci in all lobes. These foci are commonly found in rats. There were no macroscopic abnormalities in any other test or control rat. There were also no microscopic abnormalities observed that were considered treatment-related.

Conclusion: The acute inhalation median lethal concentration (LC_{50}, 4 hours) in the rat was greater than 4.16 mg/L of air. This was the highest attainable analytical concentration under the test conditions, the exposure level achieved was far in excess of any exposure levels that could be generated accidentally.

Only a transient effect on bodyweight with no other effects was observed, and therefore according to the criteria in Council Directive 67/548/EEC classification is not required.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding acute inhalation toxicity is not required.

3.3.2 Human data

No relevant studies.

3.3.3 Other data

No relevant studies.

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 Anonymous (1994a)

Reference: Skin irritation to the rabbit of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 66 PXA (TOX2001-279) / TKS '17/932160/SE
Guideline(s): OECD 404 (1992)
GLP: Yes
Deviations from OECD (2002): No
Acceptability: Yes

Material and Methods:
Test Material: Pethoxamid; Batch TB 930727; Purity: 95%.
Test Animals: Six New Zealand white male rabbits, 10 to12 weeks of age, 2.3 to 2.7 kg of weight, from Froxfield (U.K.) Ltd. Petersfield, Hampshire, England.

Test method: The rabbits received each 0.5 mL of the test substance which was administered as supplied to the shaved skin of each animal. A 2.5 x 2.5 cm gauze pad was then placed over the area and covered with an elastic adhesive dressing (semi-occlusive) for four hours.

Results:
Clinical findings: Very slight erythema was seen in all animals on Day 1. This persisted in one animal only, accompanied by very slight oedema on Days 2 and 3. All reactions had completely resolved by Day 5.

There were no signs of toxicity or ill health in any rabbit during the observation period.

Table 3.4.1-1: Individual and mean skin irritation scores

	Erythema						Oedema					
	Days					Mean score	Days					Mean score
Animal no.	0.5h	24h	48h	72h	96h	24-72h	0.5h	24h	48h	72h	96h	24-72h
2575	1	0	0	0	0	0	0	0	0	0	0	0
2576	1	0	0	0	0	0	0	0	0	0	0	0
2577	1	0	0	0	0	0	0	0	0	0	0	0
2578	1	0	0	0	0	0	0	0	0	0	0	0
2579	1	1	1	1	0	1	0	1	1	0	0	0.67
2580	1	0	0	0	0	0	0	0	0	0	0	0

Conclusion: On the basis of the skin reactions observed (mean skin irritation scores 24-72 hours after removal of the test article are 0.0-1) and the criteria specified in Council Directive 67/548/EEC, the test compound does not have to be classified as a skin irritant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid as skin irritant is not required.

3.4.2 Human data

No relevant studies.

3.4.3 Other data

No relevant studies.

3.5 Serious eye damage/eye irritation

3.5.1 Animal data

3.5.1.1 Anonymous (1994b)

Reference: Eye irritation to the rabbit of NSK-68
Author(s), year: Anonymous, 1994
Report/Doc. number: 67 PXA (TOX2001-280) / TKS '18/932199/SE
Guideline(s): OECD 405 (1987)
GLP: Yes
Deviations from OECD (2012): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-930727; Purity: 95%.

Test animals: Six New Zealand white rabbits, 13 to 15 weeks of age, 3.1 to 3.6 kg of weight, from Froxfield (U.K.) Ltd., Petersfield, Hampshire, England.

Test method: The rabbits each received 0.1 mL of the test substance, by a singular ocular instillation without irrigation to the lower everted lid of one eye of each animal.

Results:
Clinical findings: The eye findings are tabulated below. The well-defined conjunctival irritation occurring in all animals and the dulling of the cornea (5/6 rabbits) were confined to the one hour assessment. Remaining slight reactions had resolved one or two days after instillation.

There were no signs of toxicity or ill health in any rabbit during the observation period.

Table 3.5.1-1: Individual and mean eye irritation scores

Animal no.	Region of eye	1h	24h	48h	72h	4 days	7days	Mean score 24-72h
4203	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	1	0	0	0	0	0.33
	discharge	2	0	0	0	0	0	0
2624	Cornea density	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	0	0	0	0	0	0
	discharge	2	0	0	0	0	0	0
2625	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	1	0	0	0	0	0.33
	discharge	3	0	0	0	0	0	0
2626	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	0	0	0	0	0	0
	chemosis	2	0	0	0	0	0	0
	discharge	2	0	0	0	0	0	0
2501	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	1	0	0	0	0	0.33
	discharge	2	0	0	0	0	0	0
2506	Cornea density	D	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0
	Conjunctiva - redness	2	1	0	0	0	0	0.33
	chemosis	2	0	0	0	0	0	0
	discharge	2	0	0	0	0	0	0

Conclusion: Instillation of the test compound into the rabbit eye elicited a transient well-defined conjunctival irritation. On the basis of the eye reactions observed (mean eye irritation scores 0.0-0.33) and the criteria specified in Council Directive 67/548/EEC and Regulation 1272/2008, the test compound does not have to be classified as an eye irritant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid as eye irritant is not required.

3.5.2 Human data

No relevant studies.

3.5.3 Other data

No relevant studies.

3.6 Respiratory sensitisation

3.6.1 Animal data

No relevant studies.

3.6.2 Human data

No relevant studies.

3.6.3 Other data

No relevant studies.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 Anonymous (1998)

Reference: TKC-94 : Skin Sensitisation in the Guinea-Pig, (incorporating a positive control using hexyl cinnamic aldehyde (HCA))
Author(s), year: Anonymous, 1998
Report/Doc. number: 68 PXA (TOX2001-281) / TON '017/983095/SS
Guideline(s): OECD 406 (1992)
GLP: Yes
Deviations: No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch TB-960306; Purity: 95%.

Test animals: Sixty male albino guinea pigs of Dunkin/ Hartley strain, four to seven weeks old, 444 to 538 g of weight, from D. Hall Newchurch, Staffordshire, England.

Test method: The guinea pigs were allocated to four groups:
1. Control for group 2; 20 animals
2. Pethoxamid group; 20 animals
3. Control for group 4; 10 animals
4. Hexyl cinnamic aldehyde group (HCA; positive control); 10 animals

Based on the results of a preliminary study, the following dose levels were selected:

Study phase	Pethoxamid	HCA, positive control
Induction intradermal injection	0.5% v/v in Alembicol D	10% v/v in Alembicol D
Induction topical application	As supplied	As supplied
Topical challenge	25 and 12.5% v/v in Alembicol D	As supplied and 50% v/v in Alembicol D

The induction and challenge applications were held in place with semi-occlusive dressing for 48 and 24 hours respectively. After removal of the patches, the challenge sites were evaluated after 24 and 48 hours.

Results:
Clinical findings: No signs or effects on body weight were recorded. Dermal reactions were seen following the induction applications. After the challenge, dermal reactions were seen in nineteen of the twenty test animals.

Table 3.7.1-1: Number of animals exhibiting skin reactions after challenge with pethoxamid

Group	**Treatment**	**Incidence of erythema**				**Incidence of oedema**			
		24 hours		**48 hours**		**24 hours**		**48 hours**	
		anterior	**posterior**	**anterior**	**posterior**	**anterior**	**posterior**	**anterior**	**posterior**
Control	Freund's treated controls	3/20	2/20	3/20	2/20	0/20	0/20	0/20	0/20
Test	Pethoxamid	20/20	19/20	20/20	19/20	19/20	14/20	19/20	16/20
Control	Freund's treated controls	0/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10
Positive control	HCA	10/10	10/10	10/10	10/10	9/10	4/10	9/10	2/10

Conclusion: Due to the positive responses seen in 19 out of 20 test animals, pethoxamid does have to be classified as sensitizing and labelled with R43 according to the criteria specified in Council Directive 67/548/EEC.

According to Annex VI of Regulation (EC) 1272/2008, ATP01 (Regulation (EC) 790/2009), pethoxamid is classified as **Skin Sens. Cat.1 and labelled with H317, "May cause an allergic skin reaction".**

3.7.2 Human data

No relevant studies.

3.7.3 Other data

No relevant studies.

3.8 Germ cell mutagenicity

3.8.1 In vitro data

3.8.1.1 Anonymous (1994)

Reference: Pethoxamid: Bacterial mutation assay
Author(s), year: Anonymous, 1994
Report/Doc. number: 75 PXA (TOX2001-290) / TKS '22/941449
Guideline(s): OECD 471 (1983), OECD 472 (1983)
GLP: Yes
Deviations from OECD 471 (1997): - no inclusion of S. typhimurium TA102 or E.coli WP2 uvrA
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %; Solvent: DMSO.

Test method: Four strains of *Salmonella typhimurium* (TA 1535, TA 1537, TA 98 and TA 100) were dosed with a single application of 0.1 mL/plate of the test substance, with and without S9 mix (rat Aroclor). From a preliminary test, it was concluded that the test compound was not toxic up to 5000 mg/plate and therefore this dose level was chosen as top dose level in the main test. Dose levels used in the main test were as follows: 0, 50, 150, 500, 1500, 5000 mg/plate in triplicate. Positive controls were included. Revertant colonies per plate following a 72 hour incubation period were counted. The main test was repeated.

Results: None of the bacterial strains, tested with the test substance, showed any significant increases in the number of revertant colonies at any dose level, in the presence or absence of the S9 mix. Toxicity was observed following treatment with 5000 mg/plate in the first main test only in the presence of S9 mix, but in the second main test both in the presence and absence of S9 mix. All positive control compounds induced marked increases in the number of revertants.

Conclusion: The test material was found to be non-mutagenic under the conditions of this *in vitro* bacterial system.

3.8.1.2 Anonymous (2012)

Reference: Reverse Mutation Assay using Bacteria (Salmonella typhimurium and Escherichia coli) with Pethoxamid Technical

Author(s), year: Anonymous, 2012

Report/Doc. number: 647 PXA / BSL 115522

Guideline(s): OECD 471 (1997)

GLP: Yes

Deviations from guideline: - none

Acceptability: Yes

Material and Methods:
Test material: Pethoxamid technical

Lot/batch number: P1351-BKA-65

Purity: 93.5% (w/w) (dose calculation was not adjusted for purity)

Stability of test item: 17 October 2012 (stored at ambient temperature). NB: stable during the conduct of the study.

Storage conditions: At room temperature.

Test method: The test item pethoxamid was investigated for its potential to induce bacterial gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and tester strain E. coli WP2 uvrA.

In two independent experiments several concentrations of the test item were used. Each assay was conducted with and without metabolic activation. The concentrations, including the controls, were tested in triplicate.

The following concentrations were used in experiments I and II: 3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate.

Revertant colonies were automatically counted using a ProtoCol counter. For tester strains where the spontaneous mutant frequency is low (TA1535 and TA1537) revertant colonies were counted manually. Statistical analysis was not deemed necessary.

Results: No precipitation of the test item was observed in any tester strain used in experiment I and II (with and without metabolic activation). In experiment I toxic effects of the test item were observed at concentrations of 1000 µg/plate and higher (without metabolic activation) and at concentrations of 2500 µg/plate and higher (with metabolic activation), depending on the particular tester strain. In experiment II toxic effects of the test item were noted at concentrations of 316 µg/plate and higher (without metabolic activation) and at concentrations of 1000 µg/plate (with metabolic activation), depending on the particular tester strain.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with pethoxamid at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II. The reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiments.

Conclusion: In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, pethoxamid did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used. Therefore, pethoxamid is considered to be non-mutagenic in this bacterial reverse mutation assay.

3.8.1.3 Anonymous (1994)

Reference: Pethoxamid: Metaphase chromosome analysis of human lymphocytes cultured in vitro
Author(s), year: Anonymous, 1994
Report/Doc. number: 76 PXA (TOX2001-291) / TKS 12/931249
Guideline(s): OECD 473 (1983)
GLP: Yes
Deviations from OECD 473 (1997): none
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %; Solvent: DMSO.

Test method: Cultured human lymphocytes were exposed to the test substance, in the presence and absence of S9 mix (rat Aroclor). Without S9 mix, cells were exposed continuously for 18 hours. In the presence of S9 mix, exposure was limited to three hours, cells were harvested 15 hours after exposure. The following concentrations were selected for the duplicate tests:

- Experiment 1 without S9-mix: 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 µg/mL
- Experiment 1 with S9-mix: 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 µg/mL
- Experiment 2 without S9-mix: 3.75, 7.5, 15, 20, 25, 37.5, 50, 75, 100, 150 µg/mL
- Experiment 2 with S9-mix: 3.75, 7.5, 15, 30, 45, 60, 80, 100, 200 µg/mL

Solvent controls and positive controls were included. The mitotic index was recorded. The dose level causing approximately 50% decrease, was used as highest dose for metaphase analysis. Additionally, an intermediate and low dose level was selected.

Findings: In the first test no cells survived, both in the presence and absence of S9 mix, at concentrations of 62.5 µg/mL and above. In the second test, death of all cells was seen at a concentration of 75 µg/mL and above in the absence of S9 mix, and at 200 µg/mL in the presence of S9 mix.

In the first test, in the absence of S9 mix, a statistically significant increase in chromosomal aberrations occurred at the highest dose level, indicative of a clastogenic activity. In the presence of S9 mix, a statistically significant increase in chromosomal aberrations (gaps only) occurred at the intermediate dose level.

In the second test in the absence of S9 mix, statistically significant increases in chromosomal aberrations occurred at the intermediate and high dose levels. In the presence of S9 mix, a statistically significant increase in chromosomal aberrations occurred at all dose levels analysed.

The positive control compounds produced clear increases in chromosome aberrations.

Table 3.8.1-1: Summary of cytogenetic tests

Expt	**Treatment (dose in µg/mL)**	**S9-mix**	**Mean MI (%)**	**Cells with aberrations including gaps (%)**			**Cells with aberrations excluding gaps (%)**		
				range	**mean**	**statistical significance**	**range**	**mean**	**statistical significance**
1	DMSO	-	100	4-8	5.5	-	3-8	5.25	-
	Pethoxamid (2)	-	116	4-4	4.0	ns	4-4	4.0	ns
	Pethoxamid (7.8)	-	100	4-4	4.0	ns	4-4	4.0	ns
	Pethoxamid (15.6)	-	65	13-10	11.5	**	12-10	11.0	**
	EMS (500)	-	nd	17-24	20.5	***	23-17	20.0	***
	DMSO	+	100	1-5	2.5	-	1-5	2.5	-
	Pethoxamid (3.9)	+	102	4-2	3.0	ns	2-4	3.0	ns
	Pethoxamid (15.6)	+	98	6-6	6.0	*	5-6	5.5	ns
	Pethoxamid (31.3)	+	61	2-9	5.5	ns	2-8	5.0	ns
	CP (10)	+	nd	37-27	32.0	***	37-26	31.5	***
2	DMSO	-	100	0-3	1.0	-	1-0	0.25	-
	Pethoxamid (3.75)	-	100	1-2	1.5	ns	2-1	1.5	ns
	Pethoxamid (20)	-	49	4-9	6.5	***	4-7	5.5	***

	Pethoxamid (37.5)	-	41	16-16	16.0	***	15-16	15.5	***
	EMS (500)	-	nd	11-13	12.0	***	13-7	10.0	***
	DMSO	+	100	4-1	2.5	-	1-4	2.5	-
	Pethoxamid (7.5)	+	111	6-6	6.0	*	6-6	6.0	*
	Pethoxamid (45)	+	95	11-18	14.5	***	11-17	14.0	***
	Pethoxamid (80)	+	55	42-42	42.0	***	42-42	41.5	***
	CP (10)	+	-	43-39	41.0	***	43-39	40.5	***

MI: Mean mitotic index compared to negative control values; EMS: Ethylmethanesulphonate; CP: Cyclophosphamide

ns not significant; * p<0.05, ** p<0.01, *** p<0.001; nd: not determined;

Conclusion: Pethoxamid demonstrated **clastogenic activity** both in the absence and presence of S9 mix in this *in vitro* mammalian cytogenicity assay.

3.8.1.4 Anonymous (2015)

Reference: In vitro Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/TK+/-) in Mouse Lymphoma L5178Y Cells with Pethoxamid technical
Author(s), year: Anonymous, 2015
Report/Doc. number: 1449 PXA / Eurofins BioPharma Product Testing study No: 150778
Guideline(s): OECD 476 (1997)
GLP: Yes
Deviations from guideline: No
Acceptability: Yes

Materials and methods:
Test material: Pethoxamid technical

Lot/batch number: P1351-BKA-65

Purity: 94.5% (w/w) (dose calculation was not adjusted for purity)

Stability of test item: 27 February 2017 (stored at ambient temperature). NB: stable during the conduct of the study

Storage conditions: At room temperature

Test method: The test item Pethoxamid technical was assessed for its potential to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The selection of the concentrations used in the main experiments was based on data from the pre-experiments up to a maximum concentration of 10 mM. Experiment I without and with metabolic activation and experiment II with metabolic activation were performed as a 4 h short-term exposure assay. Experiment II without metabolic activation was performed as a 24 h long-term exposure assay.

The test item was investigated at the following concentrations:
Experiment I without metabolic activation: 25, 50, 100, 150, 200, 250, 300 and 350 µM
Experiment I with metabolic activation: 50, 100, 150, 200, 250, 300, 350, 400 and 450 µM
Experiment II without metabolic activation:0.5, 1, 5, 10, 25, 50, 75 and 100 µM
Experiment II with metabolic activation:60, 120, 170, 230, 280, 330, 360 and 390 µM

According to OECD Guidelines at least 8 concentrations of the test item were set up in the experiments without and with metabolic activation.

The positive controls used for experiments without metabolic activation were EMS (ethylmethanesulfonate) and MMS (methylmethanesulfonate), and for experiments with metabolic activation B[α]P (benzo[α]pyrene).

The test item is considered mutagenic if the following criteria are met:
-The induced mutant frequency (IMF) meets or exceeds the Global Evaluation factor (GEF) of 126 mutants per 10^6 cells. The GEF is defined as the mean of the negative/vehicle mutant frequency plus one standard deviation; data are gathered from ten laboratories. For the microwell method the GEF was defined to be 126.
-A dose-dependent increase in mutant frequency is detected.

Besides, combined with a positive effect in the mutant frequency, an increased occurrence of small colonies (≥40% of total colonies) is an indication for potential clastogenic effects and/or chromosomal aberrations.

A test item is considered to be negative if the induced mutant frequency is below the GEF and the trend of the test is negative.

Results:
Toxicity: No precipitation of the test item was noted in the main experiments.

Growth inhibition was observed in experiment I and II without and with metabolic activation.

In experiment I without metabolic activation the relative total growth (RTG) was 16.7% for the highest concentration (350 µM) evaluated. The highest concentration evaluated with metabolic activation was 450 µM with a RTG of 8.7%.

In experiment II without metabolic activation the relative total growth (RTG) was 11.6% for the highest concentration (100 µM) evaluated. The highest concentration evaluated with metabolic activation was 390 µM with a RTG of 10.0%.

Mutagenicity: In experiments I and II no biologically relevant increase of mutants was found after treatment with the test item (without and with metabolic activation). The Global Evaluation Factor (GEF; defined as the mean of the negative/vehicle mutant frequency plus one standard deviation; data gathered from ten laboratories) was not exceeded by the induced mutant frequency (IMF) at any concentration.

EMS, MMS and B[α]P were used as positive controls and showed distinct and biologically relevant effects in mutation frequency.

Clastogenicity: Colony sizing was performed for the highest concentrations of the test item and for the negative and positive controls. An extension of the GEF by the induced mutant frequency in combination with an increased occurrence of small colonies (defined by slow growth and/or morphological alteration of the cell clone) is an indication for potential clastogenic effects and/or chromosomal aberrations.

In experiment I (without and with metabolic activation) and experiment II (without metabolic activation), colony sizing showed no clastogenic effects. In experiment II with metabolic activation a higher number of small colonies induced by the test item was noted. However, since no mutagenicity was found at that dose, all dose groups were considered as not clastogenic.

The positive controls MMS (8 and 10 µg/mL) and B[α]P (3.5 µg/mL) induced a significant increase in mutant frequency and a biologically significant increase of small colonies (≥40%), thus proving the ability of the test system to indicate potential clastogenic effects.

Table 3.8.1-2: Summary table: Experiment I and II, without metabolic activation

Experiment	**Group**	**Concn (µM)**	**RCE[a] (%)**	**RTG[b] (%)**	**MF[c] (mutants/10^6 cells)**	**IMF[d] (mutants/10^6 cells)**	**GEF[e] exceeded**	**Statistically significant increase [f]**	**Precipitate**
I	C1	0	100.0	100.0	62.2	/	/	/	-
	C2	0	100.0	100.0	52.1	/	/	/	-
	3	25	103.3	91.9	60.3	3.1	-	-	-
	4	50	122.7	110.6	51.9	-5.3	-	-	-
	5	100	100.0	50.6	57.6	0.5	-	-	-
	6	150	87.1	48.0	61.3	4.2	-	-	-
	7	200	105.0	50.6	100.9	43.8	-	+	-
	8	250	116.2	45.1	78.8	21.6	-	-	-
	9	300	92.5	19.5	104.1	47.0	-	+	-
	10	350	108.5	16.7	161.3	104.1	-	+	-
	EMS	300 µg/mL	77.4	58.5	670.0	612.8	+	+	-
	MMS	10 µg/mL	74.2	54.1	514.8	457.6	+	+	-
II	C1	0	100.0	100.0	53.3	/	/	/	-
	C2	0	100.0	100.0	52.7	/	/	/	-
	3	0.5	109.4	109.4	53.1	0.1	-	-	-
	4	1	93.2	81.1	60.8	7.8	-	-	-
	5	5	121.5	87.1	38.1	-14.9	-	-	-
	6	10	96.1	86.3	63.5	10.5	-	-	-
	7	25	121.5	93.2	34.6	-18.4	-	-	-
	8	50	107.6	49.2	33.5	-19.5	-	-	-
	9	75	100.8	29.1	59.3	6.3	-	-	-
	10	100	94.7	11.6	62.9	9.9	-	-	-
	EMS	200 µg/mL	57.9	30.5	1901.3	1848.3	+	+	-
	MMS	8 µg/mL	49.4	33.8	1018.6	965.6	+	+	-

C: Negative Controls

a: Relative Cloning Efficiency, RCE = [(CE $_{\text{dose group}}$ / CE $_{\text{of corresponding controls}}$) x 100]. Cloning Efficiency, CE = ((-LN (((96 - (mean P1,P2)) / 96)) / 1.6) x 100)

b: Relative Total Growth, RTG = (RSG x RCE)/100. Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]

c: Mutant Frequency, MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non-selective medium)]}x800

d: Induced Mutant Frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls

e: Global Evaluation Factor, GEF (126); +: GEF exceeded, -: GEF not exceeded

f: statistical significant increase in mutant frequency compared to negative controls (Mann Whitney test , $p<0.05$).

+: significant; -not significant

EMS: Ethylmethanesulfonate [200 and 300 µg/mL]

MMS: Methylmethanesulfonate [8 and 10 µg/mL]

Table 3.8.1-3: Summary table: Experiment I and II, with metabolic activation

Experiment	**Group**	**Concn (µM)**	**RCE[a] (%)**	**RTG[b] (%)**	**MF[c] (mutants /10^6 cells)**	**IMF[d] (mutants /10^6 cells)**	**GEF[e] exceeded**	**Statistically significant increase[f]**	**Precipitate**
I	C1	0	100.0	100.0	71.0	/	/	/	-
	C2	0	100.0	100.0	77.5	/	/	/	-
	4	50	102.3	96.6	57.9	-16.3	-	-	-
	5	100	146.9	119.4	26.5	-47.8	-	-	-
	6	150	112.1	70.4	42.0	-32.2	-	-	-
	7	200	112.1	71.3	55.8	-18.4	-	-	-
	8	250	117.5	68.7	97.0	22.8	-	-	-
	9	300	84.7	35.0	114.2	39.9	-	+	-
	10	350	112.1	41.5	73.1	-1.1	-	-	-
	11	400	105.4	24.9	108.0	33.8	-	-	-
	12	450	72.4	8.7	188.5	114.3	-	+	-
	B[α]P	3.5 µg/mL	76.6	37.2	546.9	472.7	+	+	-
II	C1	0	100.0	100.0	89.1	/	/	/	-
	C2	0	100.0	100.0	56.4	/	/	/	-
	2	60	90.2	84.5	69.4	-3.3	-	-	-
	3	120	119.0	94.3	85.5	12.7	-	-	-
	4	170	83.7	58.4	94.2	21.4	-	-	-
	5	230	91.6	48.6	98.8	26.0	-	-	-
	6	280	99.0	43.5	128.8	56.0	-	+	-
	7	330	88.9	22.0	152.9	80.2	-	+	-
	8	360	76.7	15.3	158.8	86.0	-	+	-
	9	390	85.0	10.0	153.5	80.7	-	+	-
	B[α]P	3.5 µg/mL	88.9	47.3	666.9	594.1	+	+	-
C: Negative Controls									

a: Relative Cloning Efficiency, RCE = [(CE $_{\text{dose group}}$ / CE $_{\text{of corresponding controls}}$) x 100]. Cloning Efficiency, CE = ((-LN (((96 - (mean P1,P2)) / 96)) / 1.6) x 100)

b: Relative Total Growth, RTG = (RSG x RCE)/100. Relative Suspension Growth, RSG = [(value SG / value SG of corresponding controls) x 100]

c: Mutant Frequency, MF = {-ln [negative cultures/total wells (selective medium)] / -ln [negative cultures/total wells (non-selective medium)]}x800

d: Induced Mutant Frequency, IMF = mutant frequency sample – mean value mutant frequency corresponding controls

e: Global Evaluation Factor, GEF (126); +: GEF exceeded, -: GEF not exceeded

f: statistical significant increase in mutant frequency compared to negative controls (Mann Whitney test , $p<0.05$).

+: significant; -not significant

B[*a*]P: Benzo[*a*]pyrene [3.5 µg/mL]

Conclusion: In conclusion, in the described mutagenicity test under the experimental conditions reported, the test item pethoxamid is considered to be non-mutagenic in the *in vitro* mammalian cell gene mutation assay (thymidine kinase locus) in mouse lymphoma L5178Y cells.

3.8.1.5 Anonymous (1992)

Reference: Mutagenicity study of pethoxamid in mammalian cells (V79) in vitro (HGPRT-test)
Author(s), year: Anonymous, 1992
Report/Doc. number: 77 PXA (TOX2001-292) / LPT 7328/92
Guideline(s): OECD 476 (1984)
GLP: Yes
Deviations from OECD 476 (1997): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: 0592; Purity: 98.6 %; Solvent: DMSO.

Test method: Cultured mammalian cells (V79). The cells were exposed to the test substance, both in the presence and absence of S9 mix (rat Aroclor) in two independent experiments. Without S9 mix, cells were exposed for 24 hours, while in the presence of S9 mix, the exposure was limited to two hours. From a preliminary test, it was concluded that the test compound was completely cytotoxic from 30 mg/ml onwards without S9 mix, and from 1000 mg/mL onwards with S9 mix. The following concentrations were used: 0, 1, 3, 10, 20, 30 µg/mL (triplicate cultures without S9 mix) and 0, 10, 30, 100, 200, 300 µg/mL (triplicate cultures with S9 mix). Positive controls were included.

Results: Cytotoxicity was observed starting at concentrations between 20 and 30 mg/mL without (between 100 and 200 mg/mL) and with metabolic activation. The V79 cells tested with the test substance showed mutation frequencies within the normal range of the solvent controls at any dose level, in the presence or absence of S9 mix. No significance was observed according to the criteria for assay evaluation. The positive control compounds produced clear increases in mutation frequency.

Table 3.8.1-5: Results of HGPRT test

Pethoxamid concentration (µg/mL)	S9-mix	Plating efficiencies				Mutation frequency[1] x10^{-6}	
		Experiment 1		Experiment 2		Experiment 1	Experiment 2
		PE_1	PE_2	PE_1	PE_2		
0	-	0.87	0.70	0.96	0.89	7.4	4.7
1	-	1.18	0.98	0.79	1.08	6.9	4.3
3	-	0.72	0.53	0.74	0.97	3.4	4.7

10	-	0.70	0.69	0.62	0.80	4.3	4.8
20	-	1.01	0.73	0.73	0.94	3.8	4.0
30	-	0.34	0.64	0.17	0.69	3.1	12.2
EMS (600)	-	<0.10	0.82	0.05	0.42	250.7	1002.4
EMS (700)	-	<0.10	0.71	0.11	0.34	206.8	1281.6
0	+	1.08	0.83	0.73	0.65	2.4	5.5
10	+	0.80	0.54	0.72	0.82	8.1	4.9
30	+	0.88	0.88	0.83	0.97	7.5	8.9
100	+	0.92	0.90	0.88	0.61	5.6	6.6
200	+	0.69	0.78	0.77	0.81	7.2	8.4
300	+	0.41	0.90	0.33	0.85	5.3	11.5
DMBA (20)	+	0.61	0.66	0.72	0.73	341.2	301.4
DMBA (30)	+	0.54	0.74	0.54	0.68	201.1	226.8

PE1: Plating efficiency at the end of the exposure time; PE2: Plating efficiency at the time of selection; [1] Mutation frequency defined as total number of thioguanine-resistant cells; EMS: Ethyl methanesulphonate (positive control); DMBA: 9,10-dimethyl-1,2-benzanthracene (positive control), HGPRT: Hypoxanthine-guanidinephosphoribosyl-transferase

Conclusion: Pethoxamid did not show any evidence of exerting mutagenic effects in the HGPRT-test with V 79 cells (forward mutation system).

3.8.2 Animal data

3.8.2.1 Anonymous (1994)

Reference: Pethoxamid: Mouse micronucleus test
Author(s), year: Anonymous, 1994
Report/Doc.number: 78 PXA (TOX2001-293) / TKS '25/941500
Guideline(s): OECD 474 (1983)
GLP: Yes
Deviations from OECD 476 (1997): - The presence of micronuclei in 1000 polychromatic erythrocytes instead of at least 2000 was evaluated
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %.

Test animals: Four groups of 15 female and 15 male CD-1 mice of Swiss origin, specific pathogen free, 22-26 grams of weight, approximately 35 days old, from Charles River (UK) Limited, Margate, Kent, England.

Test method: Based on the results from a preliminary test, single doses of 0, 320, 640 and 1280 mg/kg bw were used. The mice were dosed by intragastric gavage and groups of 5 female and 5 male mice were killed 24, 48 and 72 hours after dosing. The mice of the positive control group were sacrificed after 24 hours. The incidence of micronucleated cells and the proportion of polychromatic erythrocytes was assessed for each animal.

Results:

Mortality: Eight female and 3 male mice died after treatment with the high dose. At post-mortem examination, none of these animals showed signs of misdosing. As at the highest dose 5 extra female

and 5 extra male mice were treated, 10 animals were replaced as follows: 5 females, 2 males instead of 8 females and 3 males.

Clinical signs: Within the first hour after dosing in all groups, included hunched posture and piloerection. Recovery for the low and intermediate dose groups was within 3 hours. The high dose group also showed lethargy and ptosis. Recovery was complete in all mice by the end of Day 1.

Erythrocyte count: No significant increase in the number of micronucleated immature erythrocytes was observed at 24, 48 or 72 hours. No effect was observed on the proportion of immature erythrocytes, indicating no bone marrow toxicity. The positive control group did show an increased frequency of micronucleated cells.

Table 3.8.2-1: Results of the micronucleus test

Treatment	**Dose level (mg/kg bw)**	**Sampling time**	**Frequency of micronucleated polychromatic cells[1]**		**P:M ratio**	
			mean incidence	**range**	**mean ratio**	**range**
Methylcellulose	1%	24 h	0.9	0-2	0.817	0.464-1.343
Pethoxamid	320		0.8	0-2	0.894	0.591-1.270
	640		0.6	0-2	0.974	0.645-1.574
	1280		1.2	0-5	0.940	0.495-1.242
Mitomycin C	12		55.1*	30-95	0.839	0.474-1.087
Methylcellulose	1%	48 h	0.7	0-3	0.932	0.616-1.440
Pethoxamid	320		0.5	0-2	0.843	0.618-1.098
	640		0.6	0-2	0.820	0.488-1.197
	1280		0.8	0-	0.768	0.328-1.352
Methylcellulose	1%	72 h	0.6	0-2	0.993	0.644-1.415
1.415Pethoxami0.451-1.783d	320		1.3	0-4	0.946	0.451-1.783
	640		1.1	0-3	1.163	0.554-2.146
	1280		0.4	0-2	0.937	0.533-1.477

[1] Number of micronucleated cells observed per 1000 immature erythocytes examined; P:M: Proportion of immature (polychromatic) erythrocytes to mature (normochromatic) erythrocytes; Methylcellulose (vehicle); Mitomycin C (positive control);

Statistical significance: * p<0.001

Conclusion: Pethoxamid did not show any evidence of chromosomal or other damage leading to micronucleus formation in this *in vivo* test.

3.8.2.2 Anonymous (1994)

Reference: Pethoxamid: In vivo rat liver DNA repair test;
Author(s), year: Anonymous, 1994
Report/Doc. number: 79 PXA (TOX2001-294) / TKS '25/941500
Guideline(s): OECD 482 (1986), OECD 486 (1997, Draft)
GLP: Yes
Deviations from OECD 486 (1997): No
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-930727; Purity: 95 %.

Test animals: Albino Hsd/Ola Sprague-Dawley male rats, specific pathogen free, 140-149 grams of weight and five weeks old, from Harlan Olac (UK), LTD., Bicester, Oxon, England.

Test method: From the results of a preliminary toxicity study, in which minor toxicity at the maximum dose level of 2000 mg/kg bw was observed, single doses of 600, 1200 (provisional group, not evaluated for unscheduled DNA synthesis) and 2000 mg/kg bw were used. Groups of 4 rats were administered the test compound orally by intragastric gavage. Hepatocytes were isolated at 2 and 14 hours after the administration. Positive controls were included. Gross nuclear grain counts (silver grains overlying the nucleus) and net nuclear grain counts (cytoplasmatic grain count subtracted from gross nuclear grain count) were assessed. On completion of the grain count analysis, the stained autoradiographs from the 14 hours expression were recorded for S-phase cells (for each animal 1000 hepatocytes from several randomly fields of view).

Results:
Mortality and clinical signs: No mortalities occurred. For the 2 hours expression time group within the first hour after dosing, slight piloerection was observed at 600 and 2000 mg/kg bw. Recovery was complete by the second hour. No clinical signs were recorded for any of the rats in the 14 hours expression time group.
Grain count: The test compound did not cause any significant increase in either the gross or net nuclear grain count at both dose levels at the 2 hours expression time At the 14 hours expression time, statistically significant increases in gross nuclear grain counts were obtained at dose levels of 600 and 2000 mg/kg bw but not in net nuclear grain counts, i.e. not indicative for unscheduled DNA synthesis. Positive control group animals showed a significant increase in the gross and net nuclear grain count.

During analysis, it was noted that some slices had an unusually high number of S phase cells (easily recognizable by their heavily grained appearance). Therefore for all groups (including the provisional group) the S-phase cells after 14 hours of expression were determined. Only the group which received 600 mg/kg bw showed statistical significance. The increases of S-phase cells are indicative of an increased cell proliferation.

Table 3.8.2-2: Results of the liver DNA repair test

Treatment	Dose level (mg/kg bw)	Expression time	Nuclear grain count		S-phase cells (cells/1000 hepatocytes)
			Mean gross	Mean net	
Methylcellulose	1%	2 h	15.5	-3.7	nd
Pethoxamid	600		15.6	-2.6	nd
	2000		16.2	-4.1	nd
Dimethylnitrosamine	4		53.8**	39.00**	nd
Methylcellulose	1%	14 h	8.7	-2.7	9.3

Pethoxamid	600		12.4**	-2.0	98.8**
	1200		nd	nd	47.0
	2000		11.3*	-2.3	47.3
2-Acetylaminofluorene	50		26.3**	15.9**	20.0[1]

Methylcellulose (vehicle); Dimethylnitrosamine (positive control); 2-Acetylaminofluorene (positive control); nd: not determined;

[1] not statistically evaluated because of insufficient variance to allow analysis;

Statistical significance: * p<0.01, ** p<0.001

Conclusion: Pethoxamid did not elicit unscheduled DNA synthesis in the rat liver in this *in vivo* test system.

3.8.3 Human data

No relevant studies.

3.8.4 Other data

No relevant studies.

3.9 Carcinogenicity

3.9.1 Animal data

3.9.1.1 Anonymous (2000a)

Reference: TKC-94 Pethoxamid: Potential tumorigenic and toxic effects in prolonged dietary administration to rats
Author(s), year: Anonymous., 2000a
Report/Doc. number: 80 PXA (TOX2001-295) / TON 6/974064
Guideline(s): OECD 453 (1981)
GLP: Yes
Deviations from OECD 453 (2009): Haematology: mean corpuscular hemoglobin (MCH) not tested; No organ weight of uterus; No preservation of the coagulating gland, peripheral nerve
Acceptability: Yes

Reference: Amendment 1 toTKC-94 Pethoxamid: Potential Tumorigenic and Toxic Effects in Prolonged Dietary Administration to Rats
Author(s), year: Anonymous., 2000-amdt-1
Report/Doc. number: 80 PXA amdt-1 (TOX2001-295) / TON 6/974064
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

In addition to the usual investigations, electron microscopical investigations of liver tissue were performed on satellite groups (0 and 1600ppm). Results were integrated in the main study report (L. Anonymous (2000a, 80 PXA (TOX2001-295) / TON '6/974064))

Reference: Supplement 1 to TKC-94/Pethoxamid - TON 6/974064: Organ weight data report
Author(s), year: Anonymous, 2003

Report/Doc. number: 80 PXA suppl-1 (TOX2001-295)
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Raw data of individual organ weights of animals used in Anonymous (2000a, 80 PXA (TOX2001-295) / TON '6/974064).

Reference: Supplement 2 to Historical histopathology data and historical organ weight data
Author(s), year: Anonymous, 2016
Report/Doc. number: 80 PXA suppl-2
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Historical Control Data is presented for studies starting between 1996 and 1999 which is considered relevant for Anonymous (2000a;80 PXA). Data on neoplastic findings in thyroid and pancreas, as well as organ weight of thyroids are presented.

Reference: Position paper - Pethoxamid: EU-Wirkstoffprüfung zur Aufnahme von Wirkstoffen in Anhang I der Richlinie 91/414/EWG - Neuer Wirkstoff: Pehoxamid, Kenn-nr. WNL 005072-00/00 : Erstellung eines Monographie-Addendums (Sektion Toxicologie und Metabolismus)
Author(s), year: Anonymous, 2003
Report/Doc. number: Cheminova A/S Report No.: 203 PXA Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Calculations of thyroid weights relative to body weights were presented. Furthermore, statistical analyis of pancreas islet cell tumour rates were shown (pair wise comparison; trend test)

Deviations from study protocol: On occasions, the temperature and humidity exceeded the pre-set limits, which were generally transient. Diet usage records indicate that some rats from the 25 ppm group may have been fed small amounts of the diet containing 400 ppm for a maximum of 3 days. These deviations were not considered to affect the integrity and/or validity of the study.

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306; Purity: 95.0 %.

Test animals: Four groups of 80 male and 80 female Crl:CD BR (IGS) rats, 43 days old, 176-260 g (males) and 128-189 g (females) of weight, from Charles River Laboratories, Manston Road, Margate, Kent, UK. The main groups (50 rats/sex/group) received the test material by dietary administration at concentrations of 0, 25, 400 and 1600 ppm for 104 weeks. The doses were based on the 13 weeks study (Report no. TKS 24/961565). From the satellite groups (30/sex/group), up to 10 rats/sex/group were pathologically examined after the completion of 26, 52 and 78 weeks of treatment.

In order of increasing doses the treated rats ingested the equivalent of 1.0, 17.0 and 70 mg/kg bw/day for males and 1.4, 23.3 and 99 mg/kg bw/day for females.

All animals were subjected to body weight, clinical sign, food and water consumption, ophthalmoscopy, haematology, biochemistry, urinalysis, organ weight, macro- and microscopic pathology assessments.

Furthermore, electron microscopy was performed with liver tissue from satellite groups sacrificed after 26 and 52 weeks of treatment.

The homogeneity and stability of the formulation was regularly checked by chemical analysis.

Analytical chemistry: The mean concentrations of pethoxamid in the test diet formulations were between +9.5% (one exception +15.6%) and -13 % of nominal concentrations which were within the acceptable limits (+10%/-15%).

Mortality, clinical and ophthalmologic findings: Neither treatment related deaths nor treatment related clinical and ophthalmologic signs occurred.

Body weight, body weight gain and food consumption: The body weights at the terminal kill were not significantly different from control values. Lower body weight gains were measured from 400 ppm, significant at 1600 ppm. For females, a treatment-related effect at 400 ppm cannot be excluded in view of the consistency between the main and satellite groups and the magnitude of differences from control values.

At 400 ppm, the males showed a significant lower food intake only during the first 4 weeks of the study. At 1600 ppm, the lower food intake was slight but consistent throughout the study. At this dose, the females consumed less food only during Week 1 to 4.

Table 3.9.1-1: Body weight, body weight gain and food consumption data

Parameter	Dose (ppm)							
	Males				Females			
	0	25	400	1600	0	25	400	600
Body weight terminal kill (g)	771	785	748	722	466	526	509	440
Body weight gain (g/rat) Week 0-4 (main)	176	178	170	149**	75	77	74	64**
Body weight gain (g/rat) Week 0-88 (main)	631	635	612	547**	376	377	336	290**
Body weight gain (g/rat) Week 0-104 (main)	560	579	540	506	308	375	349	283
Body weight gain (g/rat) Week 0-4 (satellite)	182	178	170	150**	72	78	71	66*
Food consumption (g/rat) Week 1-4 (main)	851	837	814**	779**	591	577	568	556**
Food consumption (g/rat) Week 1-104 (main)	21387	21034	20860	20121**	16483	16640	16140	15950
Food consumption (g/rat) Week 1-4 (satellite)	847	825	824	792**	577	581	563	550**
Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$								

Haematology: At 1600 ppm males showed consistently lower mean reticulocyte values. However, in the absence of a similar finding for females or any corroborative blood film or microscopic findings, this difference in males was not considered to be of toxicological importance.

Clinical chemistry and urinalysis: At 1600 ppm in all weeks of investigations, both sexes showed statistically significantly higher mean cholesterol values, except for males in Week 78. Males showed

statistically significantly higher g-GT activities values in all weeks of investigation and females showed slightly, but consistently higher mean globulin and concomitant total protein values, statistical significance being attained for the globulin values except for Weeks 78 and 104.

At urinalysis, no treatment-related findings were observed.

Table 3.9.1-2: Clinical chemistry

Parameter	**Week**	**Dose (ppm)**							
		Males				**Females**			
		0	25	400	1600	0	25	400	1600
Cholesterol (mg/dL)	13	87	75	84	122**	81	93	85	108**
	26	98	82	90	119*	90	105	102	127**
	52	108	95	100	130*	107	114	109	156**
	78	121	111	125	141	122	114	129	178*
	104	111	128	139	158*	128	123	153	187*
γ-glutamic transferase (mU/mL)	13	<1	<1	<1	<4**	1	1	1	1
	26	<2	<1	<2	<3**	2	1	1	2
	52	<2	<1	<2*	4**	2	2	2	2
	78	<1	<1	<1	<4**	1	1	1	2
	104	<1	<2	<2	<8**	1	1	1	1
Globulin (g/dL)	13	3.8	3.8	3.7	4.0	3.5	3.7	3.5	3.8*
	26	3.8	3.8	3.7	3.8	3.5	3.6	3.5	3.8*
	52	4.1	3.9	4.1	4.1	3.8	3.9	3.9	4.3**
	78	4.3	4.1	4.0	4.1	3.9	3.8	3.9	4.1
	104	3.9	3.9	4.0	3.9	4.0	3.9	4.1	4.3
Total protein (g/dL)	13	6.8	6.8	6.7	7.0	6.9	7.1	6.0	7.2
	26	6.7	6.6	6.6	6.6	7.0	7.3	7.0	7.3
	52	7.1	7.1	7.2	7.2	7.7	7.8	7.9	8.5**
	78	7.3	7.1	6.9	7.2	7.8	7.6	7.7	7.9
	104	6.4	6.4	6.6	6.6	7.3	7.0	7.3	7.6
Statistical significance *p<0.05; **p<0.01									

Pathological examinations:
Necroscopy: Only the male rats from the main group revealed changes at the high dose

Table 3.9.1-3: Necropsy findings (decedent and terminal sacrifice animals)

Finding	**Dose (ppm)**							
	Males				**Females**			
	0	25	400	1600	0	25	400	1600
Liver, enlarged	2/50	4/50	1/50	6/50	2/50	1/50	4/50	2/50

Thyroid, enlarged	4/50	8/50	6/50	12/50	4/50	2/50	3/50	2/50

Organ weights: The higher thyroid weights in treated animals at some occasions - thus not consistent with time **-** were not supported by any microscopic or biochemical changes. In contrast, the generally increased liver weights at the high dose were accompanied by the typical histopathological findings.

Table 3.9.1-4: Organ weights

Organ		**Week**	**Dose (ppm)**							
			Males				**Females**			
			0	25	400	1600	0	25	400	1600
Liver	adjusted (g)	27	24.5	23.5	24.3	26.9* +10%	11.8	11.8	12.2	13.8** +17%
		53	25.1	25.0	24.4	28.1* +12%	14.6	13.6	12.5	14.9 +2%
		79	25.0	23.6	26.3	29.1 +16%	16.7	16.2	14.9	18.7 +12%
		105	25.6	25.1	25.8	28.5 +11%	17.5	17.7	18.0	20.3** +16%
Thyroid	absolute (mg)	53	29.6	38.3 +30%	35.3 +19%	38.7 +30%	25.7	27.2	29.5	24.3
	adjusted (mg)		26.9	37.1** +38%	37.5** +39%	40.3** +50%	25.6	26.2	28.8	26.0
	relative (%)		3.7	5.0 +35%	5.1 +38%	5.5 +49%	6.8	6.9	7.5	7.0
	absolute (mg)	79	35.9	35.8	50.0 +39%	44.5 +24%	38.1	30.0	31.0	34.4
	adjusted (mg)		34.4	36.3	51.4* +49%	45.1** +31%	n.a	n.a	n.a	n.a
	relative (%)		4.4	4.7	6.7 +52%	5.8 +32%	7.5	6.1	7.1	8.4
	absolute (mg)	105	61.6	45.1	49.9	51.7	41.8	43.2	36.9	36.6
	adjusted (mg)		n.a	n.a	n.a	n.a	42.2	41.4	35.8	37.9
	relative (%)		8.0	5.7	6.7	7.2	9.0	8.2	7.2	8.3
n.a: not applicable Statistical significance: *≤ 0.05, **≤ 0.01										

Table 3.9.1-5: Historical Control Data - Range of thyroid weights from performing laboratory

Organ weight	Week	Male	Female
Absolute weight (mg)	52	31.3-35.0	27.2-32.0
Realative weight (%)	52	4.3-5.1	7.1-7.6
Absolute weight (mg)	104	40.0-61.3	35.0-44.8
Realative weight (%)	104	5.5-8.9	7.2-8.9
Week 52 data derived from 2 studies performed in 1997 and 1999 Week 104 data derived from 10 studies performed between 1996 and 1999			

Histopathology, interim kills: At the high dose, both sexes showed a statistically significant increased incidence of centrilobular hepatocyte hypertrophy in Week 26, males also in Weeks 52 and 78. Males also showed an increased incidence of concentric intracytoplasmic inclusions (mainly periportal zones) in Weeks 26, 52 and 78. The light microscopic findings were supported by electron microscopy, done at Weeks 26 and 52, in which an increase of smooth endoplasmic reticulum was found (centrilobular: minimal for both sexes; periportal: minimal for females and moderate for males) and in which the inclusions in males were recognized as multilamellar.

Histopathology, terminal kill - neoplastic findings: At 1600 ppm, a slightly higher incidence of follicular cell adenomas of the thyroid was seen in male rats. The incidence falls outside the background control range. The test for trend was statistically significant. The pairwise comparison between control and high dose groups (adenoma and carcinoma combined) was not statistically significant. The incidence of follicular carcinoma was two in the control and zero in all treated groups. The adenomas were all within a similar morphological and size range and occurred generally singly in the affected animals.

Slightly higher incidences of pancreas islet cell tumours were seen in main group male rats from the 25ppm and 1600ppm groups compared to controls. However, no statistically significant differences were noted in the incidences and distribution, and the incidences were not dose dependent. Though the findings were slightly above the range of historical control data, they were considered to be incidential and not related to treatment.

Histopathology, terminal kill - non-neoplastic findings: The main groups - as the satellite groups - showed hepatocyte hypertrophy. Associated findings were focal clear cell hepatocytes, focal cystic degeneration, and hepatocytes with concentric intracytoplasmic inclusions (mainly in periportal zones).

In the thyroid, a small and not significant increase in the incidence of follicular cell hyperplasia and follicular cell cystic hyperplasia was seen in male rats at the high dose.

No treatment related effects were recognized to be contributory to death.

Table 3.9.1-6: Histopathology findings

Finding	Dose level (ppm)							
	Males				Females			
	0	25	400	1600	0	25	400	1600
Neoplastic findings								
Thyroid								

- Follicular cell adenoma, main[1]	2/50 4%	2/50 4%	2/49 4.1%	9/50[2] 18%	1/50	0/50	2/50	2/50
- Follicular cell carcinoma, main[1]	2/50	0/50	0/49	0/50	0/50	0/50	0/50	0/50
Pancreas								
- Islet cell adenoma, main[1]	4/49 8.2%	8/50 16%	6/50 12%	8/50 16%	3/50	3/40	2/33	2/50
- Islet cell carcinoma, main[1]	0/49 0%	2/50 4%	1/50 1%	2/50 4%	0/50	0/40	0/33	0/50
Non-neoplastic findings								
Thyroid								
- Follicular cell hyperplasia	0/50	0/50	1/49	4/50	0/50	0/50	0/50	0/50
- Follicular cell cystic hyperplasia	4/50	1/50	4/49	7/50	1/50	0/50	0/50	1/50
Liver								
- Centrilobular hepatoctic hypertrophy, main	0/50	0/50	0/50	11/50**	0/50	0/50	0/50	8/50**
- Centrilobular hepatoctic hypertrophy, satellite, week 26	0/10	0/10	0/10	7/10	0/10	0/10	0/10	5/10
- Centrilobular hepatoctic hypertrophy, satellite, week 52	0/10	0/10	0/10	6/10	0/10	0/10	0/10	0/10
- Centrilobular hepatoctic hypertrophy, satellite, week 78	0/10	0/9	0/4	3/8	0/7	0/8	0/8	0/10
- Concentric intracytoplasmic inclusions, main[1]	0/50	0/50	0/50	10/50**	0/50	0/50	0/50	0/50
Concentric intracytoplasmic inclusions, week 26	0/10	0/10	0/10	4/10	0/10	0/10	0/10	0/10
- Concentric intracytoplasmic inclusions, main[1], week 52	0/10	0/10	0/10	5/10	0/10	0/10	0/10	0/10
- Concentric intracytoplasmic inclusions, main[1], week 78	0/10	0/9	0/4	2/8	0/7	0/8	0/8	0/10
- Cystic degeneration, main[1]	12/50	5/50	14/50	24/50*	1/50	2/50	0/50	0/50
- Clear cell hepatocytes, main[1]	6/50	6/50	6/50	15/50*	3/50	5/50	4/50	2/50
[1] Decedents and terminal sacrificed								

[2] Trend test statistically significant; Statistical significance: *≤ 0.05, **≤ 0.01

Table 3.9.1-7: Historical control data from performing laboratory

Finding	Males		Females	
	Totals (affected/examined)	Range	Totals (affected/examined)	Range
Thyroid				
- Follicular cell adenoma	30/954; 3.14%	0.0-12%	16/951; 1.68%	0.0-8.3%
- Follicular cell carcinoma	7/954; 0.73%	0.0-5.1%	4/951; 0.42%	0.0-1.7%
Pancreas				
- Islet cell adenoma	85/954; 8.91%	1.7-13.8%	not available – not necessary	
- Islet cell carcinoma	30/954; 3.14%	0.0-11.7%	not available – not necessary	
Historical control data derived from 16 studies performed between 1996 and 1999				

Conclusion: The NOAEL is considered to be 25 ppm (1.0 mg/kg bw/d), based on the decreased body weight gain in females at 400 ppm (approximately 11% compared with the control group; weeks 0 - 88) which has been evaluated as an adverse effect. The increase of thyroid weights in males (weeks 53 and 79) is considered to be caused by a rodent-specific mechanism which is not relevant to humans. This effect is therefore not considered for setting a NOAEL for this study.

The only evidence of tumourigenicity was a higher incidence of thyroid follicular cell adenoma in males at the high dose level of 1600 ppm. Mechanistic studies have postulated a phenobarbitone-like mode of action for pethoxamid leading to increased incidences of thyroid follicular cell tumours, particularly in the male rat. Due to differences in thyroid physiology between rodents and humans, thyroid tumours in rodents consequent to the phenobarbitone-like mode of action are not considered relevant to humans.

According to the criteria specified in Regulation (EC) 1272/2008, **classification of pethoxamid regarding carcinogenicity is not required.**

3.9.1.2 Anonymous (2000b)

Reference: Pethoxamid: Carcinogenicity study by administration to CD-1 mice for at least 80 weeks
Author(s), year: Anonymous, 2000b
Report/Doc. number: 82 PXA (TOX2001-296) / TON 014/973848
Guideline(s): OECD 453 (1981)
GLP: Yes
Deviations from OECD 453 (2009):
- slight exceedance of weight variation in females
- no organ weights of spleen, uterus
- no preservation of the coagulating gland, peripheral nerves
- haematology: only blood smears were prepared
- no clinical biochemistry measurements conducted

Acceptability: Yes

Reference: Supplement 1, Historical histopathology data CD-1 Mice studies of 78 to 106 weeks duration

Author(s), year: Anonymous, 2016
Report/Doc. number: 82 PXA suppl-1
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

Historical Histopathology Data is presented for studies starting between 1996 and 1999 which is considered relevant for Anonymous (2000b; 82 PXA). Data on neoplastic findings in the liver of male mice are presented.

Reference: TKC-94 : Carcinogenicity Study by Administration to CD-1 Mice for at least 80 Weeks. Photomicrographic Report
Author(s), year: Anonymous, 2001
Report/Doc. number: 83 PXA (TON 082/000175)
Guideline(s): -
GLP: Yes
Deviations: -
Acceptability: Yes

The objective of this study was to illustrate microscopic findings in the duodenum and jejunum of selected animals from the carcinogenicity study by administration to CD-1 mice for at least 80 weeks. Low and high magnification photomicrographs of the duodenum and jejunum of selected animals, killed at termination, are presented.

Reference: Position paper. Carcinogenicity in mice statement
Author(s), year: Anonymous, 2001
Report/Doc. number: 1484 PXA
Guideline(s): -
GLP: -
Deviations: -
Acceptability: Yes

A statement is given regarding the gastro-intestinal effects in the long-term toxicity study in mice (Anonymous (2000b); 82 PXA). It is stated that the metabolism study revealed that 33 to 66% of Pethoxamid is excreted by faeces, therefore a huge amount of metabolites is passing the gastro-intestinal tract. As the same findings are observed in the satellite group (terminated at week 52) and the main group (terminated at week 95/92) the severity of effects has not been increased between termination of the satellite and the main group. Moreover, no cancer could be observed in that region. This occurrence did not shorten the life of animals. It is further stated that Pethoxamid is assumed to be a Phenobarbital type inducer and this reaction might be an adaptive response to the hepatic enzyme induction. Furthermore, this is claimed a rodent-specific effect and not of concern for humans.

Reference: TKC-94 : Additional study on cell proliferation in the liver to Carcinogenicity Study by dietary Administration to CD-1 Mice for at least 80 Weeks
Author(s), year: Anonymous, 2001
Report/Doc. number: 1241 PXA / IET 00-0138
Guideline(s): -
GLP: Yes
Deviations: -
Acceptability: Yes

In order to evaluate the effect of pethoxamid on hepatic cell proliferation, paraffin sections of the liver obtained from the original paraffin blocks used in the carcinogenicity study of the test substance by dietary administration to CD-1 mice for at least 80 weeks (TON 014) were subjected to immunohistochemistry for proliferating cell nuclear antigen (PCNA). PCNA labeling index (LI) was determined for each animal by counting the number of PCNA-positive (S-phase) cells per approximately 1000 hepatocytes. Cell proliferation was evaluated on the basis of PCNA LI for each dose group at 0, 30, 400, and 5000 ppm after 52 and 95 weeks of treatment. There were no significant changes in PCNA LI considered to be treatment-related in any dose group after 52 or 95 weeks of treatment. Based on the results described above, it has been suggested that TKC-94 appears to have no influence on cell proliferation in the liver when administered in feed to male mice at 30, 400 and 5000 ppm for 52 or 95 weeks.

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306 and TB-960306-C; Purity: 95.0% and 94.8%.

Test animals: Groups of 60 male and 60 female CD-1 BR mice, 6 weeks old and 25-37 g (males) and 19-30 g (females) of weight, from Charles River Laboratories, Margate, Kent, England.

Test method: The mice received the test material by dietary administration at concentrations of 0, 30, 400 or 5000 ppm. Main group animals (50/sex/group) were treated until one of the groups in each sex reached 50% survival rate, namely for up to 92 weeks (females) or 95 weeks (males) for assessment of tumorigenic potential. Satellite animals (10/sex/group) were treated for up to 52 weeks and were used for assessment of chronic toxicity. In order of increasing doses the treated mice ingested the equivalent of 4.0, 56.8 and 982 mg/kg bw/d for males and 5.0, 68 and 1068 mg/kg bw/d for females.

All animals were subjected to body weight, clinical signs, food consumption, haematology (blood smears), organ weights, macro- and microscopic pathology assessments.

The treatment levels were based on the results from the 4-week (Report no. TON 3/960337) and the 13 week study (Report no. TON 5/971279).

The homogeneity and stability of the formulation was checked by chemical analysis.

Results:

Analytical chemistry: Analysis indicated that the samples taken from the formulations were within 10% deviation of nominal values.

Mortality and clinical signs: There was no effect of treatment on survival. No clinical signs were observed, indicative of a reaction to treatment.

Body weight, body weight gain and food consumption: The body weights were significantly lower in the animals of the high dose groups at the end of the study. As well, throughout the treatment period, the body weight gains were significantly decreased. This was accompanied for males by a statistically significant higher food consumption.

Table 3.9.1-8: Body weight development and food consumption

Parameter	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Body weight (g)[1]	50	53	51	42**	41	42	44	34**

Body weight gain (g/mouse) – week 0-92/95 (main)[1]	19.1	22.2	19.2	11.5**	17.2	18.0	19.2	11.3**
Body weight gain (g/mouse) – week 0-52 (satellite)	23.0	18.1	24.6	9.1**	21.3	13.9	15.4	9.4**
Food consumption (g/mouse)	4579	4512	4687	5527**	4365	4025	4348	4598
[1] Males at Week 95, females at Week 93 Statistical significance: *p<0.05; **p<0.01								

Pathological examinations:
Necroscopy: A higher incidence of liver masses, granulation of the kidneys and kidney cysts were noted in males at 5000 ppm at the end of the study. No treatment-related findings were noted at the interim kill.

Table 3.9.1-9: Macroscopic pathology, terminal kill

Finding	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Liver								
- masses	11/27	13/28	12/25	25/34	1/25	2/30	0/31	0/32
Kidneys								
- granulation	0/27	1/28	2/25	17/34	0/25	0/30	1/31	0/32
- cysts	4/27	2/28	6/25	9/34	0/25	0/25	0/25	0/25

Organ weights: From 400 ppm, the adjusted liver and kidney weights were increased and additionally at 5000 ppm, the thyroid weights were increased in both sexes and the adrenal weights in males.

Table 3.9.1-10: Organ weights

Parameter	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Liver (g), terminal, adjusted[2]	2.70	2.88 +7%	3.09 +14%	4.78** +77%	2.01	2.02	2.22* +10%	2.73** +36%
Liver, interim, adjusted[2]	2.64	2.61	2.60	3.37** +28%	1.97	2.04	2.21 +12%	2.58* +31%
Kidney (g), term., adjusted[2]	0.915	0.890	0.951	0.992	0.511	0.522 +2%	0.569** +11%	0.629** +23%
Kidney, interim, adjusted[2]	0.930	1.029	0.893	0.982	0.496	0.558 +13%	0.552 +11%	0.592* +19%
Thyroid (mg), terminal, absolute	7.0	6.7	7.2	8.5*	7.8	8.0	7.9	9.7

				+21%				+24%
Thyroid, terminal, adjusted[2]	na	na	na	na	7.8	7.8	7.6	10.1** +29%
Adrenals (mg), terminal, adjusted[2]	7.0	6.7	7.0	8.8* +26%	na	na	na	na
[2] Adjusted for body weight; na not applicable Statistical significance: *p<0.05; **p<0.01								

Histopathology, interim kill: Hepatocyte hypertrophy was observed in females at 400 ppm. At the high dose, it was seen in both sexes. Some males showed at 30 ppm intestinal swelling and rarefaction of villous epithelial cells in the duodenum and jejunum. At the higher doses, the finding occurred in both sexes and was in males accompanied by hypertrophy. From 400 ppm, a not dose related and not significant increase in follicular cell hypertrophy in the thyroid was observed.

Table 3.9.1-11: Microscopic pathology, interim kill

Finding	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Liver								
hepatocyte hypertrophy, generalised	0/8	0/8	0/10	9/9***	0/10	0/8	0/10	0/10
hepatocyte hypertrophy, periportal	0/8	0/8	0/10	0/9	0/10	0/8	2/10	9/10***
Duodenum								
swelling/rarefaction of villous epithelium	0/8	5/8*	8/10**	9/9***	0/10	0/8	6/10*	10/10***
villous hypertrophy	0/8	0/8	2/10	7/9**	0/10	0/8	0/10	7/10**
Jejunum								
swelling/rarefaction of villous epithelium	0/8	4/8	6/10*	8/9***	0/10	0/8	5/10*	10/10***
villous hypertrophy	0/8	0/8	3/10	6/9**	0/10	0/8	0/10	7/10**
Thyroids								
follicular cell hypertrophy	2/8	0/8	5/10	5/9	2/10	1/8	2/10	7/10
Statistical significance: *p<0.05; **p<0.01; *** p<0.001								

Histopathology, terminal kill - neoplastic findings: At 5000 ppm, treatment-related hepatocellular adenomas appeared in male mice. There was also a slightly higher but not statistically significant number of malignant hepatocellular tumors.

Table 3.9.1-12: Microscopic pathology – neoplastic findings, terminal kill

Finding	**Dose (ppm)**	
	Males	**Females**

	0	30	400	5000	0	30	400	5000
Liver number examined	50	50	50	50	50	50	50	50
- hepatocellular adenoma	19 (38%)	15 (30%)	18 (36%)	34** (68%)	3	1	3	0
- hepatocellular carcinoma	3 (6%)	3 (6%)	4 (8%)	6 (12%)	1	0	0	0

Histopathology, terminal kill - non-neoplastic findings: At 5000 ppm, hepatocyte hypertrophy (generalized in males and periportal in females) was seen in both sexes. It is likely that the hypertrophy was due to the induction of drug metabolizing hepatic enzymes. At both kills, the severity of this finding was mainly moderate (no evidence of progression). The incidence of vascular pooling in centrilobular areas, commonly occurring in ageing mice, was only increased in males.

In the kidney, a higher incidence of some findings, which commonly occur in older mice, was found. Males additionally showed an increased incidence of cortical tubular cell hypertrophy (slight), cortical cysts, and cortical fibrosis with tubular collapse and basophilia.

The incidence of swelling and rarefaction of villous epithelial cells of the duodenum and jejunum was increased at all doses. At higher doses this finding was accompanied by villous hypertrophy. The severity of these findings in animals killed at the end of the treatment period was not increased compared to that in mice killed after 52 weeks of treatment.

Table 3.9.1-13: Microscopic pathology, non-neoplastic findings - terminal kill

Finding	**Dose (ppm)**							
	Males				**Females**			
	0	30	400	5000	0	30	400	5000
Liver number examined	50	50	50	50	50	50	50	50
- hepatocyte hypertrophy – generalised	0	0	0	39***	0	0	0	0
- - hepatocyte hypertrophy - periportal	0	0	0	1	0	0	0	42***
- vascular pooling in centrilobular areas	0	2	0	10	4	4	6	0
Duodenum number examined	48	47	47	49	45	50	50	49
- swelling/rarefaction of villous epithelium	0	6*	29***	42***	0	3	12***	18***
- villous hypertrophy	0	0	9**	27***	0	0	0	5
Jejunum number examined	48	47	48	49	46	50	50	49
- swelling/rarefaction of villous epithelium	0	4	25***	35***	0	2	7*	14***
- villous hypertrophy	0	0	8**	16***	0	0	0	2
Kidney number examined	50	50	50	50	50	50	50	50

- cortical tubular cell hypertrophy (slight)	0	0	0	8**	0	0	0	0
- cortical fibrosis with tubular collapse and basophilia	13	13	15	37***	8	4	6	4
- cortical cysts	20	15	15	31*	7	4	7	5
- cortical tubules - basophilic	33	26	35	43*	12	13	14	41***
- medullary tubules dilated with eosinophilic casts	27	23	30	42**	14	14	13	32***
- cortical mineralisation	32	34	40	46**	3	0	1	26***
- medullary mineralisation	6	8	13	44***	0	0	0	31***
- papillary mineralisation	11	4	10	36***	3	6	1	30***
Statistical significance: *p<0.05; **p<0.01; *** p<0.001								

Table 3.9.1-14: Historical control data for hepatocellular adenomas and carcinomas in males

Finding	Totals (affected/examined)	Range
Hepatocellular adenoma	199/867; 22.95%	8.3 – 42%
Hepatocellular carcinoma	70/867; 8.07%	3.6 – 22%
Historical control data derived from 16 studies performed between 1996 and 1999		

Conclusion: The only evidence of tumourigenicity was an increased number of males showing benign hepatocellular liver tumours at the high dose of 5000ppm. The no effect level for tumourigenicity was set at 400ppm (56.8 mg/kg bw/d). Mechanistic studies have postulated a phenobarbitone-like mode of action for pethoxamid involving liver enzyme induction leading to increased liver tumour formation, particularly in males. This type of tumour formation is considered rodent-specific and not relevant to humans.

Non-neoplastic microscopic changes occurred in the duodenum and jejunum. The swelling and rarefaction of villous epithelial cells was of higher incidence in males than in females. In the duodenum, it was significantly increased in males at 30 ppm in the interim and the terminal kills. Since effects on the GIT were also noted in other toxicity studies, the LOAEL was considered to be < 30ppm (< 4 mg/kg bw/d). No NOAEL could be derived from this study.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding carcinogenicity is not required.

3.9.2 Human data

No relevant studies.

3.9.3 *In vitro* data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell transformation assays, gap junction intercellular communication tests)

No relevant studies.

3.9.4 Other data (e.g. studies on mechanism of action)

3.9.4.1 Anonymous (2001a)

Reference: TKC-94: Additional study on cell proliferation in the liver to Carcinogenicity Study by dietary Administration to CD-1 Mice for at least 80 Weeks.
Author(s), year: Anonymous, 2001a
Report/Doc. number: 1241 PXA / IET 00-0138
Guideline(s): Not applicable
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

In order to evaluate the effect of pethoxamid on hepatic cell proliferation, paraffin sections of the liver obtained from the original paraffin blocks used in the carcinogenicity study of the test substance by dietary administration to CD-1 mice for at least 80 weeks (TON 014) were subjected to immunohistochemistry for proliferating cell nuclear antigen (PCNA). PCNA labeling index (LI) was determined for each animal by counting the number of PCNA-positive (S-phase) cells per approximately 1000 hepatocytes. Cell proliferation was evaluated on the basis of PCNA LI for each dose group at 0, 30, 400, and 5000 ppm after 52 and 95 weeks of treatment. There were no significant changes in PCNA LI considered to be treatment-related in any dose group after 52 or 95 weeks of treatment. Based on the results described above, it has been suggested that TKC-94 appears to have no influence on cell proliferation in the liver when administered in feed to male mice at 30, 400 and 5000 ppm for 52 or 95 weeks.

3.9.4.2 Anonymous (2000)

Reference: Investigation of the potential effects of dietary administration of pethoxamid on thyroid function in male rats using the perchlorate discharge test
Author(s), year: Anonymous, 2000
Report/Doc. number: 94 PXA (TOX2001-304) / TON '072/002273
Guideline(s): Not applicable, non-guideline study
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

Material and Methods:

Principle of the method: The test uses the ability of perchlorate (a competitive inhibitor of thyroidal iodine transport) to discharge free ^{125}iodide from the thyroid. The presence of free ^{125}iodide within the thyroid is a consequence of the inhibition of thyroidperoxidases, which are responsible for the incorporation of ^{125}iodide into organic compounds, by substances as propylthiouracil (positive control substance). As a further positive control substance can serve phenobarbitone which acts indirectly on the thyroid by inducing hepatic microsomal enzymes including the thyroxine metabolizing UDP glucuronyl transferase (see also 3.12.1.2 and 3.12.1.4).

Test material: Pethoxamid; Batch: TB960306I; Purity: 94.8% (formulation checked by HPLC).

Test animals: Five groups of 16 male Sprague-Dawley CD albino rats each, 28 (+/-2 days) old, 14 g of weight, from Charles River UK Ltd, Margate, Kent. Only male animals were used because follicular cell adenomas were observed in this sex only in the carcinogenicity study (Report no. TON 6/974064, point 3.9.1.1).

The rats were treated for 28 consecutive days with pethoxamid by dietary administration of 0, 1600 or 5000 ppm. The dose levels and the route of administration were those previously used in toxicity studies.

The positive control groups were administered the commonly used doses of 75 mg/kg bw/d phenobarbitone or 200 mg/kg bw/d propylthiouracil by oral gavage.

Before treatment (Day 1) and on Days 12 and 24 a blood sample was taken for the measurement of the plasma concentrations of the hormones tri-iodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH). After 28 days of treatment, sodium^{125}Iodide was administered intraperitoneally to each animal and 6 hours later, potassium perchlorate or saline was administered separately to 6 animals from each group by intraperitoneal injection. Exactly two and a half minutes after the injections of potassium perchlorate or saline, each animal was anaesthetized and a blood sample was collected for the measurement of radioactivity. Immediately after sampling, all animals were sacrificed. The thyroid gland was removed by dissection and the amount of radioactivity measured.

Results:

Formulation analysis and substance intake: The mean concentrations of pethoxamid in the test diet were within 1.5% of target concentrations. The animals consumed 155 and 462 mg/kg bw/d at the administered doses of 1600 and 5000 ppm.

Mortality and clinical signs: There were no treatment-related deaths in any group.

Two rats were sacrificed on Days 17 and 20 of treatment because of corneal damage caused by blood sampling from the orbital sinus.

Pethoxamid: There were no clinical signs indicative of a reaction to treatment.

Propylthiouracil: Post-dosing salivation was recorded for the majority of the animals. Furthermore, piloerection and hair loss were noted in some animals.

Phenobarbitone: Signs included underactive behaviour, abnormal gait, hunched posture, shallow respiration and prostration occurring about 1 hour after dosing. All animals appeared to have fully recovered before the next dosing.

Body weight and food consumption:

Pethoxamid: There was a statistically significant reduction in body weight gain in comparison with controls during the first 4 days of treatment (Table 3.9.4-1). From Day 4 to 29, body weight gain continued to be lower than for the control group but to a lesser degree. This was accompanied by reduced food consumption, especially in the first week of treatment (palatability effect, also observed in other studies).

Propylthiouracil: A large reduction in body weight gain and food consumption and, after Day 11, weight loss was observed.

Phenobarbitone: Body weight gain was slightly decreased and food consumption increased.

Hormone concentrations (T3, T4, TSH):

Pethoxamid: No statistically significant differences were found for the T3 and for the T4 levels (Table 3.9.4-1). The mean TSH concentrations were slightly increased at both dose levels, but statistical significance was only reached on Day 12 in TSH level at concentration 1600 ppm.

Propylthiouracil: A marked decrease in the T3 and T4 levels was noted which was attributable to the direct effect on the thyroid. A large statistically significant increase in mean plasma concentration of TSH, apparent after treatment for 12 days, due to the resulting negative feedback was also observed.

Phenobarbitone: A statistically significant increase in the T3 levels was noted on Day 24. An increase in TSH levels over the time course is likely to be age related. From the start of treatment until Day 24, a slight increase in TSH levels was noted for the phenobarbitone treated group (6.7 ng/dl), but also for the control group (3.3 ng/dl). However, the increase was higher, although not statistically significant,

for the phenobarbitone treated animals. The significant decrease in the T4 level at Day 12 was most likely to be due to the relatively high T4 level in control animals at this day.

Thyroid weights:

Pethoxamid: A slight, but not statistically significant, increase in thyroid weight was observed, similarly to phenobarbitone treated animals.

Propylthiouracil: A large, statistically significant increase in thyroid weight was seen.

Phenobarbitone: A slight, but not statistically significant, increase in thyroid weight was observed.

Radioactivity measurements:

Thyroid radioactivity:

Pethoxamid: The total radioactivity content in the thyroid (% of dose, Table 3.9.4-1) was statistically significantly increased at the high dose (saline and perchlorate). No significant change in the thyroid radioactivity concentration (total % dose/g) was seen.

Propylthiouracil: A statistically significant decrease in thyroid radioactivity concentration was observed, especially for the perchlorate treated rats.

Phenobarbitone: A statistically significant increase in the total radioactivity content but not in the radioactivity concentration was noted (saline and perchlorate).

Whole-blood radioactivity:

Pethoxamid: A statistically significant decrease in the radioactivity concentration (% of dose per g blood, Table 3.9.4-1) was observed for the high dose, and in the total radioactivity content (% of dose) for both dose levels (saline and perchlorate; not tabulated).

Propylthiouracil: The concentration of radioactivity was statistically significantly higher (saline and perchlorate). The total radioactivity content in blood was lower (saline and perchlorate; not tabulated) because of the decreased body weight and thus blood volume.

Phenobarbitone: A statistically significant decrease was noted in the total radioactivity content and in the radioactivity concentration (saline and perchlorate).

Perchlorate administration:

Pethoxamid: No significant differences occurred between the saline and perchlorate treated animals for the determinations of radioactivity in blood as well as thyroid.

Propylthiouracil: The mean concentration and amount of radioactivity in the thyroid in saline compared with perchlorate treated animals show that about 60% of thyroid radioactivity was displaced by perchlorate. Thus, a large amount of free ^{125}iodide must have been present in the thyroid.

Phenobarbitone: The concentration and total content in blood were significantly higher in perchlorate treated groups, but this was not the case for both values for the thyroid.

Net result of the investigations:

After administration of propylthiouracil, the large displacement of thyroid radioactivity by perchlorate was a consequence of the inhibition of the peroxidases. The much lower thyroid/blood concentration ratio reflects a lower ^{125}iodide uptake and metabolization in the thyroid.

After administration of phenobarbitone, no significant discharge of thyroid radioactivity occurred by perchlorate (no inhibition of peroxidases and thus, absence of significant amounts of free ^{125}iodide). The decrease in blood radioactivity (concentration and total amount) is likely to be the result of an enhancement of the uptake and organification of ^{125}iodide by the thyroid. The enhancement could be the result of the slightly increased TSH release from the pituitary followed by some thyroid hypertrophy (indicated by the slightly increased thyroid weight).

Pethoxamid (as phenobarbitone) did not cause a significant discharge of thyroid radioactivity by perchlorate; thus, the activity of thyroid peroxidases was obviously not reduced.

The substance did not affect the T3 levels, which indicates that it does not inhibit the 5'- mono de-iodonase enzymes (conversion T4 to T3). Since there was no decline in TSH levels and no consistent elevation in T3 and T4 levels, it is unlikely that pethoxamid has an agonistic action at the TSH receptor.

Table 3.9.4-1: Administration of thyroid-active compounds to male rats - effects on parameters of general health and thyroid function (see next page)

	Control	Pethoxamid 1600 ppm	Pethoxamid 5000 ppm	Phenobarbitone 75 mg/kg bw/d	Propylthiouracil 200 mg/kg bw/d
General health parameters					
Body weight gain (g)					
Day 1-4	23.6	8.6**	-27.0**	24.8	14.4*
Day 4-29	143.7	125.9	102.0**	125.4	19.3**
Food consumption					
Week 1 (g/rat)	182	159**	86**	221**	198*
Week 2-4 (g/rat)	555	541	478**	600*	350**
Hormone levels					
T3 (ng/dl)					
Prior to treatment	131.5	134.2	124.0	112.9**	102.4**
Day 12	87.1	91.6	90.3	81.9	25.1**
Day 24	77.8	85.7[1]	87.6	94.3**	37.9[1]**
T4 (µg/dl)					
Prior to treatment	5.2	5.5	5.5	5.1	4.6*
Day 12	7.6	8.2	6.6	5.0**	0.0[2]**
Day 24	5.3	6.0[1]	4.9	5.3	0.3[1]**
TSH (ng/ml)					
Prior to treatment	5.9	6.1	6.2	5.2	5.2
Day 12	7.5	10.8*	9.0	9.5	23.7**
Day 24	9.2	12.6[1]	13.0	11.9	34.5[1]**
Thyroid weight (g)					
Saline treated	0.0146	0.0165	0.0172	0.0187	0.0452**
Perchlorate treated	0.0172	0.0186	0.0179	0.0183	0.0547**
Radioactivity measurements					
Whole-blood (% of dose/g)					
Saline treated	0.281	0.264	0.228**	0.200**	0.331**
Perchlorate treated	0.297	0.294	0.236**	0.257**	0.414**
Thyroid (total % of dose)					
Saline treated	6.24	6.82	7.58*	7.96*	3.26**
Perchlorate treated	6.25	8.38	8.12*	8.02*	1.27**
Thyroid (% of dose/g)					
Saline treated	427.4	408.0	442.7	425.7	71.9**
Perchlorate treated	364.9	461.4	454.7	440.0	23.0**

Thyroid : blood radioactivity ratio[3]					
Saline treated	1520	1549	1940**	2145**	222**
Perchlorate treated	1264	1576	1926**	1713**	56**

[1] Mean for 15 rats; [2] Values below the limit of accurate quantification (0.2 ug/dl) were taken as zero for calculation of mean and standard deviations; [3] expressed as dpm/g; Statistical significance: *p<0.05; **p<0.01

Conclusion: Comparing the data for pethoxamid with that for propylthiouracil, it suggests that pethoxamid did not directly affect the thyroid function.

The data obtained for pethoxamid at the high dose level is similar to that for phenobarbitone (TSH levels, thyroid and whole-blood radioactivity); **thus, the mechanism of action of pethoxamid is apparently similar to that of phenobarbitone.**

3.9.4.3 Anonymous (2001b)

Reference: TKC-94: 2-Week Hepatic Drug-Metabolizing Enzyme Induction and Cell Proliferation Study in Mice.

Author(s), year: Anonymous, 2001b
Report/Doc. number: 98 PXA / IET-00-0118
Guideline(s): Not applicable, non-guideline study
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

Executive summary:
In order to evaluate effects of TKC-94 on hepatic drug-metabolizing enzyme induction and cell proliferation, the test substance was administered in feed to male ICR (Crj:CD-1) mice at dose levels of 0, 30, 400 or 5000 ppm for 2 weeks. In addition, cell to cell communication in the liver was evaluated by counting the number of gap junction protein connexion 32 (Cx 32) spots per hepatocyte.

Clinically there were neither treatment-related abnormalities nor deaths in any dose group during the study. There were no significant difference in body weight change between the treated and control groups during the study. Food consumption by the 5000 ppm group was decreased (about 37% less than the control value) at the 1st week of treatment, but recovered at the 2nd week. The average food consumption by this group during the study was slightly lower (about 14% less) than that by the control group.

The average test substance intake for each group during the study was 3.92, 49.1 and 541 mg/kg bw/day for the 30, 400 and 5000 ppm dose levels, respectively.

At necropsy, enlarged and/or dark-coloured livers were frequently observed in the 5000 ppm group. Organ weight measurements revealed significant increases in both absolute and relative (ratio to body weight) liver weights in the 5000 ppm group when compared to the control group.

Analysis of hepatic microsomal samples disclosed significant increases in microsomal protein content, cytochrome P-450 content, and pentoxyresorufin O-dealkylase activity in the 5000 ppm group. In addition, P-450 isoenzyme contents of CYP1A, CYP2B, CYP3A2, and CYP4A1 were significantly increased in this group and the increase in CYP2B content was most evident. A significant increase in

pentoxyresorufin O-dealkylase activity was also noted in the 400 ppm group. In addition, a significant decrease in CYP1A was noted, but no changes in other parameters.

Measurement of cell proliferation in the liver revealed a significant increase in PCNA labelling index in the 5000 ppm group after 3 and 7 days of treatment, but not after 14 days of treatment.

Regarding cell to cell communication in the liver, a dose-dependent decrease in the number of Cx32 spots per hepatocyte was observed in the treated groups. In the 5000 and 400 ppm groups, significant decreases (20-30%) in this parameter were observed at all sampling times. In the 30 ppm group, a mild (12-17%) but significant decrease was noted after 3 and 7 days of treatment, but not after 14 days of treatment.

Based on the results observed, the overall profile of effects are suggestive that TKC-94 may be a phenobarbital-type enzyme inducer which can increase cell proliferation in the liver during the initial stages of exposure when administered at 5000 ppm in the diet. In addition, the test substance may inhibit gap junctional intercellular communication.

MATERIALS AND METHODS

Materials:

Test material:	Pethoxamid
Lot/batch number:	TB-960306 I
Purity:	95.0% (w/w) (dose calculation was not adjusted to purity)
Stability of test item:	30 September 2000 (stored at approximately 4°C in the dark) NB: stable during the conduct of the study
Storage conditions:	Refrigeration (approximately 4°C) in the dark
CAS#:	1006700-29-2

Study Design:
The objective of this study was to investigate effects of TKC-94 on hepatic drug-metabolizing enzyme induction and cell proliferation in mice following dietary administration for 2 weeks. This study was conducted as part of mechanistic studies to clarify the mechanism(s) for the development of hepatocellular tumours that increased in high-dose male mice in the carcinogenicity study.

Specific-pathogen-free (SPF) male ICR (crj:CD-1) mice were purchased from Hino Breeding Centre (Hino-cho, Gamoh-gun, Shiga) of Charles River Japan, Inc. The CD-1 mouse was chosen because this strain of mice was used in the previously conducted toxicity studies of TKC-94 in mice.

The test substance was administered orally by incorporating it into the basal diet for 2 weeks. 18 male mice/group were used. Dose levels were 0, 30, 400 or 5000 ppm in diet. After 3, 7 and 14 days of treatment, 6 animals from each group were killed and subjected to necropsy, measurement of liver weights, and measurements of cell proliferation and cell to cell communication. In addition, measurement of hepatic drug-metabolising enzymes was performed on the animals killed after 14 days of treatment.

Preparation of the treated diet was undertaken on 2 occasions at approximately a weekly interval, to fit with established stability data.

Mice were subject to daily clinical signs, weekly body weight measurement and weekly food consumption measurement. Post-life measurements entailed; liver weight recording, cell proliferation measurement, gap junction protein CX32 and hepatic drug-metabolizing enzyme measurement.

The following microsomal enzymes were measured; protein content, cytochrome P-450 content, Pentoxyresorufin O-dealkylase activity, CYP1A, CYP2B, CYP3A2, and CYP4A1.

Cell proliferation was determined by immunohistochemistry measurement of proliferating cell nuclear antigen (PCNA). Additional duodenum samples were taken and similarly processed to serve as positive controls for PCNA staining. Approximately 1000 hepatocytes for each animal were examined using an image analyser.

Frozen sections were obtained from the liver tissue embedded in a Tissue Mount for each animal at each scheduled sacrifice and subjected to immunohistochemistry for hepatic gap junction protein connexion 32(CX32) using rat monoclonal CX32 antibodies. The number of CX32 spots per hepatocyte was calculated.
Appropriate statistical analysis was undertaken on relevant data.

Results:
Achieved intake: The achieved intake is given below:

Table 3.9.4-2: Achieved intake

Dose in diet (ppm)	Average achieved intake (mg/kg bw/day)
30	3.92
400	49.1
5000	541

Clinical signs, body weight and food consumption: There were no treatment-related clinical signs observed. There were no noteworthy effects on body weight between the groups. Food consumption was lower during the first week of treatment (approximately 37%) in the high dose mice when compared with the concurrent control values but recovered at the 2nd week. The average weight gain per week was 5.1, 5.5, 5.0 and 4.4 g/mouse/day for 0, 30, 400 and 5000 ppn, respectively.

Necropsy: At necropsy, in the 5000 ppm group, enlarged and/or dark-colored livers were frequently observed after 7 and 14 days of treatment.

Organ weights: Absolute and relative liver weights in the 5000 ppm group after 3, 7 and 14 days of treatment were significantly increased when compared with concurrent controls. No similar findings were observed at <5000 ppm.

Table 3.9.4-3: Liver weights

Dose (ppm)	Absolute liver weight			Relative liver weight		
	3 days	7 days	14 days	3 days	7 days	14 days
30	104	97	102	105	95	98
400	106	93	108	105	98	107
5000	123**	131**	137**	129**	133**	136**
Values represent percentage to the control values. **: Significantly different from the control at 1% level of probability (Dunnett´s test)						

Microsomal protein content and enzyme activity: Microsomal protein content, cytochrome P-450 content, and pentoxyresorufin O-dealkylase activity which were measured for each dose group after 14 days of treatment were significantly higher in the 5000 ppm group when compared with concurrent

controls. In the 400 ppm group, pentoxyresorufin O-dealkylase activity was significantly increased, but there were no significant changes in other parameters. In the 30 ppm group, there were no significant changes in any parameter.

Table 3.9.4-4: Microsomal protein content, cytochrome P-450 content, and pentoxyresorufin O-dealkylase activity

Dose (ppm)	Microsomal protein content	Cytochrome P-450 content	Pentoxyresorufin O-dealkylase activity
30	105	93	367
400	107	100	867*
5000	121**	195**	5667**
Values represent percentage to the control values. *, **: Significantly different from the control at 5% and 1% level of probability, respectively (Dunnett´s test)			

Significant increases in cytochrome P-450 isoenzyme contents of CYP-1A (4.3 times higher), CYP2B (8.5 times higher), CYP3A2 (2.1 times higher), and CYP4A1 (1.6 times higher) were observed in the 5000 ppm group when compared to the control group. In the 400 ppm group, a significant decrease in CYP-1A content was noted, but there were no significant changes in other parameters. In the 30 ppm group, there were no significant changes in any isoenzyme content.

Table 3.9.4-5: Cytochrome P-450 isozyme contents

Dose (ppm)	Cytochrome P-450 isozyme contents			
	CYP1A	CYP2B	CYP3A2	CYP4A1
30	81	95	95	100
400	81**	105	98	120
5000	429**	845**	214**	164**
Values represent percentage to the control values. **: Significantly different from the control at 1% level of probability (Student´s t-test or Aspin-Welch test)				

Cell proliferation: In the 5000 ppm group, significant increases in PCNA LI (labelling index) were observed after 3 and 7 days of treatment, but the value after 14 days of treatment was comparable to the controls. In other dose groups, there were no significant differences in PCNA LI between the treated and control groups.

Table 3.9.4-6: PCNA labelling index

Dose (ppm)	PCNA LI on the liver		
	3 days	7 days	14 days
30	108	122	231
400	135	74	131
5000	377*	781*	163
Values represent percentage to the control values. *: Significantly different from the control at 5% level of probability (Dunnett´s test)			

In the 5000 ppm group, significant decreased (about 30% lower) number of CX32 spots were observed after 3, 7 and 14 days of treatment when compared to the controls. In the 400 ppm group, significant decreased (about 20% lower) number of CX32 spots were also observed after 3, 7 and 14 days of

treatment when compared to the controls. In the 30 ppm group, mild (12-17%) but significant decreased number of CX32 spots were noted after 3 and 7 days of treatment when compared to the controls, but not after 14 days of treatment.

Table 3.9.4-7: Number Cx32 spots per hepatocyte

Dose (ppm)	No. of Cx32 spots per hepatocyte		
	3 days	7 days	14 days
30	88*	83*	87
400	81**	82**	81*
5000	71**	69**	71**
Values represent percentage to the control values. *, **: Significantly different from the control at 5% and 1% level of probability, respectively (Dunnett´s test)			

Conclusion: Based on the results observed, the overall profile of effects are suggestive that TKC-94 may be a phenobarbital-type enzyme inducer which can increase cell proliferation in the liver during the initial stages of exposure when administered at 5000 ppm in the diet. In addition, the test substance may inhibit gap junctional intercellular communication (CJIC) in the liver. In consideration of these effects the no-observed-adverse-effect level (NOAEL) for this study was determined to be 30 ppm (3.92 mg/kg-bw/day) because there were no significant changes in any parameters in the 30 ppm group after 14 days of treatment, although a mild but statistically significant decrease in GJIC was noted after 3 and 7 days of treatment.

3.9.4.4 Anonymous (2016a)

Reference: Pethoxamid Technical: Evaluation Of Liver And Thyroid Effects And Their Potential Reversibility After Dietary Exposure In Mice And Rats
Author(s), year: Anonymous, 2016
Report/Doc. number: 1538 PXA
Guideline(s): Mechanistic study: Guidelines considered in the study design rather than adhered with. OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 407): Health Effects, Repeated Dose 28-Day Oral Toxicity Study in Rodents (2008). U.S. EPA Health Effects Test Guidelines, OPPTS 870.3050, "Repeated Dose 28-Day Oral Toxicity Study in Rodents", (1998)
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

EXECUTIVE SUMMARY:

The objective of this study was to generate liver samples from mice, and liver, thyroid, and serum samples (for thyroid hormone assessment) from rats, which were administered a diet containing pethoxamid technical. In order for changes in the liver and thyroid (including hormone levels) to be appropriately characterized, mice were dosed for a period of 7 days and rats for a period of 14 days. Liver and thyroid samples (including serum thyroid hormone levels) were used to evaluate biochemical effects and correlating macroscopic and microscopic toxicological effects on these organs. BrdU (5-bromo-2'-deoxyuridine) was administered to enable assessment of DNA replication (indicative of cell division) while potential reversibility was evaluated after a 42-day recovery period. The Recovery group animals in both species were provided with basal diet during the 42-day recovery period.

Mice in Groups 1 through 5 and the first eight rats in Groups 6 through 10 were fitted with an osmotic pump, containing 15 mg/mL BrdU in sterile saline, implanted subcutaneously between the shoulder blades. The

pumps, calibrated to deliver 1 µL/hr for seven days, were surgically implanted in all mice from Groups 1-3 on Day 0 and in Groups 4-5 on Day 42. Rats in Groups 6-8 (0, 400, and 1600 ppm) received 10 µL/hr BrdU in sterile saline from Day 7 to Day 14 (scheduled sacrifice). Rats in Groups 9-10 (0 and 1600 ppm, respectively) received the same dosing regimen from Day 49 (pump implantation) to Day 56. Incorporation of BrdU into hepatocytes was then measured using a mouse monoclonal anti-BrdU antibody and light microscopy.

At terminal sacrifice (Day 7 for Groups 1-3, Day 14 for Groups 6-8, Day 49 for Groups 4-5, and Day 56 for Groups 9-10); all animals were euthanized by CO_2 asphyxiation and subjected to a gross necropsy. The liver was harvested from all mice in Groups 1-5. The mouse liver samples were collected, weighed and stored for enzyme induction analysis, general histopathology, and IHC evaluation. The liver and thyroid glands were harvested from the first eight rats in Groups 6-10. A section of the rat livers was sent for enzyme induction analysis at another laboratory under a separate study number. The thyroids were collected, fixed overnight, weighed and stored for general histopathology and IHC evaluation. Serum was collected from all rats (Groups 6-10) for thyroid hormone analysis.

There were no mortalities during this study. There were no clinical signs considered attributable to the administration of pethoxamid technical.

There were no changes in body weight, mean daily body weight gain or food consumption considered attributable to pethoxamid technical administration seen in mice.

An initial slight decrease in bodyweight gain and food consumption (Days 0-7), that did not impact on the overall body weight values during the dosing period, was observed in rats consuming diet containing 1600 ppm pethoxamid technical (Groups 8 and 100). As the lower initial food consumption did not have any noteworthy impact on body weights, this observation was considered to be likely related to treated diet palatability and of limited toxicological relevance.

Macroscopic findings in mice were limited to ‘Pale liver’ observed in one animal treated at 5000 ppm for 14 days. This finding correlated microscopically with hepatocyte hypertrophy, grade 2. There were no test substance-related macroscopic observations in rats.

Increased absolute and relative liver weights were observed in mice treated at 5000 ppm for 14 days. Liver weights (absolute and relative to body weight) were slightly higher than concurrent controls in mice after a 42-day recovery period; however, there were no microscopic correlates, the difference was minor and only the liver-to-body weight ratio achieved statistical significance. This observation is therefore considered of limited toxicological significance.

In rats, increased absolute and relative thyroid weights were observed in animals treated at 400 and 1600 ppm for 14 days. Thyroid weight values after a 42-day recovery period were comparable to concurrent controls. No test substance-related liver weight changes were observed in rats.

Thyroid hormone levels (TSH, T4, and Free T3) for rats treated with pethoxamid technical were generally comparable to the respective control group.

Evaluation on Day 7 of liver from mice treated with pethoxamid technical at 5000 ppm demonstrated hepatocellular hypertrophy and an increase of the number of BrdU positive cells when compared with mice treated with either basal diet or pethoxamid technical at 400 ppm. These increases were completely reversible in recovery animals evaluated on Day 49.

Rat thyroid glands evaluated on Day 14, treated with pethoxamid technical at 1600 ppm, demonstrated an increase in the BrdU labeling index when compared to the rats treated with basal diet or pethoxamid technical at 400 ppm. This increase appeared to be completely reversible in recovery animals evaluated on Day 56. Thyroid follicular epithelium hypertrophy (grade 1) was observed in 2 out of 8 animals treated with 1600 ppm pethoxamid technical on Day 14, while this change was not observed in any of the recovery animals regardless of treatment consistent with full reversibility.

Under the conditions of this study and based on the toxicological and histopathological endpoints evaluated, in addition to increased absolute and relative liver weights, pethoxamid technical administered to mice in their diet at concentrations of 5000 ppm caused reversible changes in

centrilobular hepatocytes including hypertrophy as well as increased hepatocyte proliferation. Pethoxamid Technical administered to rats in their diet at concentrations of 1600 ppm caused increased absolute and relative thyroid weights and reversible increases in follicular thyroid cell proliferation. There were no noteworthy effects of pethoxamid on circulating thyroid hormone levels in rats. Only selected minor effects across the end-points assessed were observed at the low, non-oncogenic dose level of 400 ppm, in both rats and mice.

MATERIALS AND METHODS:

Materials:

Test material:	Pethoxamid technical
Lot/batch number:	P1351-JaK-T2-23-6
Purity:	92.6% (w/w) (dose calculation was not adjusted to purity)
Stability of test item:	28 November 2015 (stored at ambient temperature) *NB: stable during the conduct of the study*
Storage conditions:	At room temperature, protected from light

Forty healthy male mice and seventy-five healthy male rats were selected for the test and distributed into 10 groups in two cohorts of animals (Main Toxicity and Recovery). The Main Toxicity cohort consisted of six groups (three per species) and the Recovery cohort consisted of four groups (two per species). The mice were provided with treated diet at dietary levels of 0 ppm (Groups 1 and 4, basal diet control, main and recovery, respectively), 400 ppm (Group 2), and 5000 ppm (Groups 3 and 5, main and recovery, respectively) of pethoxamid technical. The rats were provided with treated diet at dietary levels of 0 ppm (Groups 6 and 9, basal diet control, main and recovery, respectively), 400 ppm (Group 7), and 1600 ppm (Groups 8 and 10, main and recovery, respectively) of pethoxamid technical. All treated groups were fed the treated diet for 7 (mice) or 14 days (rats). The control groups were fed the basal diet for the entirety of the study. The Recovery group animals in both species were provided with basal diet during the 42-day recovery period.

Forty (40) mice were randomly assigned to one of the following groups:

Group	No. mice/Group	Treatment - Dietary Concentration (ppm)	% in Diet	Target Dosage (mg/kg/day)[a]
1	8	Basal Diet (Control) – 0	0	0
2	8	Pethoxamid Technical – 400	0.04	107
3	8	Pethoxamid Technical – 5000	0.5	1333
4	8	Basal Diet (Control) – 0 (Recovery)	0	0
5	8	Pethoxamid Technical – 5000 (7 days dosing, 42 days Recovery)	0.5	1333

[a] Based on 30 gram male CRL: CD 1 (ICR) mice eating 8 g/day. Doses calculated without correcting for purity.

Seventy-five (75) rats were randomly assigned to one of the following test groups:

Group	No. Rats/Group	Treatment - Dietary Concentration (ppm)	% in Diet	Target Dosage (mg/kg/day)[a]
6	15	Basal Diet (Control) – 0	0	0
7	15	Pethoxamid Technical – 400	0.04	33.3
8	15	Pethoxamid Technical – 1600	0.16	133

9	15	Basal Diet (Control) – 0 (Recovery)	0	0
10	15	Pethoxamid Technical – 1600 (14 days dosing, 42 days Recovery	0.16	133

[a] Based on 300 gram male Crl: CD® SD rats eating 25 g/day. Doses calculated without correcting for purity.

The test substance and control diets were presented to their respective groups on Day 0 of the study. Additional diet was provided as needed throughout the study to ensure *ad libitum* feeding. All animals were observed daily for viability, signs of gross toxicity, and behavioral changes. Body weights were recorded two times during the acclimation period, prior to test initiation (Day 0), and weekly thereafter for all surviving animals and just prior to scheduled sacrifice. Individual food consumption was also recorded to coincide with body weight measurements. Dietary intake of pethoxamid (mg/kg/day) was calculated for all test groups.

Mice in Groups 1 through 5 and the first eight rats in Groups 6 through 10 were fitted with an osmotic pump, containing 15 mg/mL BrdU in sterile saline, implanted subcutaneously between the shoulder blades. The pumps, calibrated to deliver 1 µL/hr for seven days, were surgically implanted in all mice from Groups 1-3 on Day 0 and in Groups 4-5 on Day 42. Rats in Groups 6-8 (0, 400, and 1600 ppm) received 10 µL/hr BrdU in sterile saline from Day 7 to Day 14 (scheduled sacrifice). Rats in Groups 9-10 (0 and 1600 ppm, respectively) received the same dosing regimen from Day 49 (pump implantation) to Day 56. Incorporation of BrdU into hepatocytes was then measured using a mouse monoclonal anti-BrdU antibody and light microscopy.

At terminal sacrifice (Day 7 for Groups 1-3, Day 14 for Groups 6-8, Day 49 for Groups 4-5, and Day 56 for Groups 9-10); all animals were euthanized by CO_2 asphyxiation and subjected to a gross necropsy. The liver was harvested from all mice in Groups 1-5. The mouse liver samples were collected, weighed and stored for enzyme induction analysis, general histopathology, and IHC evaluation. The liver and thyroid glands were harvested from the first eight rats in Groups 6-10. A section of the rat livers was sent for enzyme induction analysis at another laboratory under a separate study number. The thyroids were collected, fixed overnight, weighed and stored for general histopathology and IHC evaluation. Serum was collected from all rats (Groups 6-10) for thyroid hormone analysis.

RESULTS AND DISCUSSION:

Mortality and clinical signs: There were no mortalities during this study. There were no clinical signs considered attributable to the administration of pethoxamid technical.

Body weights and food consumption: There were no changes in body weight, mean daily body weight gain or food consumption considered attributable to pethoxamid technical administration seen in mice.

An initial slight decrease in bodyweight gain and food consumption (Days 0-7), that did not impact on the overall body weight values during the dosing period, was observed in rats consuming diet containing 1600 ppm pethoxamid technical (Groups 8 and 10). As the lower initial food consumption did not have any noteworthy impact on body weights, this observation was considered to be likely related to treated diet palatability and of limited toxicological relevance.

Macroscopic observations: Macroscopic findings in mice were limited to 'Pale liver' observed in one animal treated at 5000 ppm for 14 days. This finding correlated microscopically with hepatocyte hypertrophy, grade 2. There were no test substance-related macroscopic observations in rats.

Organ weight evaluation: Increased absolute and relative liver weights were observed in mice treated at 5000 ppm for 14 days. Liver weights (absolute and relative to body weight) were slightly higher than concurrent controls in mice after a 42-day recovery period; however, there were no microscopic correlates, the difference was minor and only the liver-to-body weight ratio achieved statistical significance. This observation is therefore considered of limited toxicological significance.

Table 3.9.4-8: Summary of terminal body and liver weights (mice)

Parameter		Group				
		1	2	3	4	5
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	5000 ppm	0 ppm	5000 ppm
BW (g)	Mean	31.9	31.3	30.6	36.6	39.0
	SD	1.1	2.3	1.6	1.8	3.0
	N	8	8	8	8	8
Liver Weight (g)	Mean	2.09	1.90	2.59*	1.94	2.28
	SD	0.66	0.27	0.27	0.22	0.18
	N	8	8	8	8	8
Liver-to-BW Ratio (%)	Mean	0.07	0.06	0.08*	0.05	0.06#
	SD	0.02	0.01	0.01	0.00	0.00
	N	8	8	8	8	8

*Statistically significant from Group 1 Control, $p<0.05$, by Dunn's Multiple Comparisons Test

#Statistically significant from recovery Group 4 Control, $p<0.05$, by the Mann-Whitney Test.

In rats, increased absolute and relative thyroid weights were observed in animals treated at 400 and 1600 ppm for 14 days. Thyroid weight values after a 42-day recovery period were comparable to concurrent controls. No test substance-related liver weight changes were observed in rats.

Hormone level (measured in rats only): Thyroid hormone levels (TSH, T4, and Free T3) for rats treated with pethoxamid technical were generally comparable to the respective control group.

Table 3.9.4-9: Summary of terminal body and thyroid/liver weights (rats)

Parameter		Group				
		6	7	8	9	10
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	1600 ppm	0 ppm	1600 ppm
BW (g)	Mean	327.1	321.7	319.7	451.6	454.1
	SD	16.6	13.3	15.9	43.1	57.0
	N	15	15	15	15	15
Liver Weight (g)	Mean	15.11	14.53	16.87	16.65	16.38
	SD	1.75	1.51	1.67	2.82	2.00
	N	8	8	8	8	8
Liver-to-BW Ratio (%)	Mean	0.05	0.04	0.05	0.04	0.04
	SD	0.00	0.00	0.00	0.01	0.01
	N	8	8	8	8	8
Thyroid Weight (g)	Mean	0.014	0.018**	0.020**	0.030	0.025
	SD	0.003	0.002	0.002	0.003	0.002
	N	8	8	8	8	8
Thyroid-to-BW Ratio (%)	Mean	$4.30x10^{-5}$	$5.56x10^{-5}$**	$6.23x10^{-5}$**	$6.67x10^{-5}$	$5.50x10^{-5}$
	SD	$9.54x10^{-6}$	$4.86x10^{-6}$	$5.19x10^{-6}$	$6.37x10^{-6}$	$4.38x10^{-6}$
	N	8	8	8	8	8

**Statistically significant from Group 1 Control, $p<0.01$, by Dunnett's Multiple Comparisons Test

Table 3.9.4-10: Summary of thyroid hormone assessment (rats)

Parameter		Group				
		6	7	8	9	10
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	1600 ppm	0 ppm	1600 ppm
TSH (Ng/mL)	Mean	5.788	5.194	6.507	5.345	4.927
	SD	3.482	2.896	3.132	4.132	3.373
	N	15	15	15	15	15
Total T4 (µg/mL)	Mean	4.662	5.115	5.504*	5.795	5.293
	SD	0.768	0.569	1.143	1.877	1.304
	N	15	15	15	15	15
Free T3 (pg/mL)	Mean	3.547	3.585	3.754	4.043	3.441
	SD	0.89	0.408	0.763	1.235	0.914
	N	15	15	15	15	15

*Statistically significant from Group 1 Control, $p < 0.05$, by Dunnett Multiple Comparisons Test

Histopathology: Evaluation on Day 7 of liver from mice treated with pethoxamid technical at 5000 ppm demonstrated hepatocellular hypertrophy and an increase of the number of BrdU positive cells when compared with mice treated with either basal diet or pethoxamid technical at 400 ppm. These increases were completely reversible in recovery animals evaluated on Day 49.

Table 3.9.4-11: BrDU assessment (mice)

Parameter	Animal Sequence per Group	Group				
		1	2	3	4	5
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	5000 ppm	0 ppm	5000 ppm
Individual Mean BrdU Values	1	0.3	0.3	14.4	0.0	0.1
	2	0.2	0.1	13.8	0.0	0.1
	3	0.1	1.5	14.0	0.2	0.0
	4	0.3	0.1	14.0	0.5	0.2
	5	0.2	0.9	8.8	0.2	0.0
	6	0.1	0.1	10.8	0.0	0.1
	7	0.3	0.3	13.0	0.0	0.0
	8	0.4	1.3	9.1	0.1	0.0
Mean		0.2	0.6	12.2 ***	0.1	0.1
SD		0.1	0.6	2.3	0.1	0.1
N		8	8	8	8	8

*** Statistically significant from Group 1 Control, p<0.001, by Dunn's Multiple Comparison

Rat thyroid glands evaluated on Day 14, treated with pethoxamid technical at 1600 ppm, demonstrated an increase in the BrdU labeling index when compared to the rats treated with basal diet or pethoxamid technical at 400 ppm. This increase appeared to be completely reversible in recovery animals evaluated on Day 56. Thyroid follicular epithelium hypertrophy (grade 1) was observed in 2 out of 8 animals treated with 1600 ppm pethoxamid technical on Day 14, while this change was not observed in any of the recovery animals regardless of treatment consistent with full reversibility.

Table 3.9.4-12: BrDU assessment (rats)

Parameter	Animal Sequence per Group	Group				
		6	7	8	9	10
		Treatment			Treatment (recovery)	
		0 ppm	400 ppm	1600 ppm	0 ppm	1600 ppm
Individual Mean brdU Values	1	1.8	4.1	8.8	1.6	2.7
	2	5.6	1.8	21.0	0.3	1.7
	3	2.6	3.8	5.5	0.7	0.6
	4	5.9	2.7	5.4	0.4	0.9
	5	1.8	3.4	1.7	0.9	1.8
	6	2.1	2.9	2.3	0.6	0.3
	7	2.9	1.0	5.3	2.3	0.8
	8	1.2	4.0	7.8	0.7	1.3
Mean		3.0	3.0	7.2	0.9	1.3
SD		1.8	1.1	6.1	0.7	0.8
N		8	8	8	8	8

No statistical significance between dose groups and controls.

CONCLUSION: Under the conditions of this study and based on the toxicological and histopathological endpoints evaluated, in addition to increased absolute and relative liver weights, pethoxamid technical administered to mice in their diet at concentrations of 5000 ppm caused reversible changes in centrilobular hepatocytes including hypertrophy as well as increased hepatocyte proliferation.

Pethoxamid Technical administered to rats in their diet at concentrations of 1600 ppm caused increased absolute and relative thyroid weights and reversible increases in follicular thyroid cell proliferation. There were no noteworthy effects of pethoxamid on circulating thyroid hormone levels in rats. Only selected minor effects across the endpoints assessed were observed at the low, non-oncogenic dose level of 400 ppm, in both rats and mice.

3.9.4.5 Anonymous (2016b)

Reference: Ex Vivo Evaluation of Pethoxamid as an Inducer of Liver Microsomal Cytochrome P450 and UDP Glucuronosyltransferase (UGT) Expression in Male Rats and Mice
Author(s), year: Anonymous, 2016
Report/Doc. number: 1539 PXA
Guideline(s): Non-specific; mechanistic study
GLP: Yes
Deviations from OECD 416 (2001): Not applicable
Acceptability: Yes

EXECUTIVE SUMMARY:

The objective of this study was to evaluate the effect of Pethoxamid on liver microsomal uridine diphosphate glucuronosyltransferase (UGT) activity and mRNA levels toward the thyroid hormone thyroxine (T4) in male rats and on liver microsomal cytochrome P450 (CYP) enzyme activity and mRNA levels in male mice. The effects of Pethoxamid on the specific content of liver microsomal cytochrome b_5 and cytochrome P450 were also evaluated in both male rats and male mice.

The most pronounced effects of Pethoxamid were the statistically significant and dose-dependent increases in UGT1A6 mRNA levels in male rats and the statistically significant and dose-dependent

increases in Cyp3a11/13 activity and Cyp2b10, Cyp3a11 and Cyp4a10 mRNA levels in male mice. Of the CYP end-points assessed the induction of Cyp2b10 mRNA at 5000 ppm was the highest (115-fold concurrent control). Results following the observance of a 42-day recovery period illustrated no notable persisting effects (similar to concurrent control) on the end-points assessed.

MATERIALS AND METHODS:

The objective of this study was to evaluate the effect of Pethoxamid on liver microsomal uridine diphosphate glucuronosyltransferase (UGT) activity and mRNA levels toward the thyroid hormone thyroxine (T4) in male rats and on liver microsomal cytochrome P450 (CYP) enzyme activity and mRNA levels in male mice. The effects of Pethoxamid on the specific content of liver microsomal cytochrome b_5 and cytochrome P450 were also evaluated in both male rats and male mice.

In a separate *in-vivo* study male rats were treated with a control or one of two different dosages of Pethoxamid (equivalent to 400 and 1600 ppm in diet) for 14 days. At the end of the treatment period, or following a 42-day recovery period, the animals were euthanized and livers were removed, frozen and stored at −70°C. The frozen liver tissues were shipped to the Testing Facility, where microsomes were isolated and assayed for enzyme activity of thyroxine glucuronide (UGT1A1/6). A portion of liver tissue from the same rats was harvested and stored in RNA*later* RNA stabilization reagent at 4 C overnight prior to storage at −70°C and shipped to the Testing Facility, where it was lysed with TRIzol to isolate RNA, which was analyzed by qRT PCR to assess the effect of Pethoxamid on UGT1A1 and UGT1A6 mRNA levels.

In a separate *in-vivo* study male mice were treated with a control or one of two different dosages of Pethoxamid (equivalent to 400 and 5000ppm) for 7 days. At the end of the treatment period, or following a 42-day recovery period, the animals were euthanized and livers were removed, frozen and stored at −70°C. The frozen liver tissues were shipped to the Testing Facility, where microsomes were isolated and assayed for enzyme activities known to be relatively specific markers of CYP enzymes, namely 7-ethoxyresorufin O-dealkylation (Cyp1a1/2), testosterone hydroxylation (Cyp2b10 and Cyp3a11/13) and lauric acid 12-hydroxylation (Cyp4a10/12). A portion of liver tissue from the same mice were harvested and stored in RNA*later* RNA stabilization reagent at 4°C overnight prior to storage at −70°C and shipped to the Testing Facility, where it was lysed with TRIzol to isolate RNA, which was analyzed by qRT PCR to assess the effect of Pethoxamid on Cyp1a2, Cyp2b10, Cyp3a11 and Cyp4a10 mRNA levels.

RESULTS AND DISCUSSION:

Treatment of male rats with prototypical enzyme inducers caused anticipated increases in UGT activities. Treatment with β-naphthoflavone caused an increase of 7.14-fold in UGT1A1/6 activity when compared with concurrent control values.

Treatment of male mice with prototypical enzyme inducers caused anticipated increases in CYP activities. Treatment with β-naphthoflavone caused increases of 9.82- and 11.9-fold in Cyp1a1/2 activity when compared with concurrent control values. Treatment with phenobarbital caused increases of 2.94-, 3.44- and 3.28-fold in Cyp2b10 activity, while treatment with dexamethasone caused increases of 11.5-, 11.3- and 12.4-fold in Cyp3a11/13 activity when compared with concurrent control values. Finally, treatment with clofibric acid caused increases of 4.43- and 6.02-fold and perfluorodecanoic acid (PFDA) caused increases of 11.7- and 11.4-fold in Cyp4a10/12 activity when compared with concurrent control values.

Treatment of male rats with Pethoxamid for 14 days caused statistically significant increases in cytochrome b_5 content (1.22-fold, compared with concurrent control, at 1600 ppm), cytochrome P450 content (1.17- and 1.63-fold, compared with concurrent control, at 400 and 1600 ppm), thyroxine glucuronidase (UGT1A1/6) activity (1.62-fold, compared with concurrent control, at 1600 ppm), UGT1A1 mRNA levels (1.23-fold, compared with concurrent control, at 1600 ppm) and UGT1A6 mRNA levels (1.82- and 3.78-fold, compared with concurrent control, at 400 and 1600 ppm, respectively). Following a 42 day recovery period, measured values were generally comparable between rats previously treated at 1600 ppm Pethoxamid and concurrent controls. Cytochrome b_5 content was

similar to the concurrent control even though statistical significance was achieved in previously treated rats (1.09-fold). The statistical significance may be attributed to the low variability observed in values within the treatment group rather than reflecting a test item related increase.

Treatment of male mice with Pethoxamid for 7 days caused statistically significant increases in cytochrome b_5 content (1.39-fold, compared with concurrent control, at 5000 ppm), cytochrome P450 content (1.22- and 1.50-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), 7-ethoxyreorufin-O-dealkylation (Cyp1a1/2) activity (1.69- and 1.54-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), testosterone 16β-hydroxylase (Cyp2b10) activity (1.46- and 1.70-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), testosterone 6β-hydroxylase (Cyp3a11/13) activity (4.39-fold, compared with concurrent control, at 400 ppm), Cyp1a2 mRNA levels (1.97-fold, compared with concurrent control, at 5000 ppm), Cyp2b10 mRNA levels (12.4- and 115-fold, compared with concurrent control, at 400 and 5000 ppm, respectively), Cyp3a11 mRNA levels (6.90-fold, compared with concurrent control, at 5000 ppm) and Cyp4a10 mRNA levels (9.00-fold, compared with concurrent control, at 5000 ppm), and had little or no effect on lauric acid 12-hydroxylase (Cyp4a10/12) activity. Following a 42 day recovery period, measured values were generally comparable between mice previously treated at 5000 ppm Pethoxamid and concurrent controls. Testosterone 16β-hydroxylase (Cyp2b10) activity was 0.813-fold (achieving statistical significance) that of the concurrent controls. The statistical significance may be attributed to the low level of variability in values within the treatment group rather than reflecting a test item related decrease.

Table 3.9.4-13: Summary of Results in rats and mice

End-point	**Pethoxamid concentration**	**Fold vs. concurrent controls**	**Considered notable Pethoxamid related effect[#]**	**Effects considered reversible following 42-day observation period**
Rats				
UGT1A1/6 activity	400 ppm	1.14	No	NA
	1600 ppm	1.62 §	No	NA
UGT1A1 mRNA levels	400 ppm	1.05	No	NA
	1600 ppm	1.23 §	No	NA
UGT1A6 mRNA levels	400 ppm	1.82 §	No	NA
	1600 ppm	3.78 §	Yes	Yes
Mice				
Cyp1a1/2 activity	400 ppm	1.69 §	No	NA
	5000 ppm	1.54 §	No	NA
Cyp2b10 activity	400 ppm	1.46 §	No	NA
	5000 ppm	1.70 §	No	NA
Cyp3a11/13 activity	400 ppm	4.39 §	Yes	Yes
	5000 ppm	1.24	No[*]	NA
Cyp4a10/12 activity	400 ppm	0.924	No	NA
	5000 ppm	1.22	No	NA
Cyp1a2 mRNA levels	400 ppm	1.06	No	NA
	5000 ppm	1.97 §	No	NA
Cyp2b10 mRNA levels	400 ppm	12.4 §	Yes	Yes

	5000 ppm	115 §	Yes	Yes
Cyp3a11 mRNA levels	400 ppm	1.21	No	NA
	5000 ppm	6.90 §	Yes	Yes
Cyp4a10 mRNA levels	400 ppm	2.21	Yes	Yes
	5000 ppm	9.00 §	Yes	Yes

[#]'Yes' designation reflects > 2-fold. The 2-fold criteria was used as a numerical framework for discerning notable test item related changes from expected inter-individual variability. An increase of ≥ 2-fold was applied to reflect the EMA 2013 guidance that states that the observation of a ≥ 100% increase in enzyme level can routinely be considered as a positive indication of enzyme induction. It is acknowledged that the EMA guidance primarily relates to *in-vitro* test systems.§ Significantly different from the vehicle control (0 ppm Pethoxamid) as a result of One-way Analysis of Variance ($p < 0.05$).
[*]The reason for the apparent inverse dose-response relationship remains uncertain within the context of this study.
NA: Not applicable

CONCLUSION: The most pronounced effects of Pethoxamid were the statistically significant and dose-dependent increases in UGT1A6 mRNA levels in male rats and the statistically significant and dose-dependent increases in Cyp3a11/13 activity and Cyp2b10, Cyp3a11 and Cyp4a10 mRNA levels in male mice. Of the CYP end-points assessed the induction of Cyp2b10 mRNA at 5000 ppm was the highest (115-fold concurrent control). Results following the observance of a 42-day recovery period illustrated no notable persisting effects (similar to concurrent control) on the end-points assessed.

3.9.4.6 Anonymous (2019a)

Reference: TKC-94 : Pethoxamid technical: I*n-vitro* inhibition of non-juvenile male SD rat thyroperoxidase (TPO)-catalysed guaiacol oxidation
Author(s), year: Anonymous, 2019
Report/Doc. number: 2018TOX-PXA4481 / CLS4_0023_0001
Guideline(s): None
GLP: Yes
Acceptability: Yes

JUSTIFICATION FOR TEST SYSTEM SELECTION: The guaiacol assay of TPO activity was used for this study (***Chang and Doerge, 2000, Paul et al., 2013 and Paul et al., 2014***). The known TPO inhibitor, PTU, was used as a positive control substance. The potency of pethoxamid, and the positive control, as inhibitors of TPO were quantified in terms of half maximal concentrations (IC_{50}) values, where applicable. Rat thyroid microsomes are a well-established source used for the evaluation of TPO activity.

EXECUTIVE SUMMARY
The objective of this study was to assess the potential for pethoxamid to inhibit thyroperoxidase (TPO) activity in pooled thyroid microsomes prepared from male Sprague Dawley rats. The guaiacol assay of TPO activity was used for this study. The known TPO inhibitor, 6-propyl-2-thiouracil (PTU), was used as a positive control substance.

The positive control, PTU, was assayed at the following concentrations: 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50, 100 and 200 µM. PTU was a potent inhibitor of TPO activity, exhibiting a half maximal inhibitory concentration (IC_{50}) value of 3.4 µM, 95% CI: 2.5 to 5.2 µM. This estimate is similar to a previously published estimate of 1.3 µM, 95% CI: 0.5 to 3.2 µM (***Paul et al., 2013***), validating the performance of the assay.
Pethoxamid was assayed at the following concentrations: 0, 0.01, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 µM. Pethoxamid did not inhibit TPO activity at any of the tested concentrations.

Conclusion: Pethoxamid is not an inhibitor of TPO activity.

MATERIALS AND METHODS

Materials:

Test Material:	Pethoxamid
Description:	Brown liquid
Lot/Batch number:	21082018
Purity:	97.7 %

Vehicle / positive control: DMSO / 6-propyl-2-thiouracil (PTU).

Study Design and Methods:
Experimental dates: Start: 06 December 2018, End: 12 December 2018.

Test item and positive control stock preparations: A solubility test was carried out to confirm that a 100 mM stock solution could be prepared for assaying the test item. The test item was made up fresh on the day of the assay by performing serial dilutions from a freshly prepared 100 mM stock solution. Positive control concentrations were made up fresh on the day of the assay by performing serial dilutions from a 100 mM stock solution. In each case there were a total of 10 concentrations and a blank.

Characterization of Test System: The test system, rat thyroid microsomes, was prepared from a pool of non-juvenile male Sprague Dawley rat thyroid glands (rats between 10 and 16 weeks of age) Thyroids were stored at approximately -80°C.

Protein Determination: The protein concentration of the pooled thyroid microsomes was determined in aqueous solutions using a modification of the method of ***Lowry et al., (1951)*** and bovine serum albumin standards. Samples were analysed using a Hitachi UV-Vis spectrophotometer.

TPO Activity: This method exploits the ability of TPO to catalyse the oxidation of guaiacol (a naturally occurring organic compound described as a colourless to light yellow and pink liquid) to a coloured product. The rate of production of the coloured product was determined spectrophotometrically (OD_{450}) and TPO activity was then expressed in units of ΔOD_{450}/min/mg protein. Thyroid microsomal samples were analysed in 6 replicates per concentration of test item or positive control, using a Hidex Sense Microplate reader. Test item or positive control (2 µL) was administered directly into each well of a 96-well plate containing 198 µL of microsomal assay preparation.

Acceptability of the assay: Of the six replicates performed, at least 3 are required for analysis (all 6 used if possible). The CV of replicate activity measurements will not exceed 15%. The IC_{50} value for PTU must be approximately 2 µM (similar to previously published value in ***Paul et al., 2013***).

Statistical Analysis: TPO activity (expressed as a percentage of control activity) was determined as a function of Test Item or positive control concentration, and IC50 parameters were estimated by fitting a four-parameter logistic model to the resulting data set. Model fitting was performed using GraphPad Prism® (Version 7.04, GraphPad Software Inc, San Diego, California, USA).

Calculation of TPO Enzyme Activity: TPO enzyme activity was calculated and expressed as ΔOD_{450}/min/mg protein. For each well, the plot of OD_{450} versus time was examined to establish the period of time the OD_{450} changed in a linear manner. The start of this period was defined as T_1 (in seconds) and the end of this period was defined as T_2 (in seconds); the corresponding OD_{450}s were defined as OD_1 and OD_2, respectively. TPO enzyme activity was then calculated according to the following equation:

$$\text{TPO activity} \quad \frac{\frac{\Delta OD_{450}}{\text{min}}}{\text{mg protein}} = \frac{(60 \times (OD_2 - OD_1)) \times \frac{1000}{C}}{T_2 - T_1}$$

Where C = amount of microsomal protein (in µg) in the incubation.

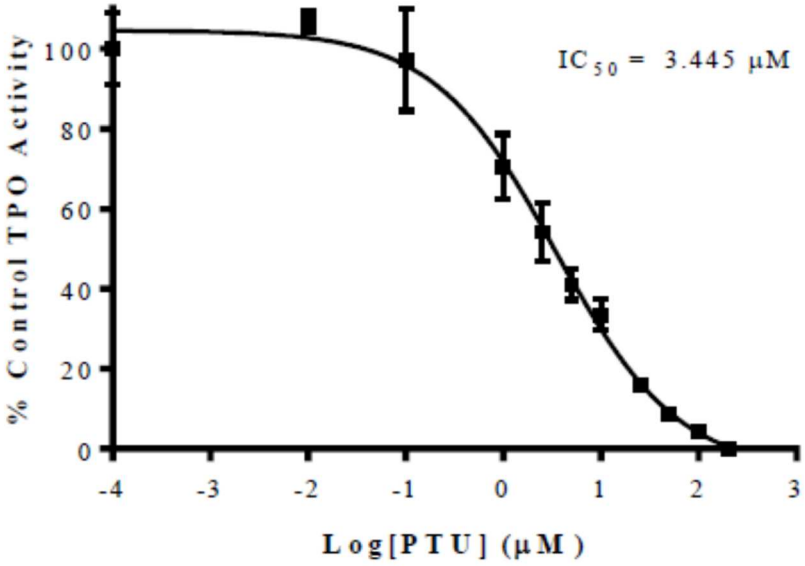
Data were then further processed and expressed as a percentage of control; these data were then used to estimate IC_{50} values using GraphPad Prism®.

RESULTS AND DISCUSSION

The positive control, PTU, was assayed at the following final concentrations: 0, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50, 100 and 200 µM. PTU was a potent inhibitor of TPO activity, exhibiting an IC_{50} value of 3.4 µM, 95% CI: 2.5 to 5.2 µM. This estimate is similar to a previously published estimate of 1.3 µM, 95% CI: 0.5 to 3.2 µM (***Paul et al., 2013***) validating the performance of the assay.

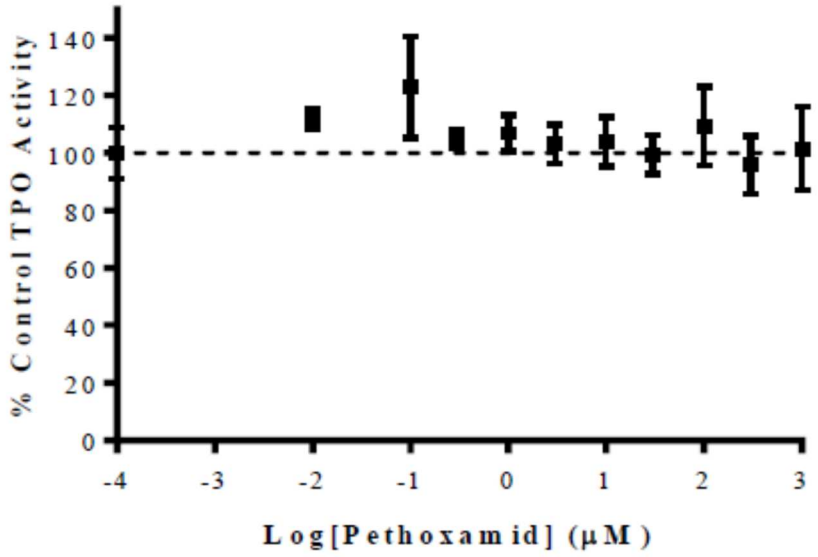
Pethoxamid was assayed at the following final concentrations: 0, 0.01, 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 µM. Pethoxamid did not inhibit TPO activity at any of the tested concentrations.

Figure 3.9.4-1: Inhibition of rat TPO activity by PTU



TPO activity (expressed as a percentage of control activity) plotted as a function of PTU concentration. Data are presented as mean ± SD of up to 6 replicates per concentration. A four parameter logistic model was fitted to the data (best-fit curve shown). Error bars are small and in general are obscured by the data symbols.

Figure 3.9.4-2: Inhibition of rat TPO activity by pethoxamid



TPO activity (expressed as a percentage of control activity) plotted as a function of pethoxamid concentration. Data are presented as mean ± SD of up to 6 replicates per concentration. The dotted line is to highlight the scatter of the data points around the 100% value.

CONCLUSION: Pethoxamid is not an inhibitor of TPO activity.

References:

Chang, H.C. and Doerge, D.R. (2000). Dietary genistein inactivates rat thyroid peroxidase in vivo without apparent hypothyroid effect. *Toxicol. Appl. Pharmacol.* **168**: 244-252.

Lowry OR, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**, 265-275.

Paul KB, Hedge JM, Macherla C, Filer DL, Burgess E, Simmons SO, Crofton KM and Hornung MW (2013). Cross-species analysis of thyroperoxidase inhibition by xenobiotics demonstrates conservation of response between pig and rat. *Toxicol.* **312**: 97-107.

Paul KB, Hedge JM, Rotroff DM, Hornung MW, Crofton KM and Simmons SO (2014). Development of a thyroperoxidase inhibition assay for high-throughput screening. *Chem. Res. Toxicol.* **27:** 387-399.

3.9.4.7 Anonymous (2020)

Reference: Pethoxamid: A 90-Day Oral (Dietary) Thyroid Mechanistic Study in Rats **Author(s), year**: Anonymous, 2020 **Report/Doc. number**: Laboratory Project ID: 00206020 / Report No.: 2018TOX-PXA4560 **Guideline(s)**: None **GLP**: Yes **Acceptability**: Yes

Justification for test system selection: The Crl:CD(SD) rat was chosen as it is an accepted rodent species for preclinical toxicity testing by regulatory authorities. In addition, this strain was used in an earlier chronic/carcinogenicity study and, therefore, enables a comparison of outcome. The number of animals was the minimum required to properly characterize the effects as, at this time, studies in

laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals do not currently exist. Oral administration was selected as this is the most likely route of human exposure.

EXECUTIVE SUMMARY

This study was designed to evaluate potential mechanisms underlying thyroid gland changes seen when pethoxamid was administered via the diet to male Sprague Dawley rats for at least 27 or at least 90 days.

Three groups of 20 male rats were fed diet containing 400, 1600 or 5000 ppm pethoxamid. An additional group of 20 male rats received basal diet and served as controls and another group of 20 male rats received 1000 ppm phenobarbital and served as positive controls. The rats were fed their diets for 29 or 92/93 consecutive days. Five rats/group were killed after 29 days. Clinical signs, body weights, body weight gains, food consumption, thyroid hormone assessment, gross necropsy findings, liver and thyroid weights, UDP-glucuronyl transferase (UGT) enzyme induction and histopathology of liver, thyroid and pituitary were evaluated during the study.

The overall mean daily intake of pethoxamid was 24, 96 and 308 mg/kg bw/day for the 400, 1600 and 5000 ppm groups, respectively.

There were no test-substance related mortalities, clinical observations, effects on food consumption or macroscopic findings.

At 5000 ppm pethoxamid, mean body weight gains were generally lower throughout the study which resulted in lower mean body weights and lower cumulative body weight gains.

There was a higher mean TSH value at 1600 and 5000 ppm on days 15, 29, 57 and 89 and a time-dependent decrease in total T4 relative to pretreatment values during the first 29 days of the study. There was no effect on total T3 or rT3.

There were higher mean thyroid/parathyroid weights at 5000 ppm on days 30 and 93/94 and higher mean liver weights at 5000 ppm on day 30 and at 1600 and 5000 ppm on day 93/94.

Test substance-related microscopic findings included follicular cell hypertrophy at 1600 and 5000 ppm on day 93/94 and hepatocellular hypertrophy at 5000 ppm on days 30 and 93/94.

T_4-glucuronidation activity was increased at 1600 and 5000 ppm on days 30 and 93/94. T_3-glucuronidation activity was increased at 1600 and 5000 ppm on day 30 and at 5000 ppm on day 92/93.

Thyroid follicular cell hypertrophy/hyperplasia and liver centrilobular hypertrophy, associated increased mean thyroid/parathyroid and liver weights were seen in the positive controls, along with increased TSH values on days 15, 29, 57 and 89, increased total T3 and rT3 values on day 89, lower mean total T4 values on days 15 and 29 and increased T_4- and T_3-glucuronidation activity on days 30 and 93/94. These changes are consistent with well recognised phenobarbital-related changes.

Conclusion: Based on the results of this study, dietary administration of pethoxamid to Crl:CD(SD) rats at dosage levels of 1600 and 5000 ppm for a minimum of 90 days resulted in liver enzyme induction leading to an increase in T4 glucuronidation and clearance of T4 which elicited a feedback response on the thyroid via an increase in TSH. It can be concluded from the hormone data that the increased TSH and associated thyroid follicular cell hypertrophy resulted in functional compensation by the thyroid in the Pethoxamid treated rats.

MATERIALS AND METHODS

Materials:

Test Material:	Pethoxamid (IN-45263-006)
Description:	Technical material, dark brown solid
Lot/Batch number:	21082018
Purity:	99.4%
CAS#:	Not reported
Stability of test compound:	Expiry date 13 February 2022 (stored at 18-24ºC)
Positive control:	Phenobarbital sodium salt
Description:	White powder
Lot/Batch number:	SLBX0037
Purity:	99.9%
Stability of test compound:	Retest date March 2020 (stored at 18-24ºC)

Vehicle: Basal Diet, test substance administered via the diet.

Test Animals:	
Species	Rat
Strain	Crl:CD(SD)
Age/weight at dosing	7-8 weeks / 210-272 g
Source	Charles River Laboratories Inc., Raleigh, NC, USA.
Housing	2-3/cage in solid-bottom cages with appropriate bedding
Acclimatisation period	13 days
Diet	Treated or basal PMI Nutrition International, LLC Certified Rodent LabDiet®5002 (meal) *ad libitum*.
Water	Municipal tap water from the public supply *ad libitum*.
Environmental conditions	Temperature: 20-26°C
	Humidity: 30-70%
	Air changes: at least 10/hour
	Photoperiod: 12 hours light and 12 hours dark

Study Design and Methods:

In-life dates: Start: 04 March 2019, End: 05 June 2019.

Dose level selection rationale: The dose levels corresponded to levels used in an earlier subchronic and chronic/carcinogenicity studies with pethoxamid. It was anticipated that the highest concentration would show test substance related effects but not produce mortalities sufficient to prevent meaningful evaluation.

Test item and control preparations: Test and positive control dietary preparations were prepared as follows. An appropriate quantity of test substance together with an appropriate amount of acetone and basal diet was mixed in a Hobart mixer to form a pre-mix. The remainder of the basal diet to achieve the desired concentration on a w/w basis was mixed with the pre-mix in a V blender. The diet was blended to achieve a total batch of homogeneous diet at the appropriate concentration/group; the acetone was evaporated overnight prior to feeding the diet to the rats. Test diets, containing pethoxamid, were prepared weekly and stored at 18-24°C. Positive control diets were prepared daily for the initial 5 days

and every 3 days thereafter and stored at 18-24°C. An appropriate quantity of basal diet was weighed out approximately weekly for the controls and stored at 18-24°C.
Samples of the diets were collected and analysed by HPLC, using a validated analytical procedure, to determine achieved concentrations, homogeneity and stability of the test substance in diet at the following times:

Day	Achieved concentration	Homogeneity	Stability
-1	all groups	all groups except control	n/a
1	n/a	n/a	positive controls
5	n/a	positive controls	n/a
28	all groups except positive control	n/a	n/a
29	positive control	n/a	n/a
84	all groups except positive control	n/a	n/a
89	positive control	n/a	n/a
n/a = not applicable			

For concentration analyses, a single (50 g) sample of each dietary preparation was collected; duplicate samples were analysed. Concentration results were considered acceptable if mean sample concentration results were 100±15% of theoretical.

For homogeneity analyses, a single (50 g) sample of each dietary preparation was collected from each stratum; duplicate samples from each stratum were analysed. Homogeneity results were considered acceptable if the relative standard deviation of the mean concentration value at each sampling location was ≤ 10% and if mean sample concentration results were 100±15% of theoretical.

Stability of pethoxamid in diet was not assessed as dietary preparations have been shown previously to be stable for at least 10 days at room temperature (18 to 24°C) and frozen (-10 to -50°C). Stability of positive control dietary preparations were determined after 1, 3 and 7 days at 8-24°C. Samples (50 g) from each stratum were combined; duplicate samples were analysed. Stability results were considered acceptable if analysis results were ≥ 90% of pre-storage concentrations.

Analytical results: The dietary preparations contained 95.1-114% of the target concentrations and were homogeneous. Test substance dietary preparations have been shown previously to be stable and homogeneous over the range of concentrations (300 and 6000 ppm) used on this study for at least 8 days at 18-24°C.

The analysed dietary formulations contained 88.8-111% of the target concentration of the positive control (1000 ppm phenobarbital) and were homogeneous. Positive control formulations, however, were not stable at 18-24°C for 7 days but were for 3 days and, therefore, were prepared every 3 days.

No test substance was detected in control diet.

Animal assignment and treatment: The rats were assigned to groups by a stratified randomisation scheme designed to achieve similar group mean body weights. Basal diet (controls) or dietary preparations containing 400, 1600 or 5000 ppm pethoxamid or 1000 ppm phenobarbital (positive controls) was administered to groups of 20 male rats for 29 or 92/93 consecutive days. Five rats/group were killed after 29 days.

Table 3.9.4-14: Study design

Group	Treatment	Dietary concentration (ppm)	Number of males
1 (control)	basal diet	0	20
2 (low)	pethoxamid	400	20
3 (mid)		1600	20
4 (high)		5000	20
5 (positive control)	phenobarbital	1000	20

Mortality and clinical observations: Animals were checked for general health/mortality and moribundity twice daily, once in the morning and once in the afternoon. Cage-side observations were recorded daily throughout the study starting on day 1. The animals were removed from their cages, and a detailed clinical observation was performed 1 week (±2 days) prior to randomisation, on the day of randomisation, on day 1 (prior to dosing), weekly (±2 days) during the study period and on the day of scheduled termination.

Body weight: Animals were weighed 1 week (±2 days) prior to randomisation, on the day of randomisation, on day 1 (prior to dosing), weekly (±2 days) during the study period, on the day prior to scheduled termination and on the day of scheduled termination.

Food consumption and achieved dose: Food consumption was measured once following randomisation (all groups) and weekly (±2 days) during the study period (groups 1-4) or daily (group 5). The mean amounts of basal diet and dietary preparations containing the test or positive control substance consumed (mg/kg bw/day) per group were calculated.

Thyroid hormone assessment: Blood samples were collected from all animals, via the jugular vein, without anticoagulant on days -3, 15, 29/30, 57 and 89. The blood was allowed to clot for 30 minutes and then all samples were centrifuged (3000 rpm; 2056xg) for 10 minutes at 4°C within 2 hours of collection and divided into aliquots. Samples were stored at -55 to -85°C prior to analysis.

Analyses to determine total T3 and T4 concentrations were conducted using a validated UPHLC/MS/MS assay. Analyses to determine TSH (rTSH [Rat Thyroid Stimulating Hormone] RIA Kit and rT3 (rT3 RIA Kit) concentrations were conducted using qualified radioimmunoassays.

Termination and pathology: A necropsy was conducted on the one rat that died on study and specified tissues were saved. Animals surviving until scheduled termination (days 30 or 93/94) were fasted overnight, weighed and killed by carbon dioxide inhalation followed by exsanguination.

Macroscopic findings: Animals were subjected to a complete necropsy examination, which included evaluation of all external surfaces and orifices; the cranial cavity and external surfaces of the brain; and thoracic, abdominal and pelvic cavities with their associated organs and tissues.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

liver pituitary	thyroid with parathyroids (post-fixation)

Paired organs were weighed together. Organ to body weight ratios, using terminal body weights, were calculated.

Tissue submission: Representative samples of the following tissues were collected from all animals and preserved in 10% neutral buffered formalin:

pituitray	abnormal tissue
liver	thyroid with parathyroids [1]

[1] parathyroids were examined if in the plane of section and in all cases where a gross lesion of the organ was present

Microscopic examination: All tissues listed above were processed, sectioned and stained with hematoxylin and eosin and examined histopthologically.

Determination of UDP-glucuronosyltransferase (UGT) enzyme induction: Liver was collected at the interim and terminal necropsies for UGT analysis from all animals/group. Two (2-3 g) samples of liver were excised from all animals/group and saved separately in RNAse-free tubes and immediately snap-frozen in liquid nitrogen for analysis of UGT. Two additional (20-30 mg) samples were excised from the left lobe and saved separately in RNAse-free tubes and immediately snap-frozen in liquid nitrogen for possible future analysis.

UGT induction *ex vivo*: The enzymatic activity in liver collected from all animals at the interim necropsy and 5 animals/group at terminal necropsy was evaluated.

Enzymatic activity	Substrate	Metabolite
T3-glucuronidation	T3	T3-glucuronide
T4-glucuronidation	T4	T4-glucuronide

Liver samples were thawed at room temperature and homogenised in ice-cold 0.1 M Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose. The homogenate was centrifuged for 30±1 minute at 12000 g (4°C). The resulting supernatant was centrifuged at 105000 g for 75±1 minutes (4°C). Subsequently the microsomal pellet was resuspended in 0.01 M Tris buffer pH 7.4 containing 3 mM EDTA. Microsomes were stored in aliquots of 0.05 mL and 0.25 mL (for protein and the UGT activity determinations, respectively) at ≤75°C.

Protein concentrations were measured in liver microsomes from each animal, in duplicate, by the method of Lowry (***Lowry et al, 1951***) using bovine serum albumin (BSA) as a standard.

Stock solutions of T3 and T4 substrates were prepared. Incubation mixtures were prepared on ice, in duplicate, by mixing 0.1 M Tris-HCl buffer pH 7.4 with rat liver microsomes and UGT reaction mixtures. After shaking, the mixtures were pre-incubated for 15±1 minutes on ice. Subsequently, the mixtures were pre-incubated for 5±1 minutes at 37±1°C in a water bath. The reaction was started by the addition of the appropriate (T3 or T4) spiking solution. After incubation at 37±1°C for a defined time, the samples were transferred to ice and a protein precipitation solution was added to precipitate proteins. Subsequently, a sample pre-treatment procedure was used to remove precipitated proteins and the samples were analysed by UPLC-PDA-MS.

Statistics: All statistical tests were conducted at the 5% significance level. All pairwise comparisons used two-sided tests and were reported at the 1% and 5% levels. Numerical data collected were analysed as indicated at each interval. Inferential statistics were performed when possible, but excluded semi-quantitative data, and any group with <3 observations. A parametric (WTDMS^{TM}) method was used for body weight, body weight gain, food consumption, TSH and rT3 data and total T3 vand T4 data. A parametric (Provantis or Nevis) method was used for organ weights and organ weights relative to body weight. Pairwise comparisons of groups 2, 3, 4 and 5 versus group 1 (controls) were made. Datasets were compared using an overall one-way ANOVA *F*-test. If the overall *F*-test was found to be significant, then the above pairwise comparisons were conducted using Dunnett's test.

RESULTS AND DISCUSSION

Mortality and clinical observations: One 1600 ppm rat was found dead on day 30 shortly after blood collection. The cause of death was acute haemorrhage and was not test substance related. There were no other mortalities.

There were no test substance related clinical observations.

Body weights: Treatment-related lower body weights were noted at 5000 ppm throughout the study and were statistically significantly different from control values during weeks 2-5, 7-10 and 12-13. Mean body weight was 7.5% lower than controls at the end of the study.

Treatment-related lower body weight gains were noted at 5000 ppm throughout the study and were statistically significantly different from control values during weeks 1-2, 4-5 and 6-7. Statistically significantly lower mean cumulative body weight gains, compared to controls, were noted at 5000 ppm throughout the study (14% lower), most notably during weeks 1-5 and 5-9 (20.7% and 19% lower, respectively).

There were no treatment-related effects on body weight at 400 or 1600 ppm pethoxamid or in the positive control group.

Table 3.9.4-15: Intergroup comparison of bodyweights and body weight change - selected timepoints

Week	Dietary Concentration pethoxamid (ppm)				Positive control (1000 ppm phenobarbital)
	0 (control)	400	1600	5000	
Body weights [g]					
1	240	240	240	240	238
2	303	304	301	279**	301
4	389	388	381	358**	385
8	492	499	490	454*	500
12	556	563	550	509*	561
14	569	581	568	527	584
Body weight changes [g]					
1-2	63	63	61	39**	63
4-5	43	46	42	35**	41
6-7	29	29	28	21**	30
Cumulative body weight changes [g]					
1-5	193	194	183	153**	188
1-9	275	281	268	229**	280
1-14	332	342	327	285*	341
5-9	88	88	83	71*	90
9-14	57	61	59	56	61

* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test)

** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test)

Food consumption and achieved dose: There were no test substance related effects on food consumption.

The mean dose received (based on nominal dietary levels of pethoxamid) is shown below.

Table 3.9.4-16: Overall mean dose received (mg/kg/day)

Group	Treatment	Dietary concentration (ppm)*	Mean test substance consumption (mg/kg bw/day)
1 (control)	basal diet	0	0
2 (low)	pethoxamid	400	24
3 (mid)		1600	96
4 (high)		5000	308
5 (positive control)	phenobarbital	1000	62

*No correction factor was used for test substance purity.

Thyroid hormone assessment: Test substance related higher mean TSH values were noted at 1600 and 5000 ppm on days 15, 29, 57 and 89 and increased in a time-dependent manner on day 15 and 29. The changes were statistically significant compared to the controls, with the exception at 1600 ppm on day 89.

There were no statistically significant and/or biologically relevant differences in total T3 levels between test substance and control groups.

There was a trend towards slightly higher total T4 levels at 5000 ppm compared to controls on days 15, 57 and 89. However, the pre-treatment values for total T4 at 5000 ppm were higher relative to controls ($P<0.01$). T4 levels at 5000 ppm decreased in a time-dependent manner on days 15 and 29 relative to pre-test values and returned to near pre-test values on day 57. The time course related decrease in total T4 levels at 5000 ppm correlated with the increase in TSH over the same time interval. As there was no impact on T3 levels at 5000 ppm, the changes in T4 were considered not to be adverse.

In the positive control group, higher mean TSH values were noted on days 15, 29, 57 and 89 and increased in a time-dependent manner on days 15 and 29. Statistically significant higher mean total T3 and rT3 values were noted on day 89. Total T4 levels decreased in a time-dependent manner on days 15 and 29, compared to pre-treatment values, and corresponded to the increase in TSH levels during the same time interval. These changes were statistically significantly different from the control group values on day 29.

There were no other test substance-related effects on thyroid hormones. Other values that achieved statistical significance were considered not test or positive control-related as they were prior to test substance initiation, there was a lack of dose-response and most values were within the range of concurrent control values.

Table 3.9.4-17: Mean thyroid hormone parameters (% difference from controls)

Day	Dietary Concentration pethoxamid (ppm)				Positive control (1000 ppm phenobarbital)
	0 (control)	400	1600	5000	
TSH (ng/mL)					
-3	4.9	8.1* (65.3)	6.6 (34.7)	8.9** (81.6)	8.5* (73.5)
15	7.3	8.3 (13.7)	14.3* (95.9)	17.8** (143.8)	18.6** (154.8)
29	11.7	14.9 (27.4)	22.1* (88.9)	24.6** (110.3)	26.5** (126.5)
57	7.9	9.9 (25.3)	16.6* (110.1)	20.8** (163.3)	24.4** (208.9)
89	12.0	12.3 (2.5)	19.3 (60.8)	24.3** (102.5)	27.2** (126.7)
Total T3 (pg/mL)					
-3	874	897 (2.6)	869 (-0.6)	866 (-0.9)	803 (-8.1)
15	686	648 (-5.5)	559** (-18.5)	678 (-1.2)	601 (-12.4)
29	709	678 (-4.4)	663 (-6.5)	720 (1.6)	642 (-9.4)
57	769	655 (-14.8)	795 (3.4)	810 (5.3)	756 (-1.7)
89	582	610 (4.8)	578 (-0.7)	677 (16.3)	729* (25.3)
Total T4 (pg/mL)					
-3	62995	60665 (-3.6)	71940 (14.3)	82600** (31.2)	78225** (24.3)
15	51195	50690 (-1.0)	52010 (1.6)	61485** (20.1)	45390 (-11.3)
29	58840	52350 (-11.0)	52250 (-11.2)	52720 (-10.4)	35590** (-39.5)
57	65993	67820 (2.8)	72043 (9.2)	79620** (20.6)	55513 (-15.3)
89	36493	38840 (6.4)	35171 (-3.6)	50287** (37.8)	36900 (1.1)
rT3 (ng/mL)					
-3	0.179	0.148* (-17.3)	0.142** (-20.7)	0.181 (1.1)	0.171 (-4.5)
15	0.141	0.094** (-33.3)	0.092** (-34.8)	0.144 (2.1)	0.108* (-23.4)
29	0.091	0.141** (54.9)	0.133** (46.2)	0.106 (16.5)	0.101 (11.0)
57	0.105	0.091 (-13.3)	0.078* (-25.7)	0.133* (26.7)	0.110 (4.8)
89	0.103	0.098 (-4.9)	0.076 (-26.2)	0.134 (30.1)	0.199** (93.2)

* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test)

** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test)

Figures in bold = values considered to be test substance-related

Macroscopic findings: No test or positive control substance-related macroscopic findings were seen on day 30 or day 93/94. Macroscopic findings observed were incidental, of the type commonly seen in this strain and age of rat and/or were of similar incidence in control and treated animals.

Organ weights:

Day 30: Increased liver and thyroid/parathyroid weights (absolute and/or relative to body weight) were seen at 5000 ppm pethoxamid and were considered to be test substance-related. The increased liver weight correlated with microscopic findings of hepatocellular hypertrophy.

Increased liver and thyroid/parathyroid weights (absolute and/or relative to body weight) were also seen in the positive controls and correlated with microscopic findings of hepatocellular hypertrophy and thyroid follicular cell hypertrophy.
No other test or positive control substance-related changes were seen. There were other isolated organ weight values that were statistically significantly different from controls but there were no patterns, trends or correlating data to suggest these values were toxicologically relevant and they were, therefore, considered incidental.

Day 93/94: The increased liver weights (absolute and relative to body weight) in the 1600 and 5000 ppm test substance groups and the increased thyroid/parathyroid weight (absolute and relative to body weight) in the 5000 ppm test substance group were considered to be test substance-related. The increased liver and thyroid/parathyroid weights at 5000 ppm correlated with microscopic findings of hepatocellular hypertrophy and thyroid follicular cell hypertrophy, respectively.

Increased liver and thyroid/parathyroid weights (absolute and/or relative to body weight) were also seen in the positive controls and correlated with microscopic findings of hepatocellular hypertrophy and thyroid follicular cell hypertrophy/hyperplasia.

No other test or positive control substance-related changes were seen. There were other isolated organ weight values that were statistically significantly different from controls but there were no patterns, trends or correlating data to suggest these values were toxicologically relevant and they were, therefore, considered incidental.

Table 3.9.4-18: Summary of mean organ weight data (% difference from controls)

	Dietary Concentration pethoxamid (ppm)				**Positive control (1000 ppm phenobarbital)**
	0 (control)	**400**	**1600**	**5000**	
Day 30					
No. animals/group	5	5	5	5	5
Terminal body weight (g)	432.4	416.6 (-3.7)	397.0 (-8.2)	350.6*** (-18.9)	384.4* (-11.1)
Liver – absolute weight (g)	17.6174	16.5890 (-5.8)	17.7314 (0.6)	18.6318 (5.8)	20.6154* (17.0)
Liver - % of body weight	4.07300	3.98076 (-2.3)	4.46751 (9.7)	5.31128** (30.4)	5.36565** (31.7)
Thyroid/parathyroid – absolute weight (g)	0.0156	0.0172 (10.3)	0.0170 (9.0)	0.0204* (30.8)	0.0238*** (52.6)
Thyroid/parathyroid - % body weight	0.00364	0.00411 (13.0)	0.00426 (17.1)	0.00576** (58.3)	0.00615** (69.2)
Day 93/94					
No. animals/group	15	15	14	15	15
Terminal body weight (g)	550.9	558.6 (1.4)	547.4 (-0.6)	504.5* (-8.4)	552.3 (0.2)
Liver – absolute weight (g)	17.0991	18.4860 (8.1)	19.7418* (15.5)	23.0585***(34.9)	25.9685***(51.9)
Liver - % of body weight	3.10376	3.30747 (6.6)	3.61093** (16.3)	4.57668** (47.5)	4.71391** (51.9)
Thyroid/parathyroid – absolute weight (g)	0.0183	0.0205 (12.4)	0.0183 (0.1)	0.0231** (26.6)	0.0248*** (35.8)

Thyroid/parathyroid - % body weight	0.00332	0.00365 (10.1)	0.00334 (0.5)	0.00460** (38.6)	0.00451** (35.8)

* Statistically significant difference from control group mean, p<0.05 (Dunnett's test)

** Statistically significant difference from control group mean, p<0.01 (Dunnett's test)

*** Statistically significant difference from control group mean, p<0.001 (Dunnett's test)

Histopathology:

Day 30: Liver centrilobular hypertrophy was seen at 5000 ppm pethoxamid and was considered to be test substance-related.

Thyroid follicular cell hypertrophy, liver centrilobular hypertrophy and pituitary gland hypertrophy of the pars distalis (one animal only) seen in the positive control group were considered to be treatment-related and were consistent with well-recognised phenobarbital effects.

Other microscopic findings seen were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence and severity in control and treated animals and were, therefore, considered unrelated to treatment.

Day 93/94: Thyroid follicular cell hypertrophy was seen at 1600 and 5000 ppm and liver centrilobular hypertrophy was seen at 5000 ppm and were considered to be treatment-related. Pituitary gland hypertrophy of the pars distalis was seen in one animal at 5000 ppm was potentially test substance-related.

Thyroid follicular cell hypertrophy and hyperplasia, liver centrilobular hypertrophy and pituitary gland hypertrophy of the pars distalis (one animal only) seen in the positive control group were considered to be treatment-related and were consistent with well-recognised phenobarbital effects.

Other microscopic findings seen were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence and severity in control and treated animals and were, therefore, considered unrelated to treatment.

Table 3.9.4-19: Summary of incidence of selected microscopic findings

		Dietary Concentration pethoxamid (ppm)				**Positive control (1000 ppm phenobarbital)**
		0 (control)	**400**	**1600**	**5000**	
Day 30						
No. tissues examined/group		5	5	5	5	5
Pituitary – hypertrophy, pars distalis	minimal	0	0	0	0	1
	total	0	0	0	0	1
Thyroid – hypertrophy, follicular cell	minimal	0	0	0	0	2
	total	0	0	0	0	2
Liver – hypertrophy, centrilobular	mild	0	0	0	3	0
	moderate	0	0	0	0	3
	marked	0	0	0	0	2
	total	0	0	0	3	5
Day 93/94						
No. tissues examined/group		15	15	14	15	15

Pituitary – hypertrophy, pars distalis	minimal	0	0	0	0	1
	mild	0	0	0	1	0
	total	0	0	0	1	1
Thyroid – hypertrophy, follicular cell	minimal	0	0	3	1	0
	moderate	0	0	0	4	0
	severe	0	0	0	10	15
	total	0	0	3	15	15
Thyroid – hyperplasia, follicular cell	minimal	0	0	0	0	3
	mild	0	0	0	0	2
	total	0	0	0	0	5
Liver – hypertrophy, centrilobular	minimal	0	0	0	4	0
	mild	0	0	0	1	0
	moderate	0	0	0	0	12
	marked	0	0	0	0	3
	total	0	0	0	5	15

UDP-glucuronosyltransferase (UGT) induction: Dose-dependent increases in T_4-glucuronidation activity were observed in the pethoxamid groups and were statistically significantly different from controls at 1600 and 5000 ppm at days 30 and 93/94 (4.9 and 3.2-fold increases at 5000 ppm compared to control values, respectively).

Increases in T_3-glucuronidation activity were also noted in the pethoxamid groups, although the dose-response was not as prominent as it was for T_4-glucuronidation activity and was statistically significant at 1600 ppm at day 30 and at 5000 ppm at day 93/94 (1.5 to 1.9-fold increase at day 30 and 1.0 to 1.93-fold at day 93/94).

A statistically significant increase in liver microsomal protein content was observed at 5000 ppm at day 30 (1.5-fold increase compared to controls) and was consistent with induction of liver metabolising enzymes.

T_4-glucuronidation activity (4.1 and 2.4-fold) and T_3-glucuronidation activity (3.7 and 1.9-fold) were statistically significantly increased in the positive controls at day 30 and day 93/94, respectively. Liver microsomal protein content was elevated at day 30 only (1.6-fold). These changes were consistent with the well-recognised increases in liver metabolising enzymes and UDP-glucuronyl transferase activity in rodents induced by phenobarbital.

Table 3.9.4-20: Protein content and enzyme activities (fold induction compared to control)

Group	**Treatment**	**Protein content**	**T_3-glucuronidation activity**	**T_4-glucuronidation activity**
Day 30				
2 (low)	400 ppm pethoxamid	1.2	1.5	2.0
3 (mid)	1600 ppm pethoxamid	1.1	1.9	2.7

4 (high)	5000 ppm pethoxamid	1.5	1.7	4.9
5 (positive control)	1000 ppm phenobarbital	1.6	3.7	4.1
Day 93/94				
2 (low)	400 ppm pethoxamid	0.9	1.4	1.5
3 (mid)	1600 ppm pethoxamid	0.9	1.0	1.9
4 (high)	5000 ppm pethoxamid	1.0	1.9	3.2
5 (positive control)	1000 ppm phenobarbital	1.0	1.9	2.4

CONCLUSION: Based on the results of this study, dietary administration of pethoxamid to Crl:CD(SD) rats at dosage levels of 1600 and 5000 ppm for a minimum of 90 days resulted in liver enzyme induction leading to an increase in T4 glucuronidation and clearance of T4 which elicited a feedback response on the thyroid via an increase in TSH. It can be concluded from the hormone data that the increased TSH and associated thyroid follicular cell hypertrophy resulted in functional compensation by the thyroid in the Pethoxamid treated rats.

Reference:
Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1): 265-275.

3.9.4.8 Anonymous (2019b)

Reference: Effect of pethoxamid on biliary excretion of [^{125}I]thyroxine and metabolites in rats
Author(s), year: Anonymous, 2019
Report/Doc. number: 2018MET-PXA4538 / 037182-1
Guideline(s): None
GLP: Yes
Acceptability: Yes

Justification for Test System Selection: The objective of this study was to determine the effect of pethoxamid on Thyroxine (T4) clearance by determining the levels of [^{125}I]Thyroxine ([^{125}I]T4) and its metabolites in serum and bile in bile-duct cannulated rats treated with pethoxamid for 7 days followed by subsequent treatment with [^{125}I]T4 at a single dose level.

EXECUTIVE SUMMARY
Bile-duct cannulated rats were pre-treated with 1% (w/v) methylcellulose with 0.5% Tween 80 in water (negative control), phenobarbital (PB, positive control), or pethoxamid once daily for 7 consecutive days. On Day 8, approximately 15 minutes prior to the [^{125}I]T4 dose, the rats were dosed with 2 mg/kg of potassium iodide. A single IV dose of [^{125}I]T4 in sterile saline was administered by intravenous injection to each male bile duct and jugular vein cannulated rats. The study design for the induction phase is summarised below.

Table 3.9.4-21: Induction phase

Group	Inducer	Inducer dose level (mg/kg)	Inducer dose concentration (mg/mL)	Inducer dose volume (mL/kg)	Number of males
1	1% (w/v) methylcellulose (MC) with 0.5% (w/v) Tween 80 in water (Negative Control)	0	0	10	6
2	Phenobarbital (PB) (Positive Control)	100	20	5	6
3	Pethoxamid (PXA)	300	30	10	6

Blood was collected from the jugular cannula for serum at 6 time points following IV dose administration. Bile was collected at 2 time points (0-2 and 2-4 hours) following IV dose administration.

The mean serum Tmax values for total radioactivity were 0.42 ± 0.14 hours, 0.40 ± 0.14 hours, and 0.25 ± 0.00 hours for Groups 1, 2, and 3, respectively. The total radioactivity Cmax and AUC0-4 values for Group 2 (PB treatment) and Group 3 (pethoxamid treatment) were decreased relative to Group 1 (control group), suggesting greater metabolism and subsequent elimination. The mean Cmax values were 0.715 ± 0.085 ng-equiv/mL, 0.427 ± 0.039 ng-equiv/mL, and 0.541 ± 0.084 ng-equiv/mL for Groups 1, 2, and 3, respectively. The mean AUC0-4 values were 1.742 ± 0.210 hr*ng-equiv/ml, 1.083 ± 0.168 hr*ng-equiv/mL, and 1.285 ± 0.186 hr*ng-equiv/mL for Groups 1, 2, and 3, respectively.

Mean liver weights in the PB and pethoxamid groups increased relative to the control group, consistent with the induction of metabolizing enzymes in these two groups

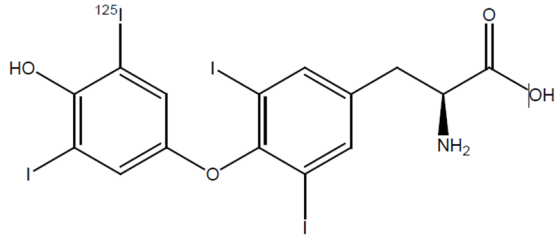
T4 was the major radioactive component in the pooled serum extracts. A peak corresponding in HPLC retention time to T4 Glucuronide was observed in the serum extracts from control rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.00% at 0.25 hours to 1.77% at 4 hours. This same peak was also observed in the serum extracts of pethoxamid -treated rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.44% at 0.25 hours to 6.76% at 4 hours. No T4 Glucuronide was found in the serum extracts from PB-treated rats, possibly due to rapid elimination in the bile.

The mean percentages of total radioactivity (expressed as the percent of administered dose or % AD) excreted in bile were 7.96 ± 0.38%, 16.13 ± 5.46%, and 11.99 ± 2.80% for Groups 1, 2, and 3, respectively. The % AD excreted in bile for Group 2 (PB-treated) and Group 3 (pethoxamid -treated) were approximately 2.0-fold higher and 1.5-fold higher, respectively, than the % AD in bile for Group 1 (control).

Bile samples from Groups 1, 2, and 3 were analyzed by HPLC/RAD to determine the distribution of [^{125}I]T4 and its metabolites. A peak corresponding in retention time to T4 Glucuronide was the major radioactive peak in the bile samples. There were greater quantities of T4 Glucuronide and T3 sulfate conjugates in bile from the PB- and pethoxamid -treated groups than in the control group. T4 Glucuronide accounted for 4.66 ± 0.30%, 10.61 ± 4.42%, and 7.44 ± 2.24% AD for Groups 1, 2, and 3, respectively. T3 Sulfate accounted for 0.62 ± 0.10%, 1.18 ± 0.15%, and 1.01 ± 0.24% AD for Groups 1, 2, and 3, respectively. Peaks corresponding in retention time to T4, T4 4'-O-Sulfate, and iodide anion were also observed. The increase in the amount of T4 Glucuronide and T3 Sulfate following PB and pethoxamid treatment was presumably due to the induction of the enzyme(s) responsible for formation of T4 Glucuronide and T3 Sulfate.

Conclusion: PB and pethoxamid treatment resulted in greater overall clearance of thyroxine due to induced T4 glucuronidation and in part induced T3 sulfation in rat liver. This induction correlated with the increase in liver weight in PB- and pethoxamid -treated rats.

MATERIALS AND METHODS
Materials:

Test Material:	[^{125}I] thyroxine ([^{125}I]T4)
Lot/Batch number:	AU52490
Specific activity:	969 Ci/mmol
Radiochemical purity:	≥95%
Structure:	

Storage:	2-10ºC
Reference standard:	Unlabeled L-thyroxine
Lot/Batch number:	BCBV2496
Purity:	≥98%
Stability:	Retest date April 2020
Reference standard:	T4 Glucuronide
Lot/Batch number:	6-EKP-111-3
Purity:	99.1%
Stability:	Retest date 31 July 2022
Reference standard:	Thyroxine 4'- *O* Sulfate
Lot/Batch number:	M17Q08157
Purity:	98.5%
Stability:	Retest date August 2019
Reference standard:	Thyroxine T_3 Sulfate
Lot/Batch number:	M17Q06160
Purity:	≥95%
Stability:	Retest date June 2019
Inducer:	Pethoxamid technical
Lot/Batch number:	21082018
Purity:	99.4%
Stability:	Expiry date 13 February 2022
Inducer:	Phenobarbital sodium salt
Lot/Batch number:	SLBX0037
Purity:	99.9%
Stability:	Retest date March 2020
Negative control:	1% methylcellulose with 0.5% Tween 80 in water solution
Vehicle::	[^{125}I]T4 was dissolved in saline

Test Animals:	
Species	Rat (bile-duct and jugular vein-cannulated for the collection of serial bile and blood samples).
Sex:	Male.
Strain	Sprague-Dawley
Age/weight at dosing	10.4 weeks/297.0-355.7 g
Source	Charles River Breeding Laboratories, Inc.
Housing	Individually housed in Nalgene metabolism cages
Acclimatisation period	1 day
Diet	Certified rodent diet *ad libitum*
Water	Tap water *ad libitum*
Environmental conditions	Temperature: 68-79°F Humidity: 30-70% Air changes: At least 10/hour

Study Design and Methods:
Experimental dates: Start: 04 April 2019, End: 07 May 2019.

Induction phase: A 1% (w/v) methylcellulose with 0.5% Tween 80 in water solution (Group 1, negative control) was administered by oral gavage (10 mL/kg) daily for 7 days to the animals in Group 1. Phenobarbital sodium salt was dissolved in 0.9% saline to yield a final concentration of 20 mg/mL (Group 2, Positive Control). This solution was administered via oral gavage (5 mL/kg, 100 mg/kg) daily for 7 days to the animals in Group 2. Pethoxamid technical was suspended in 1% (w/v) methylcellulose with 0.5% Tween 80 in water to yield a concentration of 30 mg/mL This suspension was administered via oral gavage (10 mL/kg, 300 mg/kg) daily for 7 days to the animals in Group 3.

[^{125}I]T4 Dose Level and Vehicle: [^{125}I]T4 was dissolved in saline. [^{125}I]T4 in vehicle was administered by bolus intravenous injection to each experimental animal at a dose level of 30 µCi/animal.

[^{125}I]T4 Dose Analysis: The radioconcentration (dpm/g) and homogeneity (CV) of the dose formulations were determined prior to dosing. Three aliquots of the dosing formulation were analysed for the determination of radiolabel concentration and homogeneity by gamma counting before and after dosing. Radiochemical purity of each dose formulation was determined before and after dosing.

[^{125}I]T4 Dosing Procedure: KI in sterile saline (2 mg/mL) was administered via bolus intravenous injection into the tail vein of each rat at 2 mg/kg (1 mL/kg) approximately 15 minutes prior to [^{125}I]T4 dosing to prevent uptake of [^{125}I]T4 by the thyroid. Then, [^{125}I]T4 was administered via bolus intravenous injection into the tail vein of each rat at a dose level of 30 µCi/animal.

Daily Observations: Animals were observed for viability at least 4 hours apart (once in the morning and once in the afternoon), throughout the study.

Body Weights: All animals were weighed prior to initial dose administration.

Termination: At the end of the exposure period at 4 hours post-dose, rats were killed by an overdose of CO_2 followed by exsanguination. The liver was removed from each animal weighed and stored at approximately -70°C for potential future analysis.

Sample Collection and Analyses: Blood samples (target of 250 µL per sample) were collected from the jugular cannula from each animal at 0.25, 0.5, 1, 1.5, 2, and 4 hours. The blood samples were placed in tubes without anticoagulant. Following each sample collection, the rats were infused with an equal volume of saline to maintain blood volume. Immediately following blood collection, the samples were held on wet ice for 15 minutes for coagulation before centrifugation. Samples were centrifuged within 60 minutes of collection under refrigeration (5°C for 10 minutes at 2000g). Aliquots of serum samples

were analysed for total radioactivity by gamma counting. Remaining serum samples were stored at approximately -70°C for metabolite profiling.

Bile samples were collected at 2 hours (0-2 hour collection) and 4 hours (2-4 hour collection) from the bile duct cannula. Aliquots of bile samples were analysed for total radioactivity by gamma counting. Remaining bile samples were stored at approximately -70°C for metabolite profiling.

Radioactivity in all samples was quantitated by gamma counting using a Packard Cobra II Gamma Counter. Formulation samples, bile samples, and serum extract samples were diluted with 0.5 mL of HPLC water prior to analysis. HPLC fractions were analyzed without dilution. Dose formulations, bile samples, and serum extracts were analyzed by HPLC/RAD. Unlabeled reference standards of T4 and potential metabolites were analyzed with each HPLC run. The UV retention times of the standards were determined.

Metabolite Profiling: Bile samples were diluted with HPLC mobile phase A (20 mM ammonium acetate in water, pH 4.0) and analysed by HPLC/RAD. Individual animal bile samples containing sufficient amounts of radioactivity were analysed. Serum samples from the animals were pooled by group and time point by combining equal volumes of serum from each animal. Serum samples were extracted with an equal volume of acetonitrile. The samples were centrifuged at 5°C at 2000g for 10 minutes. Aliquots of the serum extracts were analyzed by gamma counting to determine extractablity and by HPLC/RAD to detect and quantitate [^{125}I]T4 and metabolites.

Calculations: The radiolabel concentration in serum (as determined by gamma counting) was calculated as nanogram equivalents/mL by dividing the cpm/mL by the specific activity of the [^{125}I]T4 test substance. Data from the analyses of the serum collected from the animals was used to calculate the following parameters for both total radioactivity concentrations in serum: 1) the time (Tmax) to reach peak concentrations of radiolabel in the serum, 2) the concentration (Cmax) of radiolabel in the serum at Tmax, 3) the total area under the serum total radioactivity concentration versus time curve (AUC), and 4) the elimination half-life of total radioactivity. WinNonlin version 6.2 (Pharsight Corp.), operating as a validated software system was used to determine the pharmacokinetic parameters using noncompartmental analysis. The excretion of radioactivity in bile was calculated as the percentage of administered dose by dividing the quantity of radioactivity in each bile sample by the administered dose.

RESULTS AND DISCUSSION

Dose analyses: Radiochemical purity and homogeneity of the [^{125}I]T4 formulations are shown below:

Table 3.9.4-22: Radiochemical purity (%)

Group	Pre-dose	Post-dose
1, 2	90.78	90.05
3	85.62	93.11

Table 3.9.4-23: Homogeneity (cpm/µL dose formulation)

Group	Pre-dose	Post-dose	Coefficient of variation (%)
1, 2	196,473	9,966	5.07
3	204,426	10,019	4.90

Administered doses: The amounts of radioactivity dosed were 26.550 µCi for Groups 1 and 2 and 27.625 µCi for Group 3.

Serum pharmacokinetics: The mean T_{max} values for total radioactivity were 0.42 ± 0.14 hours, 0.40 ± 0.14 hours, and 0.25 ± 0.00 hours for Groups 1, 2, and 3, respectively. Terminal elimination half-lives were not reported for 2 of 3 animals in Group 1 and for all animals in Groups 2 and 3 due to extensive extrapolation (>30%) for the elimination phase.

The total radioactivity C_{max} and AUC_{0-4} values for Group 2 (PB treatment) and Group 3 (pethoxamid treatment) were decreased relative to Group 1 (control group), suggesting greater metabolism and elimination. The mean C_{max} values were 0.715 ± 0.085 ng-equiv/ml, 0.427 ± 0.039 ng-equiv/mL, and 0.541 ± 0.084 ng-equiv/mL for Groups 1, 2, and 3, respectively. The mean AUC_{0-4} values were 1.742 ± 0.210 hr*ng-equiv/ml, 1.083 ± 0.168 hr*ng-equiv/mL, and 1.285 ± 0.186 hr*ng-equiv/mL for Groups 1, 2, and 3, respectively. The results for Groups 1 and 2 are similar to those previously reported in the literature (Kato *et al.*, 2010).

Table 3.9.4-24: Serum total radioactivity concentrations (ng-eq/g) following a single IV dose of [^{125}I]T4

Time (h)	**Total radioactivity concentration (ng-eq/g)**					
	Group 1		**Group 2**		**Group 3**	
	1% MC/0.5% Tween 80 in Water (Negative Control)		**PB (Positive Control) 100 mg/kg bw/day**		**Group 3, Pethoxamid 300 mg/kg bw/day**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0.25	0.668	0.125	0.389	0.080	0.541	0.084
0.5	0.662	0.100	0.345	0.145	0.480	0.057
1.0	0.553	0.084	0.328	0.065	0.366	0.058
1.5	0.418	0.052	0.293	0.025	0.313	0.048
2.0	0.411	0.047	0.208	0.070	0.251	0.034
4.0	0.236	0.045	0.190	0.015	0.240	0.046

n=3 for Groups 1 and 3 and n=5 for Group 2.

T4 was the major radioactive component in the pooled serum extracts. A peak corresponding to iodide anion (I^-), presumably formed by radiolytic decomposition of [^{125}I]T4, was observed in all serum extract samples. A peak corresponding in HPLC retention time to T4 Glucuronide was observed in the serum extracts from control rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.13% at 0.25 hours to 1.77% at 4 hours. This same peak was also observed in the serum extracts of pethoxamid-treated rats, and the amount of the metabolite (as a percentage of HPLC peak area) increased with time, ranging from 0.44% at 0.25 hours to 6.76% at 4 hours. No T4 Glucuronide was found in the serum extracts from PB-treated rats.

Table 3.9.4-25: Quantitation of T4 and metabolites in pooled serum extracts

Group	**Treatment**		**% HPLC**					
			0.25 h	**0.5 h**	**1 h**	**1.5 h**	**2 h**	**4 h**
1	1% MC/0.5% Tween 80 in water (negative control)	Iodide	18.12	28.29	31.03	29.16	29.79	31.82
		T4 glucuronide	0.13	0.08	0.47	0.33	0.63	1.77
		T4	81.11	71.10	67.94	69.87	68.78	64.72
2	PB (Positive Control) 100 mg/kg bw/day	Iodide	31.03	32.68	30.11	32.31	31.28	37.74
		T4 glucuronide	0.00	0.02	0.00	0.00	0.00	0.00
		T4	68.70	66.93	68.80	67.13	68.10	61.59
3	Pethoxamid 300 mg/kg bw/day	Iodide	24.85	25.84	29.40	30.64	27.42	26.72
		T4 glucuronide	0.44	0.77	1.98	2.70	3.23	6.76
		T4	74.37	72.01	67.68	65.67	68.48	65.18

Liver weight: Mean liver weights in the PB and pethoxamid groups increased relative to the control group.

Table 3.9.4-26: Mean liver weights (g)

Mean liver weight (g)					
Group 1		**Group 2**		**Group 3**	
1% MC/0.5% Tween 80 in Water (Negative Control)		**PB (Positive Control) 100 mg/kg bw/day**		**Group 3, Pethoxamid 300 mg/kg bw/day**	
mean	**SD**	**mean**	**SD**	**mean**	**SD**
13.9302	0.9773	17.0702	0.6986	19.7548	1.7509

n=3 for Groups 1 and 3 and n=5 for Group 2.

Excretion of Administered Radioactivity in Bile: The mean percentages of administered dose (% AD) excreted in bile were 7.96 ± 0.38%, 16.13 ± 5.46%, and 11.99 ± 2.80% for Groups 1, 2, and 3, respectively. The % AD excreted in bile for Group 2 (PB-treated) and Group 3 (pethoxamid -treated) were approximately 2.0-fold higher and 1.5-fold higher, respectively, than the % AD in bile for Group 1 (control).

Table 3.9.4-27: Percentage Recovery of the Administered Radiolabeled Dose ([^{125}I]T4) in Bile

Time (h)	**Percentage of administered radiolabeled dose**					
	Group 1		**Group 2**		**Group 3**	
	1% MC/0.5% Tween 80 in Water (Negative Control)		**PB (Positive Control) 100 mg/kg bw/day**		**Group 3, Pethoxamid 300 mg/kg bw/day**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
2	3.89	1.43	7.77	3.50	6.96	1.61
4	4.06	1.10	8.35	2.12	5.02	1.32
Total	7.96	0.38	16.13	5.46	11.99	2.80

n=3 for Groups 1 and 3 and n=5 for Group 2.

Bile samples from Groups 1, 2, and 3 were analyzed by HPLC/RAD to determine the distribution of [^{125}I]T4 and metabolites in the bile samples. A peak corresponding in retention time to T4 Glucuronide was the major radioactive peak in the bile samples. There was significantly more T4 Glucuronide in bile from the PB- and pethoxamid-treated groups than for the control group. T4 Glucuronide accounted for 4.66 ± 0.30%, 10.61 ± 4.42%, and 7.44 ± 2.24% AD for Groups 1, 2, and 3, respectively. T3 Sulfate accounted for 0.62 ± 0.10%, 1.18 ± 0.15%, and 1.01 ± 0.24% AD for Groups 1, 2, and 3, respectively. The increase in the amount of T4 Glucuronide and T3 Sulfate following PB and pethoxamid treatment was presumably due to the induction of the enzyme(s) responsible for formation of T4 Glucuronide and T3 Sulfate.

Table 3.9.4-28: Quantitation of T4 and metabolites in bile samples

Group	**Treatment**	**Time (h)**		**% AD**	
				mean	**SD**
1	**1% MC/0.5% Tween 80 in Water (Negative Control)**	Total % AD	Bile	7.96	0.38
			Iodide	0.46	0.12
			T4 glucuronide	4.66	0.30
			T3 sulfate	0.62	0.10
			T4 4'-*O*-Sulfate	0.61	0.10
			T4	0.54	0.16
2	**PB (Positive control) 100 mg/kg bw/day**	Total % AD	Bile	16.13	5.46
			Iodide	1.30	0.38
			T4 glucuronide	10.61	4.42
			T3 sulfate	1.18	0.15
			T4 4'-*O*-Sulfate	0.88	0.25
			T4	0.70	0.24
3	**Pethoxamid 300 mg/kg bw/day**	Total % AD	Bile	11.99	2.80
			Iodide	0.79	0.16
			T4 glucuronide	7.44	2.24
			T3 sulfate	1.01	0.24

			T4 4'-*O*-Sulfate	0.59	0.15
			T4	0.95	0.16

n=3 for Groups 1 and 3 and n=5 for Group 2.

CONCLUSION: PB and pethoxamid treatment resulted in greater clearance of thyroxine due to induced T4 glucuronidation in rat liver. The induction of T3 sulfation by PB and pethoxamid was also observed. This induction correlated with the increase in liver weight in PB- and pethoxamid -treated rats.

Reference:
Y. Kato, *et al.*, *Toxicology and Applied Pharmacology*, **249 (2010)**, 238-246

3.9.4.9 Anonymous (2019c)

Reference: Pethoxamid technical: mRNA and DNA-synthesis induction in cultured mouse and human hepatocytes
Author(s), year: Anonymous, 2019
Report/Doc. number: 2018TOX-PXA4482 / CLS4_0023_0006
Guideline(s): None
GLP: Yes
Acceptability: Yes

Justification for Test System Selection: The hypothesis under investigation was that the mode of action (MoA) for pethoxamid-induced mouse liver tumor formation was similar to that of phenobarbital (PB). PB is an activator of the constitutive androstane receptor (CAR) and to a weaker extent pregnane X receptor (PXR) (***Elcombe et al, 2014***). The test system used was male CD-1 mouse primary hepatocytes and primary cryopreserved hepatocytes from three male human donors.

EXECUTIVE SUMMARY
This study was designed to test the hypothesis that pethoxamid induces mouse liver tumors by a phenobarbital (PB)-like mode of action (MoA) (***Elcombe et al., 2014***). CYP2B and CYP3A expression is induced by the constitutive androstane receptor (CAR) and to a weaker extent pregnane X receptor (PXR). In rodents, activation of CAR by PB leads to hepatocellular tumors that are not evident in hamsters, guinea pigs or primates including humans (***Elcombe et al., 2014***). While CAR/PXR induced gene expression is conserved across species, differences in replicative DNA synthesis (RDS) [S-phase of the cell cycle], a key event associated with liver tumor formation, occurs in rodent but not in human hepatocytes.

Isolated primary male CD-1 mouse hepatocytes or male primary human hepatocytes (3 donors) were exposed in culture to pethoxamid, PB or epidermal growth factor (EGF) for approximately 96 hours after which cell cytotoxicity was evaluated by quantification of ATP levels, or the cells were harvested and processed for mRNA analysis of Cyp2b10 and Cyp3a11 (mouse) and CYP2B6 and CYP3A4 (human), or cells were processed for assessment of replicative DNA synthesis.

Following a preliminary cytotoxicity assessment where cytotoxicity was observed at dosing concentrations ≥ 30 µM (1.3% of control ATP content), mouse hepatocytes were exposed to pethoxamid at dosing concentrations of 1, 3, 10 and 20 µM. Human hepatocytes were exposed to pethoxamid (1, 3, 10 and 20 µM, donor 385) and (0.3, 1, 3 and 10 µM, donors 8210 and 8219). Pethoxamid caused overt cytotoxicity (as defined by a decrease in intracellular ATP content of >20% of control levels) in human donor 385 (20 µM, 70.7% of control ATP levels), donor 8210 (10 µM, 62.2% of control ATP concentration) and donor 8219 (10 µM, 75.4% of control ATP levels).

In mouse hepatocytes, PB induced Cyp2b10 and Cyp3a11 to approximately 2.1- and 4.3 fold control, but high variance resulted in a lack of statistical significance. Pethoxamid induced a somewhat lower

increase in Cyp2b10 or Cyp3a11 relative to PB of ~ 1.4-1.5-fold (at 3 µM) in mouse hepatocytes, but this response also lacked statistical significance due to variance in the data.

In all 3 human donors, PB (1000 µM) treatment resulted in statistically significant induction of CYP2B6 mRNA (donor 385, 20.2-fold; donor 8210, 5.6-fold; donor 8219, 5.0-fold. Likewise, CYP3A4 mRNA induction by PB (1000 µM) was statistically significant in all 3 donors, (donor 385, 8.8-fold; donor 8210, 9.8-fold; donor 8219, 13.8-fold).

Pethoxamid induced dose related increases in CYP2B6 in human hepatocytes, reaching 4.3-fold at 20 µM (donor 385), and 2.0- and 3.2-fold at 10 µM (donors 8210 and 8219, respectively). CYP3A4 was similarly increased reaching 1.86-fold at 20 µM (donor 385) and 2.4- and 2.53-fold at 10 µM (donors 8210 and 8219, respectively).
Overt cytotoxicity was observed for all human donor hepatocytes at either 10 (donors 8210 and 8219) or 20 µM (donor 385) pethoxamid. The only exception to this was treatment with pethoxamid at 3 µM in donor 8219, where a statistically significant induction in CYP3A4 (2.50-fold) was observed in the absence of cytotoxicity.

In cultures of primary male CD-1 mouse hepatocytes, the positive control EGF (25 ng/mL) induced RDS (6.2-fold). As expected in mouse hepatocytes, PB (1000 µM) also induced RDS (1.9-fold). Pethoxamid induced RDS at all concentrations tested (1 µM, 1.7-fold; 3 µM, 2.8-fold; 10 µM, 2.0-fold; 20µM, 2.2-fold).

In human hepatocytes, the positive control EGF (25 ng/mL) induced RDS in all 3 human hepatocyte donors (donor 385, 5.1-fold; donor 8210, 8.7-fold; donor 8219, 13.5-fold).
Neither pethoxamid nor PB caused an increase in RDS in hepatocytes from any human donor tested.

Conclusion: Pethoxamid is a weak inducer of CAR and/or PXR in vitro. Pethoxamid induced RDS in mouse hepatocytes but not in human hepatocytes. The response observed with pethoxamid is qualitatively similar to that of PB. The results in this study provide data supporting the lack of human relevance for mouse liver tumor formation following treatment with pethoxamid.

MATERIALS AND METHODS
Materials:

Test Material:	Pethoxamid
Description:	Brown solid when frozen
Lot/Batch number:	21082018
Purity:	97.7%
Stability of test compound:	Expiry date 12 October 2020 (stored at approximately -20ºC)

Control items: Phenobarbital, sodium salt (PB); Epidermal Growth Factor (EGF)

Vehicle: Dimethyl sulfoxide (DMSO) for test item the test item was formulated in DMSO and administered such that the final DMSO concentration in all cultures was 0.1% (v/v) (i.e. 1 µL DMSO/mL medium).

Study Design and Methods:
Experimental dates: Start: 10 December 2018. End: 08 August 2019.

Test item and control preparations: A solubility test confirmed that pethoxamid was soluble in dimethyl sulfoxide (DMSO) at 1M. When the subsequent dilutions were made in culture medium, pethoxamid precipitated at final concentrations at or exceeding 100µm. During the main experiments, where final Test Item concentrations did not exceed 20 mM, no undissolved Test Item was observed by microscopy any tested concentrations. The test item was formulated in DMSO and administered such that the final DMSO concentration in all cultures was 0.1% (v/v) (i.e. 1 µL DMSO/mL medium).

PB was prepared in sterile water at a concentration of 1M, filter sterilized through a 0.2 µm filter and stored at approximately 4ºC.

EGF was reconstituted according to the manufacturer's instructions to 25 ng/mL and stored at approximately -70ºC. The aliquot of EGF used for treating hepatocyte cultures was stored at approximately -20ºC.

A vehicle control with DMSO (0.1% v/v) only was included in all cultures.

Characterization of Test System; The test system used was male CD-1 mouse primary hepatocytes (*Mus musculus*) and primary cryopreserved hepatocytes from three male human donors (*Homo sapiens*). Hepatocytes were isolated from male CD-1 mice using a two-step collagenase perfusion method according to ***Mitchell et al, (1984)***. In brief, mice were terminally anesthetised using Euthatal®, the livers were perfused with Krebs Ringer phosphate buffer, followed by Krebs Ringer hydrogen carbonate buffer. Human hepatocytes were sourced from Corning Life Sciences, Fogostraat 12, 1060 LJ Amsterdam, Netherlands (donor 385) or Life Technologies, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR (donors 8210 and 8219). Viability of all cell preparations was greater than 70%.

Hepatocyte culture conditions: Primary mouse hepatocytes were cultured in CL-15 media. Freshly isolated hepatocytes were initially cultured in CL-15 media for approximately 4 hours to allow adherence to the tissue culture plates in a humidified incubator at 37ºC under atmospheric air. Cells were seeded in 96-well plates at a density of 2 x 10^4 cells per well (cytotoxicity) or 6-well plates at a density of 0.8 x 10^6 cells per well for mRNA analysis and RDS.

Human hepatocytes were cultured in HCL-15 media (CL15-media + ascorbic acid (0.3 mM). Human hepatocytes were cultured on collagen coated tissue culture plates. Human hepatocytes were thawed in Cryopreserved Hepatocytes Recovery Medium (CHRM, Life Technologies), then cultured in Cryopreserved Hepatocytes Plating Medium (CHPM, Life Technologies) for approximately 6 hours to allow adherence in a humidified incubator at 37ºC under 95% air /5% CO_2. Cells were seeded in 96-well plates at a density of 4 x 10^4 cells per well (cytotoxicity) or 6-well plates at a density of 1.6 x 10^6 cells per well for mRNA analysis and RDS.

Cytotoxicity: In the preliminary study, a preliminary cytotoxicity assessment was carried out to evaluate the potential cytotoxicity of pethoxamid and to select appropriate concentrations of pethoxamid for use in the main study for analysis of mRNA expression and replicative DNA-synthesis. Pethoxamid was evaluated at 9 concentrations (1, 3, 10, 30, 100, 300, 600, 900 and 1000 µM). A vehicle control (DMSO 0.1% (v/v)) was included. The medium including test item or vehicle control was replenished daily for 3 days following the initial dosing and cells were cultured as detailed above. Cells were cultured for a total of approximately 96 hours prior to evaluation of cytotoxicity.

In the main study, pethoxamid cytotoxicity was evaluated at 4 or 5 concentrations outlined below, while PB cytotoxicity was evaluated at 1000 µM. The vehicle control was DMSO (0.1% v/v). Mouse hepatocytes were cultured with pethoxamid (1, 3, 10 and 20 µM), PB (1000 µM) or vehicle control (DMSO 0.1% (v/v)). Human hepatocytes were cultured with pethoxamid (donor 385: 0.3, 1, 3, 10 and 20 µM; donors 8210 and 8219: 0.3, 1, 3, and 10 µM), PB (1000 µM) and vehicle control (DMSO 0.1% (v/v)). The medium including pethoxamid, PB or vehicle control was replaced daily for 3 days following the initial dosing and cells were cultured as detailed above. Cells were cultured for a total of approximately 91-96 hours prior to evaluation of cytotoxicity.

Hepatocytes were plated in 6 replicates for each concentration in 96-well plates for cytotoxicity (ATP) measurements.

Hepatocyte culture mRNA analysis: Mouse or human hepatocytes were seeded in 6 well plates (n=3 per treatment). Mouse hepatocytes were cultured with pethoxamid (1, 3, 10 and 20 µM), PB (1000 µM) or vehicle control (DMSO 0.1% (v/v)). Human hepatocytes were cultured with pethoxamid (donor 385: 0.3, 1, 3, 10 and 20 µM; donors 8210 and 8219: 0.3, 1, 3, and 10 µM), PB (1000 µM) and vehicle control (DMSO 0.1% (v/v)). To keep a consistent number of dosing concentrations for evaluation across all human donors, the 0.3 µM dosing samples for donor 385 were not processed to measures of mRNA abundance.Following approximately 93-96 hours in culture, cells were processed for mRNA analysis.

Hepatocyte culture RDS (S-phase): Mouse or human hepatocytes were seeded in 6 well plates and incubated for 96 hours (n=5 per treatment). 5-bromo-2'-deoxyuridine (BrdU) was added to the culture medium for the last 3 days of culture. BrdU is a thymidine analogue and is incorporated into newly synthesized DNA. BrdU incorporation can be determined immunocytochemically and quantified.

Mouse hepatocytes were cultured with pethoxamid (1, 3, 10 and 20 µM), PB (1000 µM), EGF (25 ng/mL) or vehicle control (DMSO 0.1% (v/v)). Human hepatocytes were cultured with pethoxamid (donor 385: 0.3, 1, 3, 10 and 20 µM; donors 8210 and 8219: 0.3, 1, 3, and 10 µM), PB (1000 µM), EGF (25 ng/mL) or vehicle control (DMSO 0.1% (v/v)).

Cytotoxicity as evaluated by intracellular ATP concentrations: Cell toxicity was assessed following approximately 91 to 96 hours of culture as indicated by ATP depletion. Cellular ATP was determined by luminometry and analyzed using a Hidex Sense Microplate reader with Hidex Sense Plate Reader Software version 0.5.55.0. The bioluminescent determination of ATP release from viable cells was carried out using an assay kit supplied by Promega (CellTiter-Glo luminescent cell viability assay) according to manufacturer's instructions. Results were expressed as a percentage of the amount of ATP released as compared to the control cells.

Taqman® Analysis of mRNA expression: RNA was extracted from cultured hepatocytes using an RNeasy mini kit according to manufacturer's instructions. cDNA was synthesized from all available RNA samples using Qiagen QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions and quality checked using ThermoFisher NanoDrop 1000. Taqman® analysis was performed on all available samples using primer/probe sets specific for murine Cyp2b10 and Cyp3a11, or human CYP2B6 and CYP3A4 (Assay-on-demand kits, Applied Biosystems). Beta-actin was used as the internal standard (Assay-on-demand kits, Applied Biosystems). Data was analyzed by generation of threshold cycle (C_T) and delta delta C_T values for both the internal standard and cytochrome P450 genes.

Replicative DNA synthesis (S-phase): The number of cells undergoing RDS in any given cell population was determined by the incorporation of BrdU and was analysed immunocytochemically. Immunostaining was performed after fixation, using a mouse monoclonal anti-bromodeoxyuridine Clone Bu20a primary antibody (Agilent, M0744, Santa Clara, California, USA) and a polyclonal rabbit anti-mouse immunoglobulins/house radish peroxidase secondary antibody (Agilent, P0260, Santa Clara, California, USA). The number of hepatocytes in S-Phase was assessed by manual counting of four fields of view from five independent wells and recorded as the labelling index [(number of labelled hepatocyte / total number of hepatocytes) x 100]. Cells deemed morphologically abnormal at any concentration were not counted. Typically, 300 – 400 total cells were counted per field of view.

Statistical analysis: Statistical comparisons between hepatocytes treated with test item and the vehicle control group were undertaken using a one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. Statistical comparisons between hepatocytes treated with each control item (PB or EGF) and the vehicle control group were undertaken using a student's t-test. Analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION
Mouse hepatocytes:

Cytotoxicity: The preliminary investigation of pethoxamid cytotoxicity in male CD-1 mouse hepatocytes demonstrated that pethoxamid was tolerated up to a concentration of 10 µM, as cytotoxicity was observed at dosing concentrations ≥ 30 µM (1.3% of control ATP content). Therefore, concentrations of 1, 3, 10 and 20 µM pethoxamid was tested in the subsequent experiments and no significant decreases in cell viability were observed.

Table 3.9.4-29: Effect of pethoxamid on ATP levels in primary male CD-1 mouse hepatocytes (preliminary study)

Test Item & Concentration	**ATP Content (Luminescence Units)**	
	Mean ± SD	**Fold Control Mean ± SD**
Vehicle control (0.1% [v/v] DMSO)	348000 ± 59000 (100 ± 17)	-
Pethoxamid 1 µM	405000 ± 11000 (116.4 ± 3.1)***	1.16 ± 0.20
Pethoxamid 3 µM	407400 ± 9500 (117.1 ± 2.7)***	1.17 ± 0.20
Pethoxamid 10 µM	410000 ± 20000 (117.9 ± 5.9)***	1.18 ± 0.21
Pethoxamid 30 µM	4510 ± 600 (1.30 ± 0.17)***	0.0130 ± 0.0028
Pethoxamid 100 µM	515 ± 34 (0.1481 ± 0.0097)***	0.00148 ± 0.00027
Pethoxamid 300 µM	371 ± 74 (0.107 ± 0.021)***	0.00107 ± 0.00028
Pethoxamid 600 µM	298 ± 60 (0.086 ± 0.017)***	0.00086 ± 0.00023
Pethoxamid 900 µM	245 ± 20 (0.0704 ± 0.0057)***	0.00070 ± 0.00013
Pethoxamid 1000 µM	216 ± 13 (0.0621 ± 0.0036)***	0.00062 ± 0.00011
ATP values are Mean ± SD, n = 6 (expressed as percentage in parentheses). The error estimates in the fold control values were calculated by error propagation from the SD associated with the numerator and denominator values used to calculate the mean fold control value. In all cases, the number of significant figures reflects the error in the measurement. A one way-ANOVA was performed on the results followed by a Dunnett's multiple comparison test; *** statistically different from control P < 0.001.		

Induction of Cyp2b10 and Cyp3a11 in primary male mouse CD-1 hepatocytes: PB (1000 µM) induced Cyp2b10 (2.1-fold) and Cyp3a11 (4.3-fold) mRNA expression, but statistical significance was not reached due to variability in the data.

Pethoxamid induces a somewhat lower increase in Cyp2b10 and Cyp3a11 mRNA expression relative to PB in primary male mouse CD-1 hepatocytes. The highest induction of Cyp2b10 and Cyp3a11 was observed at 3 µM (1.41-1.508-fold, respectively). However, due to variability in the data, these responses lacked statistical significance.

Table 3.9.4-30: Taqman® analysis of Cyp2b10 and Cyp3a11 mRNA in primary mouse hepatocytes from male CD-1 mice

Test Item & Concentration	**Cyp2b10**	**Cyp3a11**
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.37	1.00 ± 0.12
PB 1000 µM	2.1 ± 1.5	4.3 ± 2.5
Pethoxamid 1 µM	0.99 ± 0.66	1.20 ± 0.49
Pethoxamid 3 µM	1.41 ± 0.54	1.508 ± 0.079

Pethoxamid 10 µM	1.22 ± 0.59	0.92 ± 0.17
Pethoxamid 20 µM	0.64 ± 0.16	0.76 ± 0.10
Data expressed as fold change over the mean vehicle control. Values are Mean ± SD; n = 3 per group. The number of significant figures is dictated by the error in the measurement. A one-way ANOVA was performed on the results followed by a Dunnett's multiple comparison test. Δ A Student's t test was performed on the results for PB treated hepatocytes. No statistical differences were observed compared to DMSO control.		

Replicative DNA synthesis (S-phase) mouse hepatocytes: In cultures of primary male CD-1 hepatocytes, the positive control EGF (25 ng/mL) induced RDS (6.2-fold, P < 0.001). As expected in mouse hepatocytes PB (1000 µM) also induced RDS (1.90-fold, P < 0.01). Pethoxamid induced RDS at all concentrations tested (1 µM, 1.71-fold, P < 0.01; 3 µM, 2.78-fold, P < 0.001; 10 µM, 2.03-fold, P < 0.001; 20 µM, 2.15-fold, P < 0.001)

Table 3.9.4-31:Effect of pethoxamid, PB or EGF on replicative DNA synthesis (S-Phase) in primary mouse hepatocytes from male CD-1 mice

Test Item & Concentration	**S-Phase Labelling Index**	
	Mean ± SD	**Mean % control ± SD**
Vehicle control (0.1% [v/v] DMSO)	0.230 ± 0.060 (100 ± 26)	–
PB 1000 µM	0.44 ± 0.11 (190 ± 50)**	1.90 ± 0.70
Pethoxamid 1 µM	0.394 ± 0.056 (171 ± 24)**	1.71 ± 0.50
Pethoxamid 3 µM	0.64 ± 0.10 (278 ± 45)***	2.78 ± 0.85
Pethoxamid 10 µM	0.468 ± 0.056 (203 ± 24)***	2.03 ± 0.58
Pethoxamid 20 µM	0.496 ± 0.057 (215 ± 25)***	2.15 ± 0.61
EGF 25 ng/mL Δ	1.43 ± 0.14 (620 ± 62)***	6.2 ± 1.7
S-Phase values are Mean ± SD, n = 5 (expressed as percentage in parentheses). The error estimates in the fold control values were calculated by error propagation from the SD associated with the numerator and denominator values used to calculate the mean fold control value. In all cases, the number of significant figures reflects the error in the measurement. A one-way ANOVA was performed on the results, followed by a Dunnett's multiple comparison test; ** statistically different from control P < 0.01; *** P < 0.001. Δ A Student's t test (two-tailed) was performed on the results for PB or EGF treated hepatocytes; ** statistically different from control P < 0.01; *** P < 0.001		

Human hepatocytes:
Cytotoxicity: The preliminary evaluation of pethoxamid toxicity in primary cultures of male human hepatocytes demonstrated cytotoxicity above 10 µM for donor 385 and above 3 µM for donors 8210 and 8219. Subsequently, pethoxamid concentrations chosen for evaluation in donor 385 were 0.3, 1, 3, 10 and 20 µM, and 0.3, 1, 3 and 10 µM for donors 8210 and 8219.

In the main experiment, overt cytotoxicity was observed in response to pethoxamid (20 µM) in donor 385 (70.7% of control ATP levels (P < 0.001)). In donors 8210 and 8219, pethoxamid (10 µM) also caused overt cytotoxicity (62.2% of control ATP (P < 0.001) and 75.4% of control ATP levels (P < 0.01), respectively). No cytotoxicity was detected at any other concentration tested in all three donors (Viability > 90%).

Table 3.9.4-32: Effects of pethoxamid or PB on ATP levels in primary human hepatocytes from three male donors (main study)

Test Item & Concentration	**ATP Content (Luminescence Units)**		
	Donor 385	**Donor 8210**	**Donor 8219**
Vehicle control (0.1% [v/v] DMSO)	587000 ± 25000 (100.0 ± 4.2)	306000 ± 24000 (100.0 ± 7.8)	274000 ± 33000 (100 ± 12)
PB 1000 µM Δ	541000 ± 28000 (92.3 ± 4.7)*	296000 ± 35000 (97 ± 11)	299000 ± 35000 (109 ± 13)
Pethoxamid 0.3 µM	604000 ± 46000 (103.0 ± 7.9)	295000 ± 26000 (96.5 ± 8.6)	296000 ± 34000 (108 ± 12)
Pethoxamid 1 µM	600000 ± 34000 (102.3 ± 5.8)	322000 ± 40000 (105 ± 13)	306000 ± 37000 (111 ± 14)
Pethoxamid 3 µM	601000 ± 39000 (102.5 ± 6.7)	303000 ± 31000 (99.0 ± 10.0)	300000 ± 32000 (109 ± 12)
Pethoxamid 10 µM	549000 ± 27000 (93.6 ± 4.7)	190000 ± 15000 (62.2 ± 5.1)***	207000 ± 15000 (75.4 ± 5.3)**
Pethoxamid 20 µM	415000 ± 33000 (70.7 ± 5.7)***	Not tested	Not tested
ATP values are Mean ± SD, n = 6 (expressed as percentage in parentheses). In all cases, the number of significant figures reflects the error in the measurement. A one way-ANOVA was performed on the results followed by a Dunnett's multiple comparison test; ** statistically different from control P < 0.01; *** P < 0.001. Δ A Student's t test (two-tailed) was performed on the results for PB treated hepatocytes; * statistically different from control P < 0.05			

Induction of CYP2B6 and CYP3A4 in human hepatocytes: PB (1000 µM) induced CYP2B6 mRNA expression in all 3 human donors (donor 385: 20.2-fold, P < 0.05; donor 8210: 5.6-fold, P < 0.05; donor 8219: 4.96-fold, P < 0.01). Pethoxamid (20 µM) induced CYP2B6 mRNA expression (4.3-fold, P < 0.01) in donor 385. In donor 8219, pethoxamid (10 µM) induced CYP2B6 mRNA expression (3.2-fold, P < 0.05). CYP2B6 mRNA expression was increased (2.0-fold) in donor 8210, although this increase was not statistically significant.

PB (1000 µM) induced CYP3A4 mRNA expression in all 3 donors (donor 385: 8.8-fold, P < 0.05; donor 8210: 9.8-fold, P < 0.01; donor 8219: 13.8-fold, P < 0.001). Pethoxamid (3 and 10µM) induced a statistically significant increase in CYP3A4 mRNA expression in donor 8219 (2.50-fold (P < 0.01) and 2.53-fold (P < 0.01), respectively). While increases in CYP3A4 were observed in donor 385 at 3, 10 and 20 µM (1.89-, 1.88-, 1.86-fold, respectively) and in donor 8210 at 1, 3, and10 µM (1.50-, 2.37-, 2.4-fold, respectively), these changes did not reach statistical significance.

Table 3.9.4-33: Taqman® analysis of CYP2B6 and CYP3A4 mRNA in human hepatocytes from three male donors

Test Item & Concentration	**Donor 385**		**Donor 8210**		**Donor 8219**	
	CYP2B6	**CYP3A4**	**CYP2B6**	**CYP3A4**	**CYP2B6**	**CYP3A4**
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.42	1.00 ± 0.45	1.00 ± 0.39	1.00 ± 0.38	1.00 ± 0.27	1.00 ± 0.24
PB 1000 µM Δ	20.2 ± 9.0*	8.8 ± 4.1*	5.6 ± 2.6*	9.8 ± 1.8**	4.96 ± 1.00**	13.8 ± 1.6***
Pethoxamid 0.3 µM	Not tested	Not tested	1.14 ± 0.46	1.18 ± 0.49	1.58 ± 0.17	1.56 ± 0.19
Pethoxamid 1 µM	1.59 ± 0.68	1.17 ± 0.57	1.32 ± 0.66	1.50 ± 0.62	1.87 ± 0.57	1.64 ± 0.42
Pethoxamid 3 µM	2.44 ± 0.58	1.89 ± 0.71	1.88 ± 0.75	2.37 ± 0.83	2.27 ± 0.27	2.50 ± 0.17**
Pethoxamid 10 µM	2.9 ± 1.2	1.88 ± 0.96	2.0 ± 1.1	2.4 ± 1.1	3.2 ± 1.5*	2.53 ± 0.79**

Pethoxamid 20 µM	4.3 ± 1.7**	1.86 ± 0.87	Not tested	Not tested	Not tested	Not tested
Data expressed as fold change over mean vehicle control. Values are Mean ± SD; n = 3 per group. The number of significant figures is dictated by the error in the measurement. A one-way ANOVA was performed on the results followed by a Dunnett's multiple comparison test; * statistically different from control P < 0.05; ** P < 0.01. Δ A Student's t test was performed on the results for PB treated hepatocytes; * statistically different from control P < 0.05; ** P < 0.01; *** P < 0.001.						

Replicative DNA synthesis (S-phase) human hepatocytes: The positive control EGF (25 ng/mL) induced RDS in all 3 human hepatocyte donors; (donor 385, 5.09-fold, P < 0.001), (donor 8210, 8.7-fold, P < 0.001) and (donor 8219, 13.5-fold, P < 0.001). PB had no effect on RDS in primary hepatocytes from any human donor.

Pethoxamid had no effect on RDS in primary hepatocytes from any human donor at all concentrations tested.

CONCLUSION: Pethoxamid is a weak inducer of CAR and/or PXR in vitro. Pethoxamid induced RDS in mouse hepatocytes but not in human hepatocytes. The response observed with pethoxamid is qualitatively similar to that of PB. The results in this study provide data supporting the lack of human relevance for mouse liver tumor formation following treatment with pethoxamid.

References:
Elcombe, C. R., Peffer, R. C., Wolf, D. C., Bailey, J., Bars, R., Bell, D., Cattley, R. C., Ferguson, S. S., Geter, D., Goetz, A., Goodman, J. I., Hester, S., Jacobs, A., Omiecinski, C. J., Schoeny, R., Xie, W. & Lake, B. G. 2014. Mode of action and human relevance analysis for nuclear receptor-mediated liver toxicity: A case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. *Crit Rev Toxicol*, 44, 64-82.

Mitchell, A. M., Bridges, J. W. & Elcombe, C. R. 1984. Factors influencing peroxisome proliferation in cultured rat hepatocytes. *Arch Toxicol*, 55, 239-46.

3.10 Reproductive toxicity

3.10.1 Animal data

3.10.1.1 Anonymous (1998a)

Reference: TKC-94 : Preliminary Study of Effects on Reproductive Performance in CD Rats by Dietary Administration
Author(s), year: Anonymous, 1998
Report/Doc. number: 84 PXA (TOX2001-297) / TON013/980047
Guideline(s): OECD 416 (1983)
GLP: Yes
Deviations from OECD 416 (2001): Dose range finding study, with low number of animals and limited parameters assessed
Acceptability: Yes; limited information

Material and Methods:
Test material: Pethoxamid, Batch: TB-960306; Purity 95.0%.

Test animals: Four groups of 6 male and 6 female of the CD strain Sprague Dawley rats, 215-291 g (males), 137-217 g (females), 7 weeks of age, from Charles River UK Limited, Margate Kent, England to form the F0 generation.

Test method: The animals received the test material by dietary administration at concentrations of 0, 25, 100, 400 or 1600 ppm (F0: 15 days prior and through pairing, gestation and lactation; selected F1: until about 6 weeks of age).

Findings and conclusion:

Mortality, clinical signs, body weight and food consumption: No treatment related clinical signs were observed and there were no deaths. At 1600 ppm, F0 females showed the lowest body weight gain before pairing. It was not affected during gestation and gains were superior to the control group during lactation. Food consumption was unaffected as well reproduction parameters and litter size and survival.

Absolute body weights of offspring at birth were lower for the groups receiving 100, 400 and 1600 ppm than for the control group. This was considered to be to the slightly larger litter size at birth in these groups. Body weight gains from Day 4 of age were lower at 400 and 1600 ppm. At the selection of the F1 generation, this resulted in lower mean body weights for the 1600 ppm group than for the control group. Body weights to six weeks of age were similar for the 1600 ppm and control groups.

Pathological examination:
Necropsy: Necroscopy revealed no treatment related findings in the F0 and F1 generations.

Organ weights: In the F1 generation, liver weights (absolute and body weight relative) were increased at 1600 ppm.

Conclusion: It was concluded, that treatment at 1600 ppm (F0: 127-172 mg/kg bw/d; F1: 240-296 mg/kg bw/d) resulted in a decreased body weight gain of the offspring and 25 ppm was estimated to be a no effect level.

3.10.1.2 Anonymous (2000)

Reference: TKC-94 : Study of Reproductive Performance in CD Rats treated continuously through two successive generations by Dietary Administration;
Author(s), year: Anonymous, 2000
Report/Doc. number: 85 PXA (TOX2001-298) / TON015/992242
Guideline(s): OECD 416 (1983)
GLP: Yes
Deviations from OECD 416 (2001): - no pituitary and thyroid weight of parental animals recorded (- no functional investigations in offspring; only *recommended* endpoint in OECD 416)
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid, Batch: TB-960306; Purity 95.0%.

Test animals: Four groups of 28 male and 28 female of the CD strain Sprague Dawley rats, 215-291 g (males), 137-217 g (females), 7 weeks of age, from Charles River UK Limited, Margate Kent, England to form the F0 generation.

Test method: Based on a preliminary study [Report no. TON 013/980047], the animals received the test material by dietary administration at concentrations of 0, 25, 200 or 1600 ppm throughout two generations. Both the F0 and F1 generation received the treated diet for a minimum of 10 weeks from selection throughout pairing, gestation and lactation.

All adult animals were subjected to a detailed necropsy and the reproductive organs and selected potential target organs were weighed and retained. Histopathological examinations were performed on

tissues from control and high dose animals. Surplus F1 offspring and F2 offspring were killed on Day 25 of age and selected organs from one pup per sex per litter were weighed and retained in fixative.

Investigated endpoints (parental):
Clinical Signs
Mortality
Body weight: Males: Beginning of treatment and weekly thereafter. Females: Beginning of treatment and weekly thereafter; then Days 0, 7, 14, 20, 21 after mating and Days 1, 4, 7, 14 and 21 of lactation
Food consumption
Pre-coital interval (females only): time between initial pairing and detection of mating
Parturition and duration of gestation
Maternal behaviour
Macroscopic pathology
Oestrous cycles (F0, F1)
Mating performance and fertility: percentage mating, conception rate, fertility index, gestation index
Sperm numbers (F0 and F1 males): sperm motility, sperm count, sperm morphology, homogenisation-resistant spermatids
Organ weights: adrenals, brain, epididymides (L+R), kidneys, liver, ovaries with oviduct (L+R), prostate, seminal vesicles and coagulation glands, spleen, testes (L+R), thymus, uterus with cervix
Histopathology: animals from control and high dose group: abnormalities, adrenal glands, brain, epididymis (R), kidneys, liver, mammary glands caudal, ovaries with oviduct (L+R), pituitary, prostate, seminal vesicles and coagulation gland, spleen, testis (R), thymus, uterus, cervix, vagina

Investigated endpoints (offspring):
Mortality and Litter size: group mean litter size, survival indices
Clinical signs
Sex ratio
Sexual maturation of F1: vaginal opening, preputial separation
Organ weights: brain, liver, kidneys (L+R), spleen, thymus
Histopathology: abnormalities, adrenal glands, brain, epididymis (L+R), kidneys, liver, ovaries with oviduct (L+R), prostate, seminal vesicles and coagulation gland, spleen, testis (L+R), thymus, uterus, cervix, vagina

Results:
F0 generation

Mortality and clinical signs: There were no treatment-related clinical signs and mortalities.

Body weight gain and food consumption: There were no effects on body weight or body weight gain before pairing. At 1600 ppm, females showed a reduced body weight gain during gestation with recovery during the lactation phase. The food consumption was not adversely affected.

Pathological examinations:
Necropsy: Necropsy revealed no treatment related effects.

Organ weights: At 1600 ppm, increases in both absolute and body weight related liver weights and decreases in absolute and body weight related spleen weights in females were observed.

Histopathology and sperm evaluation: No treatment related effects were observed.

Reproduction and litter parameters (estrus cycles, pre-coital interval, mating, fertility, gestation length, parturition, litter size, sex ratio, survival of offspring): These parameters were not influenced by the administration of the test substance.

Table 3.10.1-1: Overview of toxicity in F0 rats

Parameter	Dose (ppm)							
	Male				Female			
	0	25	200	1600	0	25	200	1600
Achieved intake (mg/kg bw/day)								
- before pairing		2.7-1.3	22-11	170-85		2.9-1.8	24-14	181-117
- during gestation						2.1-1.7	17-14	132-112
- during lactation						2.5-4.3	24-38	180-295
Body weight (g)	not applicable							
- Day 0 gestation					310	303	305	299
- Day 7 gestation					345	337	337	331
- Day 14 gestation					381	368	368	366
- Day 20 gestation					459	441	445	433
Body weight gain (g)	not applicable							
- Weeks 0-7 gestation					35	34	33	31
- Weeks 0-14 gestation					71	65	63	66
- Weeks 0-20 gestation					149	138	141	133**
Body weight (g)	not applicable							
- Day 1 lactation					328	323	322	319
- Day 4 lactation					355	336	337	338
- Day 7 lactation					364	351	349	353
- Day 14 lactation					380	367	369	364
- Day 21 lactation					365	362	354	366
Absolute organ weights								
- Liver (% control)	100	98	98	113**	100	97	99	112**
- Spleen (% control)	100	97	95	93	100	95	94	95*
Relative organ weights								
- Liver (% control)	100	100	101	118**	100	99	102	112**
- Spleen (% control)	100	100	98	97	100	96	97	90**

* statistically different from control P < 0.05; ** P < 0.01

F1 generation

Mortality and clinical signs:
There were no treatment related clinical signs and mortalities. Sexual maturation was not influenced.

Body weight gain and food consumption: At 1600 ppm, body weight was significantly decreased at day 21 in male and female pups. At 1600 ppm, during the last week before weaning (Days 14 to 21), both sexes showed a slightly but statistically significantly reduced weight gain. Then, the pups had already access to the treated diet and chemical intake is expected to be at its highest in this period. From weaning until Week 10, the body weight gains were further lower at 1600 ppm. However, the control females gained relatively more weight than the treated females in this period and showed an unusual

high body weight at Week 10 (background control data from 5 studies: 297 to 319 g, mean: 305 g, SD: 8.5 g). Thus, the body weights of all treated female groups appeared low and the lower body weight gain for females at the middle dose was considered to be due to this unusual high weight gain in control females. The divergence of the control group mean body weight from background control data continued during gestation (Day 20: 443 g, SD: 7 g (5 studies); concurrent control group 472 g, SD 42 g) and lactation phases (Day 21: 355 g, SD: 3 g (4 studies); concurrent control group 379 g, SD 33 g). Therefore, the body weights of females appeared to be low but they generally lay within the background control data.

The food consumption was not adversely affected.

Pathological examinations:
Necropsy: Necropsy of F1 pups that died before weaning revealed absence of milk in the stomach as the only consistent finding. Necropsy of F1 pups at 25 days of age revealed no evident changes that could be related to treatment. Necropsy of the F1 adults revealed no treatment related effects.

Organ weights: In female offspring, at 200 ppm, and in both sexes at 1600 ppm, liver weights were significantly increased. At the high dose, spleen weights were lower in both sexes. In the adults, at 1600 ppm, relative liver weights were increased. The absolute thymus weights were significantly decreased (compared to the control group at 200 ppm: 85% for females, 1600 ppm: 86 and 78% for males and females).

Histopathology and sperm evaluation: No treatment related effects were observed.

Reproduction and litter parameters (sexual maturation: vaginal opening and preputial separation, estrus cycles, pre-coital interval, mating, fertility, gestation length, parturition, litter size, sex ratio, survival of offspring): These parameters were not influenced by the administration of the test substance.

Table 3.10.1-2: Overview of toxicity in F1 rats

Parameter	**Dose (ppm)**							
	Male				**Female**			
	0	**25**	**200**	**1600**	**0**	**25**	**200**	**1600**
Achieved intake (mg/kg bw/day)								
- before pairing		4.3-1.5	34-12	291-97		4.7-1.9	36-16	303-123
- during gestation						2.0-1.8	17-14	138-121
- during lactation						2.4-4.5	18-37	139-312
Body weight offspring F1 (g)								
- Day 1	6.0	6.4	6.2	6.3	5.7	5.9	5.9	6.0
- Day 4 (before cull)	8.3	8.9	8.8	8.9	8.0	8.4	8.4	8.4
- Day 7	13.9	14.5	14.6	14.2	13.1	13.8	14.0	13.4
- Day 14	30.9	31.1	32.0	30.2	29.9	30.0	31.0	29.0
- Day 21	51.9	52.7	53.1	48.6*	50.1	50.3	51.3	46.7*
Body weight gain offspring F1 (g)								

- Days 1-4	2.2	2.6	2.5	2.4	2.1	2.5	2.5	2.3
- Days 1-7	7.7	8.1	8.4	7.7	7.3	7.9	8.1	7.4
- Days 1-14	24.7	24.8	25.7	23.7	24.0	24.1	25.0	23.0
- Days 1-21	45.8	46.3	46.8	42.2*	44.2	44.3	45.4	40.8*
Body weight parent F1 (g)								
- Week 0	92	88	94	86	84	80	86	81
- Week 1	146	143	151	138	128	122	130	123
- Week 2	211	204	211	198*	165	157	164	158
- Week 3	276	268	275	257**	196	186	190	185*
- Week 4	338	331	336	312**	224	212*	215	211*
- Week 5	390	381	382	356**	245	238	236	230*
- Week 6	429	421	419	392**	267	253	250*	247**
- Week 7	462	454	448	424**	283	270	268*	263**
- Week 8	491	476	471	447**	297	282	276*	
- Week 9	514	498	490	464**	315	295*	294*	285**
- Week 10	531	518	506	479**	328	305*	306*	295**
- Week 11	540	527	518	489**		-	-	-
- Week 12	556	546	534	507**	-	-	-	-
- Week 13	567	559	547	519**	-	-	-	-
- Week 14	585	577	561	530**	-	-	-	-
- Week 15	604	590	577	548**	-	-	-	-
- Week 16	617	604	592	557**	-	-	-	-
- Week 17	629	619	600	568**	-	-	-	-
- Week 18	637	627	608	573**	-	-	-	-
Body weight gain parent F1 (g)								
- Weeks 0-3	184	180	181	171*	112	107	104*	104*
- Weeks 0-10	440	430	413	393**	244	225	220*	214**
- Weeks 0-18	545	540	514	487**	-	-	-	-
Body weight F1 (g)	not applicable							
- Day 0 gestation					331	306**	309**	299**
- Day 7 gestation					362	337**	336**	326**
- Day 14 gestation					398	368**	368**	361**
- Day 20 gestation					472	441**	430**	432**
Body weight gain F1 (g)	not applicable							
- Weeks 0-7 gestation					31	31	28	27
- Weeks 0-14 gestation					67	63	59	62
- Weeks 0-20 gestation					141	136	122**	133**
Body weight F1 (g)	not applicable							

- Day 1 lactation					343	311*	314*	305**
- Day 4 lactation					362	337*	331**	318**
- Day 7 lactation					372	343**	339**	328**
- Day 14 lactation					388	361**	361**	349**
- Day 21 lactation					379	355*	362*	356*
Adults F1 Absolute organ weights								
- Liver (% control)	100	100	98	103	100	91	93	99
- Thymus (% control)	100	110	100	86*	100	87	85*	78**
Adults F1 Relative organ weights								
- Liver (% control)	100	101	104	115**	100	99	99	108*
- Thymus (% control)	100	112	105	97	100	93	89	85*
Offspring F1 absolute organ weights								
- Liver (% control)	100	103	107	110*	100	105	109*	112**
- Spleen weight (% control)	100	97	97	82**	100	103	106	87*
Offspring F1 relative organ weights								
- Liver (% control)	100	101	105	119**	100	104	106*	117**
- Spleen weight (% control)	100	96	97	89**	100	102	103	91*
Adults F0 Reproductive organs								
Absolute organ weights								
Epididymides weight (g)	1.456	1.379	1.448	1.402	-	-	-	-
Prostate (g)	0.789	0.806	0.791	0.803	-	-	-	-
Seminal vesicles (g)	2.71	2.67	2.70	2.60	-	-	-	-
Testes (g)	3.87	3.59	3.89	3.83	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.147	0.132	0.142	0.132
Uterus+cervix (g)	-	-	-	-	0.57	0.48*	0.51	0.49
Relative organ weights								
Epididymides weight (g)	0.2243	0.2181	0.2308	0.2261	-	-	-	-
Prostate (g)	0.1226	0.1281	0.1251	0.1296	-	-	-	-
Seminal vesicles (g)	0.416	0.422	0.433	0.420	-	-	-	-
Testes (g)	0.597	0.567	0.621	0.619	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.0419	0.0381	0.0420	0.0374 *
Uterus+cervix (g)	-	-	-	-	0.165	0.139*	0.149	0.140
Adults F1 Reproductive organs								
Absolute organ weights								
Epididymides weight (g)	1.405	1.328	1.401	1.366	-	-	-	-
Prostate (g)	0.733	0.690	0.713	0.659	-	-	-	-

Seminal vesicles (g)	2.39	2.39	2.54	2.33	-	-	-	-
Testes (g)	3.83	3.61*	3.84	3.77	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.144	0.125	0.135	0.117**
Uterus+cervix (g)	-	-	-	-	0.49	0.48	0.46	0.39**
Relative organ weights								
Epididymides weight (g)	0.2222	0.2157	0.2330	0.2432*	-	-	-	-
Prostate (g)	0.1155	0.1117	0.1185	0.1170	-	-	-	-
Seminal vesicles (g)	0.377	0.388	0.423*	0.414	-	-	-	-
Testes (g)	0.606	0.586	0.639	0.672**	-	-	-	-
Ovaries+ovids (g)	-	-	-	-	0.0395	0.0367	0.0389	0.0348
Uterus+cervix (g)	-	-	-	-	0.135	0.139	0.133	0.115

Statistical significance: *p<0.05; **p<0.01

F2 generation

Body weight gain and food consumption: At 1600 ppm, body weight was significantly decreased at day 21 in male and female pups. Furthermore, during the last week before weaning (Days 14 to 21), both sexes showed a slightly but statistically significantly reduced weight gain. Then, the pups had already access to the treated diet and chemical intake is expected to be at its highest in this period.

Pathological examinations:
Necropsy: Necropsy of F2 pups that died before weaning revealed absence of milk in the stomach as the only consistent finding. Necropsy of F2 pups at 25 days of age revealed no evident change that could be related to treatment.

Organ weights: At 200 ppm, the male pups and at 1600 ppm both sexes showed an increased relative liver weight.

Table 3.10.1-3: Overview of toxicity in F2 rats

Parameter	**Dose (ppm)**							
	Male				**Female**			
	0	**25**	**200**	**1600**	**0**	**25**	**200**	**1600**
Body weight offspring F2 (g)								
- Day 1	6.1	6.1	6.1	5.9	5.8	5.7	5.7	5.5
- Day 4 (before cull)	8.6	8.7	8.8	8.2	8.2	8.3	8.1	7.8
- Day 7	14.2	14.0	14.2	13.2	13.6	13.4	13.2	12.7
- Day 14	31.3	30.6	31.0	29.2	30.0	29.5	29.4	27.9
- Day 21	53.2	51.3	51.6	47.0**	50.9	49.1	49.1	45.0**
Body weight gain offspring F2 (g)								
- Days 1-4	2.5	2.6	2.5	2.3	2.4	2.4	2.4	2.4

- Days 1-7	8.0	7.9	7.9	7.3	7.7	7.6	7.4	7.2
- Days 1-14	25.1	24.4	24.8	23.3	24.1	23.6	23.6	22.4
- Days 1-21	47.0	45.2	45.3	41.2**	45.1	43.2	43.3	39.5**
Absolute liver weight (% control)	100	99	103	108	100	95	96	103
Relative liver weight (% control)	100	101	106*	119**	100	99	103	116**
Statistical significance: *p<0.05; **p<0.01								

Conclusion: Up to 1600 ppm (85 mg/kg bw/d), pethoxamid had no adverse effects upon reproductive performance of rats through two successive generations. Because of the slightly reduced body weight gain of the pups (F1, F2) at the end of the lactation phase, the NOAEL (NOEL) for offspring toxicity is considered to be 200 ppm (equivalent to 11 mg/kg bw/d for the F0- and F1-generation). This dose is also considered to be a NOAEL for parental toxicity, based on decreased body weight gain during gestation and increased liver weight and decreased weight of thymus and spleen.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (adverse effects on sexual function and fertility) is not required.

3.10.1.3 Anonymous (1996)

Reference: TKC-94 : Preliminary Study of Embryo-foetal Toxicity in the CD Rat by Oral Gavage Administration
Author(s), year: Anonymous, 1996
Report/Doc. number: 86 PXA (TOX2001-299) / TNP001/0116
Guideline(s): OECD Guideline 414 (1981)
GLP: Yes
Deviations from OECD 414 (2001): Limited number (six) of animals per group; Shorter administration period (day 6 to 15); Limited parameters retrieved (no skeletal or soft tissue examination of foetuses done)
Acceptability: Yes (limited information); range finding study

Material and Methods:
Test material: Pethoxamid; Batch: TB-951005; Purity: 95.0%.

Test animals: Four groups each of 6 pregnant female Crl:CD rats, weighing from 213 to 263 g (approx. 10 to 11 weeks of age).

Test method: The rats received the test compound at dosages of 0, 8, 80, 400 or 800 mg/kg bw/d by oral gavage from Day 6 to 15 of gestation.

Summarized findings: At 800 mg/kg bw/d, adverse substance related maternal toxicity was observed, as mortality (Day 9 and 10: 2 dams died and 1 killing *in extremis*, respectively) and reduced body weight gain. The dams which survived showed a general condition similar to the control group. All animals of the high dose group were pregnant. Apart from salivation which was observed at 80, 400 and 800 mg/kg bw/d, no treatment related effects were observed for animals receiving 8, 80 and 400 mg/kg bw/d.

No adverse effects on the litter responses and foetal development were observed.

Conclusion: A dosage between 400 and 800 mg/kg bw/d was concluded suitable as the highest dose in the main study.

3.10.1.4 Anonymous (1997a)

Reference: TKC-94 : Study of Embryo-foetal Toxicity in the CD Rat by Oral Gavage Administration **Author(s), year**: Anonymous, 1997a **Report/Doc. number**: 87 PXA (TOX2001-300)/ TNP002/0696 **Guideline(s)**: OECD Guideline 414 (1981) **GLP**: Yes **Deviations from OECD 414 (2001)**: - Shorter administration period (day 6 to 15 of gestation); - 40% (10/25) of the dams of the high dose group died. **Acceptability**: Yes, number of animals (15) in the high dose is slightly lower than the lowest number of animals (16) to be achieved according to OECD 414

Material and Methods:

Test material: Pethoxamid; Batch: TB-951005; Purity: 95.0%.

Test animals: Four groups each of 25 pregnant female Crl:CD rats, weighing from 245 to 298 g (approx. 11 to 12 weeks of age).

Test method: From a preliminary study [Report no. 96/TNP001/0116], it was concluded that the highest dose considered suitable should be between 400 and 800 mg/kg bw/d. Thus, the rats received the test compound at dosages of 0, 8, 80 or 600 mg/kg bw/d by oral gavage from Day 6 to 15 of gestation.

Results:

Maternal responses
Mortality and clinical signs: At 600 mg/kg bw/d, 10 early decedents were recorded. The animals were killed after showing a significant adverse reaction following dose administration. Signs prior killing included piloerection, unresponsiveness to stimuli and hunched posture. In all animals of the high dose group, salivation was observed after all dose administrations. Apart from that, the surviving animals showed no treatment related effects. Post-dosing salivation was also seen at some occasions following the administration of 80 mg/kg bw/d.

Bodyweight gain and food consumption: At 600 mg/kg bw/d, the body weight gain was essentially static during the first three days of treatment and was lower at Day 9 and 10 when compared to the control group. The body weight gain increased after cessation of treatment. This was not accompanied by changes in food consumption. Some decedents showed overnight weight loss.
Pathological examination: The majority of the early decedents exhibited disturbances to the gastrointestinal tract as devoid of or with reduced content and pale appearance to the liver. All dams were confirmed as pregnant.

The surviving animals did not show treatment-related effects.

Litter responses: No treatment-related findings were recorded for the number of implantations and viable young, extent of pre-and post-implantation losses, foetal and placental weights and foetal anogenital distance.

No treatment-related effects were recorded at macroscopic, visceral and skeletal examinations.

Table 3.10.1-4: Results in the rat teratogenicity study

	Dose level (mg/kg bw/day)			
	0	**8**	**80**	**600**
Number of Dams	25	25	25	25

Number surviving to Day 20 with live litters	25	24	25	15
Killed *in extremis*	0	0	0	9 (8,9,10,11,14,17)[1]
Found dead	0	0	0	1 (11)
Not pregnant	0	1	0	0
Body weight gain Days 6-9 gestation (g)	11	13	14	4**
Body weight gain Days 6-15 gestation (g)	48	51	53	48
Body weight gain Days 6-20 gestation (g)	120	131	129	125
[1] (day of death, days 9, 10 and 11 2 animals killed *in extremis*) Statistical significance: **p<0.01				

Conclusion: Significant maternal toxicity was seen at 600 mg/kg bw/d. The dose of 8 mg/kg bw/d is considered to be the maternal NOAEL, based on the salivation which occurred from 80 mg/kg bw/d onwards. The NOAEL (NOEL) for developmental toxicity was at 600 mg/kg bw/d.

Although more than 10% of the dams died at the high dose, the study is considered to be acceptable because a high number of dams (15) were available for the investigations at the end of the study, and there was no evidence of developmental toxicity. In the preliminary study, at 400 mg/kg bw/d, no substance related effects (apart from salivation) were observed on the dams and up to the highest dose (800 mg/kg bw/d), no developmental toxicity was seen.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.1.5 Anonymous (2014a)

Reference: A developmental toxicity study of pethoxamid by oral (gavage) administration in rats
Author(s), year: Anonymous, 2014
Report/Doc. number: 1138 PXA / 20039155
Guideline(s): OECD 414 (2001)
GLP: Yes
Deviations from OECD 414 (2001): -
Acceptability: Yes

Material and Methods:

Test material: Pethoxamid technical
Lot/batch number: P1351-JaK-T2-23-6
Purity: 95.80% (w/w) (dose calculation was not adjusted to purity)
Stability of test item: 06 January 2014 (stored at ambient temperature). *NB: stable during the conduct of the study*
Storage conditions: At room temperature, protected from light

Test method: From the results of a previously conducted study performed at Huntingdon Life Sciences Ltd., UK, [Report No. 96/TNP002/0695 02.12.96], it was concluded that the highest dose considered suitable should be between 400 and 600 mg/kg bw/day due to mortality observed at 600 mg/kg bw/day.

Pregnant female Crl:CD(SD) rats (25 or 30/group) were orally administered pethoxamid or the vehicle control (1% (w/v) methylcellulose (400 cP) and 0.5% (v/v) Tween® 80 in reverse osmosis membrane-

processed deionized water) once daily by oral gavage on gestation days 6 through 20 (GD 6- 20) at dose levels of 0, 10, 75 or 500 mg/kg bw/day. The dosing volume was 5 mL/kg bw. There were 25 rats per group, except at the high dose which contained 30 rats.

Due to mortality and/or adverse clinical signs of toxicity in 7 rats at the 500 mg/kg bw/day dose level within the first 3 to 10 days of dosing, the high-dose level was reduced to 350 mg/kg bw/day. Mortality occurred in 3 additional rats at the 350 mg/kg bw/day dose level, which resulted in a subsequent reduction of the dose level to 250 mg/kg bw/day. There were 2 additional deaths in the 250 mg/kg bw/day dose group that occurred on GD 21 after the last dose on GD 20.

The following parameters and end points were evaluated in this study: viability, clinical signs, body weights, food consumption, necropsy observations, uterine contents (including uterine weights) and foetal external, visceral, and skeletal alterations.

Results:

Mortality: At 500/350/250 mg/kg bw/day, there was an increase or a statistically significant increase in the total number of female rats that were either found dead or euthanized due to adverse clinical signs during the study. There was also one female rat in the vehicle control group that was euthanized due to an injury to the left hind paw. Common clinical signs in these rats included effects on gait (e.g., ataxia; low carriage; decreased motor activity; lost righting reflex), respiratory effects (e.g. hyperpnea, bradypnea, dyspnea, open-mouthed breathing, gasping), and additional adverse clinical signs (e.g. discolored faeces [light brown], paleness in the ears, eyes and extremities, hunched posture, ungroomed coat, coldness to the touch). All other female rats survived until scheduled euthanasia.

Clinical signs: There was a statistically significant increase in the number of rats in the 500/350/250 mg/kg bw/day dose group (21 to 26) observed with hunched posture; light brown faeces; mild dehydration (based on skin turgor); and the total number of rats observed with dehydration (mild, moderate and/or severe). In the 500/350/250 mg/kg bw/day dose group, there was also a statistically significant increase in the number of rats (4 to 9 per group) observed with moderate dehydration; slightly pale and/or pale ears; ungroomed coat; ptosis; thin body condition; urine staining; slight excess salivation; decreased motor activity; pale extremities (both forelimbs, both forepaws, both hind limbs and/or both hind paws); coldness to the touch; scant faeces; and ataxia. There was also an increase (1 to 3 per group) in the number of rats in the 500/350/250 mg/kg bw/day dose group observed with impaired righting reflex; bradypnea; hyperpnea; paleness in the body and/or whole body; red perivaginal area fur and/or brown-yellow perioral fur; soft or liquid faeces; urine-stained abdominal fur; low carriage; brown and dried perioral substance; open mouth breathing; splayed hind limbs; prostate; vocalization in the home cage; pale eyes; abdominal distention; red substance in the cage; active vaginal bleeding; dyspnea; gasping; tachypnea; and piloerection.

Body weight: Body weights were reduced or statistically significantly reduced in the 500/350/250 mg/kg bw/day dose group at all intervals during the dose period (GDs 6 through 21). In the 500/350/250 mg/kg bw/day dose group, there was a statistically significant reduction in body weight gain observed for the entire dose period (calculated as GDs 6 to 21) as well as the overall study period (calculated as GDs 0 to 21). Reductions or statistically significant reductions in body weight gains and/or statistically significant body weight losses were observed at all tabulated intervals (with the exception of GDs 12 to 15) in the 500/350/250 mg/kg bw/day dose group. Uterine weights in the 500/350/250 mg/kg bw/day dose group were slightly reduced (8%) in comparison with the vehicle control group value.

Food consumption: Correlating with concurrent reductions in body weight gains and body weight losses, mean absolute and relative food consumption values at 500/350/250 mg/kg bw/day were reduced. In this dose group, mean absolute and relative food consumption values were reduced or significantly reduced after the initiation of dose administration.

Maternal necropsy: There was a statistically significant reduction in the number of rats in the 500/350/250 mg/kg bw/day dose group that appeared normal at the time of necropsy. There was also a statistically significant increase in the number of rats in the 500/350/250 mg/kg bw/day dose group observed with all lobes of the liver mottled, tan, red, dark red and/or brown. In this dose group, there were also one or two rats observed with numerous pitted areas on the liver; all lobes of the lungs spongy, pale and/or dark red; intestines distended with gas; and/or small spleen.

Caesarean-sectioning and litter observations: Pregnancy occurred in 25 (100%), 23 (92.0%), 25 (100%) and 29 (96.7%) rats in Groups 1 through 4, respectively. Due to the previously described mortality, Caesarean sectioning observations on GD 21 were based on 24, 23, 25 and 17 pregnant rats in the 0 (Vehicle Control), 10, 75 and 500/350/250 mg/kg bw/day dose groups, respectively. Although more than 10% mortality of the dams occurred in the 500/350/250 mg/kg bw/day dose group, the study was considered to be acceptable because a sufficiently high number of dams (n=17) were available for the investigations at the end of the study.

A statistically significant reduction in foetal body weights (male, female and combined) occurred in the 500/350/250 mg/kg bw/day dose group. No additional test substance-related Caesarean-sectioning or litter parameters were affected by doses of the test substance as high as 500/350/250 mg/kg bw/day.

In the 10 mg/kg bw/day dose group, there was a statistically significant increase in the litter averages for resorptions, early resorptions, postimplantation loss, dams with any resorptions and percent resorbed conceptuses per litter in comparison with the vehicle control group values. These values were not considered to be test substance-related because they were not dose-dependent. There was also a slight increase in the percent postimplantation loss observed in the 500/350/250 mg/kg bw/day dose group at the time of Caesareansectioning; however, this value (7.4%) was primarily the result of one dam that had all resorbed conceptuses. When this dam was removed from the summary, the percent postimplantation loss changed from 7.4% to 1.6%, which was comparable with the vehicle control group value (2.8%). The litter averages for corpora lutea, implantations, preimplantation loss, litter sizes, live foetuses and percent live male foetuses were comparable among the four dose groups.

Foetal alterations: Foetal alterations were defined as: 1) malformations (irreversible changes that occur at low incidences in this species and strain); or 2) variations (common findings in this species and strain and reversible delays or accelerations in development). Litter averages were calculated for specific foetal ossification sites as part of the evaluation of the degree of foetal ossification.

No foetal gross external, soft tissue or skeletal alterations (malformations or variations) were considered to be test substance-related.

Table 3.10.1-5: Findings in the rat teratogenicity study

Parameter	**Dose level (mg/kg/day)**			
	0	**10**	**75**	**500/350/250**
Dam data				
Number of animals	25	25	25	30
Surviving to Day 21 with live litters	24	23	25	17
Killed *in extremis*	1	0	0	4
Found dead	0	0	0	8**
Not pregnant	0	2	0	1
Mean body weight gain (g)				

Days 6-9	11.0	12.1	13.8	-6.6**
Days 6-21	146.4	146.4	152.3	104.8**
Days 0-21	175.9	175.1	182.9	133.6**
Mean corrected[1] body weight gain (g)				
Days 6-21	41.3	44.0	45.0	12.5**
Days 0-21	70.8	72.7	75.6	41.3**
Mean food consumption (g/day)				
Days 6-21	23.5	23.1	23.7	19.5**
Days 0-21	22.6	22.3	22.8	19.8**
Litter responses				
% Postimplantation loss	2.8	6.5*	2.9	7.4
Foetal evaluation				
Foetal body weight (g)	5.65	5.56	5.58	5.08**
Foetal body weight – male (g)	5.81	5.70	5.71	5.18**
Foetal body weight – female (g)	5.48	5.42	5.44	4.96**
Foetal abnormalities	None	None	None	None

*p<0.05; **p<0.01

[1] Corrected body weight gain = gestation day 21 body weight minus the gravid uterine weight

Conclusion: The maternal NOAEL for pethoxamid is 75 mg/kg bw/day. Mortality, clinical signs, reductions in body weight, body weight gain and/or body weight losses and reductions in absolute and/or relative feed consumption values occurred in the 500/350/250 mg/kg bw/day dose group.

The developmental NOAEL is also 75 mg/kg bw/day. Reductions in gravid uterine weights and foetal body weights occurred in the 500/350/250 mg/kg bw/day dose group.

Pethoxamid is not a selective developmental toxicant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.1.6 Anonymous (1997b)

Reference: TKC-94 : Study of Tolerance in the Rabbit by Oral Gavage Administration
Author(s), year: Anonymous, 1997
Report/Doc. number: 89 PXA (TOX2001-301) / TON008/970270
Guideline(s): Not applicable
GLP: Yes
Acceptability: Yes (tolerance study)

Material and Methods:

Test material: Pethoxamid; Batch: TB-960306; Purity: 95.0%.

Test animals: Non-pregnant female New Zealand White rabbits (2 for the staircase phase weighing 3.92 and 3.98 kg and 2 for the constant dosage study weighing 4.49 and 4.64 kg) from an accredited closed colony, Froxfield Farms UK Limited, Hampshire, England.

Test method:

Staircase phase: Pethoxamid was administered by oral gavage to 2 rabbits, beginning with 50 mg/kg bw/d for 2 days. In the absence of any adverse response, the doses were doubled every two days (until Day 10, second day of dosing at 800 mg/kg bw/d).

Constant dosage study: Two females received 300 mg/kg bw/d for 7 consecutive days from Day 6 of gestation. Thereafter animals were killed and examined for reactions to the treatment.

Results:
Staircase phase: At 800 mg/kg bw/d, adverse toxicity occurred.

Constant dosage study: Body weight loss was recorded in both animals at 300 mg/kg bw/d. No other abnormalities were detected. Only one female was pregnant.

Conclusion: It was concluded that the highest dose in a preliminary teratogenicity study on rabbits should be at or less than 300 mg/kg bw/d.

3.10.1.7 Anonymous (1998b)

Reference: TKC-94 : Preliminary Embryo-foetal Toxicity Study in the Rabbit by Oral Gavage Administration
Author(s), year: Anonymous, 1998
Report/Doc. number: 90 PXA (TOX2001-302) / TON009/972410
Guideline(s): OECD Guideline 414 (1981)
GLP: Yes
Deviations from OECD 414 (2001):
- Limited number (4) of animals per group
- Shorter administration period (day 6 to 19)
- Limited parameters retrieved (no skeletal or soft tissue examination of foetuses assessed, no weight of gravid uteri recorded)

Acceptability: Yes (limited information); range finding study

Material and Methods:

Test material: Pethoxamid; Batch: TB-960306; Purity: 95.1%.

Test animals: Four groups of 4 pregnant female New Zealand White rabbits, 3.3-4.3 kg of weight and 18-26 weeks old, from an accredited closed colony, Froxfield Farms UK Limited, Hampshire, England.

Test method: The rabbits received the test compound at doses of 0, 30, 100 or 300 mg/kg bw/d, administered by oral gavage from Day 6 to 19 of gestation.

Results: The general condition was unaffected by the treatment. There were no death or treatment related pregnancy failures. The dams of the high dose group exhibited body weight loss and reduced food intake. Macroscopic examination on Day 29 of gestation revealed no effects related to treatment.

Conclusion: It was concluded that the highest dose in the main study should be between 100 and 300 mg/kg bw/d.

3.10.1.8 Anonymous (1998c)

Reference: TKC-94 : Study of Embryo-foetal Toxicity in the Rabbit by Oral Gavage Administration
Author(s), year: Anonymous, 1998
Report/Doc. number: 88 PXA (TOX2001-303) / TON012/982289
Guideline(s): OECD Guideline 414 (1981)
GLP: Yes
Deviations from OECD 414 (2001): Shorter administration period (day 6 to 19 of gestation)
Acceptability: Yes

Material and Methods:

Test material: Pethoxamid; Batch: TB-960306; Purity: 95.1%.

Test animals: Four groups of 20 pregnant female New Zealand White rabbits, 3,1-4,9 kg of weight and 18-26 weeks old, from an accredited closed colony, Charles River UK Limited, Margate, Kent, England.

Test method: Based on the effects observed in preliminary studies [Report no. TON008/970270 and TON009/972410], the rabbits received the test compound at doses of 0, 12.5, 50 or 200 mg/kg bw/d, administered by oral gavage from Day 6 to 19 of gestation. To ensure there were sufficient numbers of litters available for foetal pathology examination, a second set of four groups each of 5 pregnant rabbits were given pethoxamid at the same dosages and underwent the same procedures as conducted for Groups 1 to 4. These animals were not needed for the investigations.

Results:

Maternal reponses
General condition: One female receiving 50 mg/kg bw/d was killed for humane reasons on Day 23 of gestation following significant body weight loss. At necropsy, findings revealed a deflated lung and clear fluid within the thoracic cavity; this death was not considered to be related to treatment. One female in each of the groups receiving 12.5 or 200 mg/kg bw/d aborted; these abortions were not considered to be treatment-related as two females in the control group also aborted at about the same stage of pregnancy.

Body weight gain and food consumption: During the second half of treatment, at 50 mg/kg bw/d a stasis and at 200 mg/kg bw/d a stasis followed by a reduction in body weight gain were observed. After termination of the treatment, the body weight gain rapidly increased in both groups.

During the second half of the treatment period, the food consumption was slightly lower at 50 mg/kg bw/d and markedly lower at 200 mg/kg bw/d (approximately 76% of the control value). Once treatment had ended, diet intake recovered and was greater than for the control group at the end of the gestation period.

Pathological examination: No treatment-related findings were observed.

Litter responses: No treatment-related findings were recorded for the litter responses as assessed by the number of implantations and viable young, extent of pre- and post-implantation losses, final live litter size, foetal and placental weights.

No treatment-related findings were recorded at macroscopic, visceral and skeletal examinations.

Table 3.10.1-6: Data for dams in the rabbit teratogenicity study

Doses (mg/kg bw/d)	**0**	**12.5**	**50**	**200**
Number of dams	20	20	20	20
Number surviving to Day 29 with live litters	15	17	16	15
Number with only resorptions *in utero*	0	0	1	1
Not pregnant	3	2	2	3
Number aborting	2	1	0	1
Body weight gain (kg) Days 14-20 of gestation	0.08	0.08	0.04	-0.01
Food intake (g/rabbit/day) Days 13-19 of gestation	147	144	132	111

Conclusion: At 200 mg/kg bw/d and 50 mg/kg bw/d (the latter not that pronounced, hence not considered adverse), a reduced body weight gain and a lower food intake was observed during the treatment period. Therefore, the dose of 50 mg/kg bw/d is considered to be the maternal NOAEL.

No dose was associated with adverse effects on *in utero* survival or embryo-foetal development; thus the NOAEL (NOEL) for developmental toxicity is at 200 mg/kg bw/d.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.1.9 Anonymous (2014b)

Reference: A developmental toxicity study of pethoxamid by oral (gavage) administration in rabbits
Author(s), year: Anonymous, 2014
Report/Doc. number: 1139 PXA / Study No. 20039156
Guideline(s): OECD Guideline 414 (2001)
GLP: Yes
Deviations from OECD 414 (2001): -
Acceptability: Yes

Material and methods:

Test material: Pethoxamid technical
Lot/batch number: P1351-JaK-T2-23-6
Purity: 95.80% (w/w) (dose calculation was not adjusted to purity)
Stability of test item: 06 January 2014 (stored at ambient temperature). *NB: stable during the conduct of the study*
Storage conditions: At room temperature, protected from light

Test method: In a previously conducted study performed at Huntingdon Life Sciences Ltd., UK, [Report No. TON012/982289 October 5, 1998], a dose level of 200 mg/kg bw/day resulted in decreased body weights and reduced food consumption. No effects were observed on the foetuses. Therefore, four groups of pregnant female New Zealand white female rabbits received the test compound by gavage at daily dose levels of 0, 12.5, 50 or 200 mg/kg bw/day from Day 6 to 28 of gestation mating. There were 25 rabbits/group.

The vehicle control substance was 1% (w/v) methylcellulose (400 cP) and 0.5% (v/v) Tween® 80 in Reverse osmosis membrane-processed deionized water. The dose volume was 5 mL/kg.

The following parameters and end points were evaluated in this study: viability, maternal clinical signs, maternal body weights, maternal body weight changes, maternal food consumption, maternal gross observations, gravid uterine weights, ovarian and uterine examinations, foetal sex, foetal body weights and foetal external, visceral and skeletal morphology.

Results:

Mortality: At 200 mg/kg bw/day, there was a statistically significant increase in the number of rabbits that aborted (four rabbits). Rabbits observed to abort were subsequently euthanized during the study. There was also an additional rabbit in the 200 mg/kg bw/day dose group that delivered on GD 29 and was subsequently euthanized. Common clinical signs in the rabbits observed to abort included: scant faeces, ungroomed coat, thin body condition, mild dehydration and red substance in the cage pan. All other females survived until scheduled euthanasia.

Clinical signs: There was a statistically significant increase in the number of does in the 200 mg/kg bw/day dose group observed with scant faeces during the dose period. In the 200 mg/kg bw/day dose group, there was an increase or statistically significant increase in the number of does (3 to 6 per category) observed with thin body condition; ungroomed coat; red substance in the cage pan; red substance on the fur of the lower midline or perivaginal area; and the total amount of sparse hair coat observed on study (on the back and the underside). There was also an increase in the number of does (2) observed with no faeces in the cage pan and mild dehydration (based on skin turgor).

Body weights: Maternal body weights were statistically significantly reduced in the 200 mg/kg bw/day dose group beginning on GD 15 and continuing for the remainder of the dose period. In the 200 mg/kg bw/day dose group, there was also a statistically significant reduction in body weight gain observed for the entire dose period as well as the overall study period. Reductions or statistically significant reductions in body weight gains, reductions in body weight losses or statistically significant body weight losses were observed at all tabulated intervals in the 200 mg/kg bw/day dose group. Although not statistically significant, uterine weights in the 200 mg/kg bw/day dose group were slightly reduced (88% of the vehicle control group value). Corrected body weights (body weight on GD 29 minus the gravid uterine weight) were statistically significantly reduced in the 200 mg/kg bw/day dose group. Corrected mean body weight gains (body weights on GDs 6 to 29 and 0 to 29 minus the gravid uterine weight) were also statistically significantly reduced in the 200 mg/kg bw/day dose group.

Food consumption: Correlating with concurrent reductions in body weight gains and body weight losses, mean absolute and relative food consumption values at 200 mg/kg bw/day were also reduced. In the 200 mg/kg bw/day dose group, mean absolute food consumption values were reduced or significantly reduced at all intervals after the initiation of dose administration. The relative food consumption values were also decreased or statistically significantly decreased at all intervals during the dose period.

Maternal necropsy: There were no necropsy findings that were considered to be treatement related.

Caesarean-sectioning and litter observations: Pregnancy occurred in 22 (88.0%), 25 (100%), 23 (92.0%) and 21 (84.0%) does in the 0, 12.5, 50 and 200 mg/kg bw/day dose groups, respectively. Reflecting the four does that aborted and were subsequently euthanized and the one doe that delivered early at 200 mg/kg bw/day described previously, Caesarean-sectioning observations were based on 22, 25, 23 and 16 pregnant rabbits in Groups 1 through 4, respectively. Average foetal body weights (total, male and female) were statistically significantly reduced in the 200 mg/kg bw/day dose group.

No other Caesarean-sectioning or litter parameters were affected by doses of pethoxamid as high as 200 mg/kg bw/day. The litter averages for corpora lutea, implantations, preimplantation loss, litter sizes, live foetuses, early resorptions, late resorptions, postimplantation loss and percent male foetuses were comparable among the four dose groups. All placentae appeared normal.

Foetal alterations: Foetal alterations were defined as: 1) malformations (irreversible changes that occur at low incidences in this species and strain); or 2) variations (common findings in this species and strain and reversible delays or accelerations in development). Litter averages were calculated for specific foetal ossification sites as part of the evaluation of the degree of foetal ossification.

The 200 mg/kg bw/day dose group had statistically significant increases in the incidence of supernumerary thoracic ribs with associated statistically significant increases and decreases in the numbers of thoracic and lumbar vertebrae, respectively, a common variation observed at maternally toxic doses. Similar findings were observed in the 50 mg/kg bw/day dose group; the incidences were within historical control values but still considered to be the starting point of effects, being more severe at the high dose.

No additional foetal gross external, soft tissue or skeletal alterations (malformations or variations) were considered to be test substance related.

No additional gross external, soft tissue or skeletal alterations (malformations or variations) were considered to be test substance-related.

Table 3.10.1-7: Findings in the rabbit teratogenicity study

Parameter	**Dose Level (mg/kg bw/day)**			
	0	**12.5**	**50**	**200**
Dam Data				
Number of animals	25	25	25	25
Aborted and euthanized	0	0	0	4**
Delivered and euthanized	0	0	0	1
Number found dead	0	0	0	0
Number pregnant	22	25	23	25[1]
Clinical signs	-	-	-	scant feces, ungroomed coat, thin body condition, mild dehydration, sparse hair coat and red substance in the cage pan and on the fur
Body weight gain				
Mean (kg) Days 9-12	0.05	0.05	0.04	0.00**
Mean (kg) Days 6-29	0.35	0.33	0.38	0.09**
Mean k(g) Days 0-29	0.48	0.46	0.48	0.20**
Corrected body weight gain				
Mean (g) Days 6-21	-0.20	-0.19	-0.16	-0.40**
Mean (g) Days 0-21	-0.07	-0.07	-0.05	-0.28**
Food consumption				
Mean (g/day) Days 6-29	142.9	137.9	148.7	101.9**

Mean (g/kg/day) Days 6-29	37.5	36.5	39.4	27.9**
Litter responses	No treatment-related effects			
Foetal evaluation				
Foetal body weights (g) Total	44.05	42.42	44.11	35.81**
Males	44.98	43.29	45.15	36.43**
Females	42.81	41.80	43.49	35.09**
Foetal ossification sites/foetus/litter				
Vertebrae:				
Thoracic (±SD)				
MIN	12.43 ±0.30	12.55 ±0.27	12.63*[2] ±0.31	12.70** ±0.27
MAX	12	12	12	12
Lumbar (±SD)	13	13	13	13
MIN	6.56 ±0.31	6.44 ±0.27	6.35*[2] ±0.31	6.30** ±0.27
MAX	6	6	6	6
	7	7	7	7
Ribs (pairs) (±SD)	12.38 ±0.30	12.45 ±0.25	12.54 ±0.30	12.64** ±0.28
MIN	12	12	12	12
MAX	13	13	13	13

[1]Inclduing the does that aborted or delivered early and were euthanized.

[2]Incidence within historical control values: vertebrae thoracic range 12.40 – 12.66; vertebrae lumbar 6.34 – 6.60; ribs (pairs) 12.34 – 12.58.

*p<0.05; **p<0.01.

CONCLUSION: The maternal NOAEL for pethoxamid is 50 mg/kg bw/day. Significant maternal toxicity was seen at the highest dose level of 200 mg/kg bw/day. At this dose level, 4 rabbits aborted, and clinical signs, reduced body weight gain and reduced food consumption were observed.

The developmental NOEL is 12.5 mg/kg bw/day. In the 200 mg/kg dose group, foetuses showed reductions in foetal body weight and statistically significant increases in the incidence of supernumerary thoracic ribs with associated statistically significant increases and decreases in the numbers of thoracic and lumbar vertebrae, resp., a common variation observed at maternally toxic doses.

The developmental NOAEL is 12.5 mg/kg bw/day. In the 200 mg/kg dose group, fetuses showed reductions in fetal body weight and statistically significant increases in the numbers of ossified thoracic ribs with associated statistically significant increases and decreases ($p \leq 0.01$) in the numbers of ossified thoracic and lumbar vertebrae, resp., a common variation observed at maternally toxic doses. In the 50 mg/kg bw/day dose group, there were statistically significant increases and decreases ($p \leq 0.05$) in the numbers of ossified thoracic and lumbar vertebrae, respectively. Though the effects at 50 mg/kg were still within the historical control data of the Testing Facility, 50 mg/kg were considered to be the starting point of effects, being more severe at the high dose.

Pethoxamid is not a selective developmental toxicant.

According to the criteria specified in Regulation (EC) 1272/2008, classification of pethoxamid regarding reproductive toxicity (development of the offspring) is not required.

3.10.2 Human data

No relevant studies.

3.10.3 Other data (e.g. studies on mechanism of action)

No relevant studies.

3.11 Specific target organ toxicity – single exposure

3.11.1 Animal data

3.11.1.1 Anonymous (2014c)

Reference: An acute neurotoxicity study of Pethoxamid by oral gavage in rats.
Author(s), year: Anonymous, 2014
Report/Doc. number: 1137 PXA / Testing Facility Study No. 20039155
Guideline(s): OECD Guideline 424 (1997)
GLP: Yes
Deviations from OECD 486 (1997): No
Acceptability: Yes

EXECUTIVE SUMMARY

The overall objective of this study was to evaluate the potential of pethoxamid to cause neurotoxic effects in Crl:CD(SD) rats after a single oral exposure. The study contains two parts—Part A, range-finding study and Part B, definitive acute neurotoxicity study. The objectives of Part A of the study were as follows: 1) to determine the high dose to be tested in the definitive acute neurotoxicity study (Part B); and 2) to establish the appropriate time point based on clinical signs of toxicity for conducting Functional Observational Battery (FOB) and motor activity evaluations in the definitive study. The objective of Part B of this study was to perform an overall neurotoxicologic evaluation of the rats after acute oral exposure to pethoxamid, including FOB and motor activity assessments, at the time-of-peak effect and a neuropathologic examination centered on the central and peripheral nervous system.

In Part A, male and female rats (5/sex/group) were administered pethoxamid or the vehicle control substance once by oral gavage at dose levels of 0, 600, and 800 mg/kg bw. The vehicle used was 1% methylcellulose and 0.5% Tween® 80 in reverse osmosis deionized water. The dose volume was 5 mL/kg. For Part A of the study, the following parameters and end points were evaluated: viability, clinical signs including detailed clinical observations, body weights, food consumption, and necropsy observations.

No mortality was observed in Part A of the study. In the 800 mg/kg bw dose group, one male rat was observed with decreased motor activity, ptosis, pale right and left ears, mild and/or moderate dehydration, bradypnea and thin body condition. Body weight loss was observed in the 600 and 800 mg/kg bw dose groups in both sexes on Days 1 to 2, which was statistically significant in males at the high dose and in females at the mid and high dose compared with the vehicle control values. There was also a statistically significant reduction in absolute and relative food consumption values observed in male and female rats at 600 and 800 mg/kg bw on the day following dose administration compared with the vehicle control values. In the male rats at 800 mg/kg bw, absolute and relative food consumption values remained reduced on Days 2 to 3.

In the male and female rats, detailed clinical signs at 600 and 800 mg/kg bw that were considered to be related to the test substance included the following: hunched posture, vocalization to the touch,

chromorhinorrhea, pale ears, red urine, coldness to the touch, ptosis, mild or moderate dehydration and whole body tremors. These detailed clinical signs were most apparent between the 12- and 16-hour time points; some clinical signs remained apparent at the 24-hour time point. Based on these data, 16 hours was selected as the time-of-peak effect (TOPE) for evaluating FOB and motor activity for the definitive acute neurotoxicity study.

In Part B, male and female rats (10/sex/group) were administered pethoxamid or the vehicle control substance once by oral gavage on Day 1 at dose levels of 0, 100, 300, or 800 mg/kg bw. The vehicle used was 1% methylcellulose and 0.5% Tween® 80 in reverse osmosis deionized water. The dose volume was 5 mL/kg. The following parameters and end points were evaluated: viability, clinical signs, body weights, food consumption, necropsy observations and brain weights. An FOB, followed by motor activity evaluation was performed on all rats prior to dose administration, on the day of dose administration at the estimated TOPE, 7 days after dose administration and 14 days after dose administration. Neurohistopathological examinations were performed on selected tissues (central and peripheral nervous system, skeletal muscles and eyes with retinas and optic nerves) from 5 rats per sex from the vehicle control and the 800 mg/kg bw.

Two female rats in the 800 mg/kg bw dose group were found dead on either Days 2 or 3. The death of these rats was attributed to the test substance. The adverse clinical signs observed in these rats included the following: decreased motor activity; ptosis; mild and moderate dehydration; hunched posture; pale right and left ears and extremities; bradypnea; scant feces; and ungroomed coat.

A decrease or statistically significant decrease in body weight gain occurred in male and female rats at 300 mg/kg bw on Days 1 to 2 compared with vehicle control values. In the male and female rats at 800 mg/kg bw, a statistically significant loss in body weight occurred on Days 1 to 2. There was also a decrease or statistically significant decrease in absolute and relative food consumption values observed in male and female rats at 300 and 800 mg/kg bw on Days 1 to 2.

Adverse clinical signs during the FOB evaluation (i.e., hunched posture, bradypnea and pale right and left ears and extremities) were observed in one female rat that was subsequently found dead. Although these observations occurred in only a single female rat, they were considered to be test substance related. The other found dead female did not survive to the FOB evaluation performed on Day 2. There were no statistically significant or biologically important effects of pethoxamid on the FOB parameters in the male or female rats at any time point that were considered to be test substance related. No effects on brain weight or gross pathology of the cranial cavity were observed in either sex. No test substance-related microscopic lesions were apparent in the neurohistopathological evaluation of the central or peripheral nervous system, eyes with retinas and optic nerves and skeletal muscle.

On the basis of these data, the no-observable-adverse-effect-level (NOAEL) for systemic toxicity of pethoxamid following a single oral gavage dose is considered to be 100 mg/kg bw for both male and female rats based on body weight and food consumption effects at 300 mg/kg bw and mortality, clinical observations (in the two female rats that were subsequently found dead), body weight and food consumption effects at 800 mg/kg bw. There was no evidence of neurotoxicity in either sex at the highest dose tested; therefore, the NOAEL for neurotoxicity is considered to be 800 mg/kg bw for male and female rats.

MATERIALS AND METHODS

Materials:

Test material:	Pethoxamid technical
Lot/batch number:	P1351-JaK-T2-23-6
Purity:	95.80% (w/w) (dose calculation was not adjusted to purity)

Stability of test item:	06 January 2014 (stored at ambient temperature) *NB: stable during the conduct of the study*
Storage conditions:	At room temperature, protected from light

Study Design:

Groups of 5 male and 5 female Crl:CD(SD)BR rats were administered pethoxamid once by gavage at dose levels of 0, 100, 300 or 800 mg/kg bw. The vehicle used was 1% methylcellulose and 0.5% Tween® 80 in reverse osmosis deionized water. Dose levels and the time to peak effect (TOPE) were determined in a preliminary study. In this study, rats (10/sex/group) were administered pethoxamid once by gavage at dose levels of 0, 600 or 800 mg/kg bw. No mortality occurred; however, body weight loss and clinical signs of toxicity (including but not limited to hunched posture, vocalization, cold to touch and whole body tremors) were observed at both dose levels. The clinical signs occurred predominately between 12 and 16 hours after dose administration. Some signs were still apparent at 24 hours. Based on the results of the preliminary study, a 16-hour TOPE and dose levels of 0, 100, 300 and 800 mg/kg bw were selected for the definitive study.

RESULTS AND DISCUSSION

Mortality: 800 mg/kg bw: Two female rats were found dead, one on Day 2 and one on Day 3.

Clinical signs: 800 mg/kg bw: Clinical signs of toxicity were observed only in the two female rats that were found dead. Signs included decreased motor activity, ptosis, mild and moderate dehydration, hunched posture, pale ears and extremities, bradypnea, scant feces and/or ungroomed coat.

Body weight gain: 800 mg/kg bw: Body weight loss (statistically significant) was observed in male and female rats on Days 1 to 2 after which body weight rebounded. 300 mg/kg bw: Statistically significantly decreased body weight gain was observed in male rats from Day 1 to 2. Body weight gain was reduced in females on Day 1 to 2; however, the effect was not statistically significant.

Food consumption: 800 mg/kg bw: Absolute (g/day) and relative food consumption (g/kg/day) were statistically significantly decreased in males and females from Day 1 to 2. 300 mg/kg bw: Relative food consumption (g/kg/day) was statistically significantly decreased in both sexes from Day 1 to 2.

Functional Observational Battery: 800 mg/kg bw: At the TOPE, hunched posture, bradypnea and pale ears and extremities were observed in one female rat that was subsequently found dead.

Motor Activity: No treatment-related effects observed.

Brain weights: No treatment-related effects observed.

Macroscopic findings: No treatment-related effects found in the two females that were found dead.

Neurohistopathology: No treatment-related effects observed.

Table 3.11.1-1: Summary of acute neurotoxicity study findings-Part B—Definitive Study

Parameter	**Males**			
	Dose Levels (mg/kg bw)			
	0	**100**	**300**	**800**
Mortality	0	0	0	0
Body Weight				
Day 1	315.0	312.2	304.8	305.8
Day 2	321.6	318.6	306.8	302.9
Day 3	330.1	326.0	316.7	309.7

Body Weight Gain **Days 1 to 2**	6.6	6.4	2.0*	-2.9**
Food Consumption **Absolute Food Consumption (g/day)** **Days 1 to 2**	25.5	25.0	21.8	16.2**
Relative Food Consumption (g/kg/day) **Days 1 to 2**	80.2	79.2	72.1*	52.9**
Functional Observation Battery	No treatment-related effects			
Motor Activity	No treatment-related effects			
Neurohistopathology	No treatment-related effects			
Parameter	Females			
	Dose Levels (mg/kg bw)			
	0	100	300	800
Mortality	0	0	0	2
Body Weight **Day 1** **Day 2** **Day 3**	219.0 223.1 225.8	218.4 220.4 223.7	222.1 222.3 227.5	223.3 219.9 227.6
Body Weight Gain **Days 1 to 2**	4.1	2.0	0.2	-4.2**
Food Consumption **Absolute Food Consumption (g/day)** **Days 1 to 2**	16.0	15.5	12.7	8.8**
Relative Food Consumption (g/kg/day) **Days 1 to 2**	72.5	70.7	56.8*	39.0**
Functional Observation Battery	-	-	-	At TOPE: hunched posture, bradypnea and pale ears and extremities were observed in one female rat that was subsequently found dead
Motor Activity	No treatment-related effects			
Neurohistopathology	No treatment-related effects			

*p<0.05; **p<0.01

CONCLUSION: The no-observable-adverse-effect-level (NOAEL) for systemic toxicity for pethoxamid following a single gavage dose is considered to be 100 mg/kg bw for male and female rats based on decreased body weight gain and relative food consumption at 300 mg/kg bw and mortality, clinical observations (in the two female rats that were subsequently found dead), body weight loss and decreased absolute and relative food consumption at 800 mg/kg bw. No clear evidence of neurotoxicity was observed in either sex at the highest dose tested; therefore, the NOAEL for neurotoxicity is considered to be 800 mg/kg bw for male and female rats.

3.11.2 Human data

No relevant studies.

3.11.3 Other data

No relevant studies.

3.12 Specific target organ toxicity – repeated exposure

3.12.1 Animal data

3.12.1.1 Anonymous (1994)

Reference: Toxicity to rats by dietary administration for 4 weeks
Author(s), year: Anonymous, 1994
Report/Doc. number: 69 PXA (TOX2001-282)/ TKS '13/932511
Guideline(s): OECD Guideline 407 (1981)
GLP: Yes
Deviations from OECD 407 (2008): Yes (Haematology: reticulocytes and bile acids not examined; Pathology: no weight of thymus, prostate + seminal vesicles with coagulating glands recorded; Histopathology: no preservation of seminal vesicles with coagulating glands, peripheral nerve (sciatic or tibial); no full histopathology carried out on the preserved organs)

Acceptability: Yes

Deviations from study protocol: Due to a technical error, the water residue was not measured at the seventh day of Week 3 and the water consumption was calculated on a 6 day basis. This deviation is not considered to affect the integrity of the study.

Material and Methods:

Test material: Pethoxamid; Batch TB-930727: Purity: 95.2%.

Test animals: Groups of 5 male and 5 female Crl: CD(SD)BR rats, 28 days old, 187 g to 253 g (males), 151 g to 191 g (females), from Charles River (U.K.), Ltd, Margate, Kent, England.

The rats received the test material by dietary administration at concentrations of 0, 500, 2500, 5000 and 7500 ppm for a duration of 4 weeks. This was equal to 45.3, 227, 482 and 699 mg/kg bw/d for males and 52.9, 266, 535 and 737 mg/kg bw/d for females.

Results:

Clinical findings:

No mortalities occurred in the study. Treatment-related clinical signs or opthalmological findings were not observed.

Body weight, body weight gain, food and water consumption:

Findings on body weight, body weight gain and food consumption were observed from 2500 ppm, partly attaining statistical significance (Table 3.12.1-1). At 7500 ppm body weight losses were noted for both sexes in Week 1. Males showed a notably lower water consumption at this dose.

Table 3.12.1-1: Rat oral 28-day: Body weight, body weight gain, food and water consumption

Sex	**Male**					**Female**				
Dose (ppm)	0	500	2500	5000	7500	0	500	2500	5000	7500
Body weight, term. kill (g)	361	355	326	299*	234**	239	239	221	218*	190**
Body weight gain										
Week 0-1 (g/rat)	55	47	27**	2**	-30**	24	24	15	3**	-13**
Week 1-4 (g/rat)	89	92	83	80	47**	49	49	42	48	39
Week 0-4 (g/rat)	144	139	110**	83**	16**	73	72	56	51**	26**
Food consumption[1]										
Week 1 (g/rat)	193	179	171	166	79	133	142	139	139	93
Week 1-4 (g/rat)	776	741	691	679	557	576	611	581	573	468
Water consumption[1]										
Week 3 (g/rat/week)	212	180	197	199	159	170	169	157	152	174

[1] Only measured in Week 3, statistical analysis not possible due to there only being one cage/sex/group;

Statistical significance: *p≤0.05; **p≤0.01

Haematology:

At 5000 and 7500 ppm, both sexes showed lower mean total white blood cell counts associated with lower lymphocyte counts achieving statistical significance in males (Table 3.12.1-2). In females, the hemoglobin values were significantly lower. The decrease of the concomitant lower mean corpuscular hemoglobin concentrations was only significant at 7500 ppm.

Table 3.12.1-2: Rat oral 28-day: Haematology

Sex	**Male**					**Female**				
Dose (ppm)	0	500	2500	5000	7500	0	500	2500	5000	7500
Platelets (x10^3/mm^3)	1016	879	824*	875*	870*	950	810	621	781	820
WBC (x10^3/mm^3)	11.6	10.2	10.5	9.6	8.0*	6.9	8.7	7.8	5.9	5.4
Lymphocytes (x10^3/mm^3)	10.16	8.35	8.81	7.08**	6.47**	5.97	7.27	6.67	4.63	4.52
Hemoglobin (g/dl)	15.5	16.0	15.6	15.0	14.7	15.8	16.0	15.6	14.8*	14.7**
MCHC (%)	29.2	29.0	28.4	29.0	29.0	30.1	30.0	29.3	29.1	28.7*

WBC: White blood cells; MCHC: Mean corpuscular hemoglobin concentrations;

Statistical significance: *p≤0.05; **p≤0.01

Clinical chemistry:

The main finding was a dose related increase in mean cholesterol values from 500 ppm (Table 3.12.1-3). Values for other parameters (ALT, globulin, phosphorus and glucose) varied from the control values at 5000 and 7500 ppm.

Table 3.12.1-3: Rat oral 28-day: Clinical chemistry

Sex	**Male**					**Female**				
Dose (ppm)	0	500	2500	5000	7500	0	500	2500	5000	7500
Cholesterol (mg/dl)	58	90*	128**	152**	190**	77	84	117*	167**	210**
ALT (mU/ml)	26	24	22	27	31	21	24	20	30	35*
Glucose (mg/dl)	118	114	104	120	99*	118	113	107	99*	93**
Globulin (g/dl)	3.5	3.8	3.8	4.2**	3.9**	3.4	3.5	3.6	4.0**	4.1**
Phosphorus (mEq)	4.8	4.5	4.5	4.3**	3.9**	3.7	3.8	3.6	3.4	3.6

ALT: Alanine amino transferase; Statistical significance: *p≤0.05; **p≤0.01

Pathological examinations:

Necroscopy: No treatment related changes were found.

Organ weights: Generally, the liver weights were increased, beginning in males from 500 ppm (Table 3.12.1-4). Statistically significant was this finding for the adjusted weights only. In males, the absolute spleen weights were decreased from 500 ppm onwards (11, 17, 33% of the mean control value).

Histopathology: From 2500 ppm, histopathologic findings as centrilobular enlargement of hepatocytes and intracytoplasmic eosinophilic inclusions accompanied the increase of liver weights.

Table 3.12.1-4: Rat oral 28-day: Liver weight and liver histopathology

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**500**	**2500**	**5000**	**7500**	**0**	**500**	**2500**	**5000**	**7500**
Liver weight										
Absolute liver weight (g)	16,4	18,3 +12%	22,1 +35%	22,3 +36%	17,6 +7%	11,4	11,4 +0%	12,0 +5%	15,8 +39%	14,9 +31%
Adjusted liver weight (g)[1]	13.7	15.9 +16%	21.6** +58%	23.1** +69%	22.3** +63%	10.9	11.0 +1%	12.1 +11%	15.8** +45%	15.7** +44%
Liver histopathology										
Centrilobular enlargement of hepatocytes	0/5	0/5	1/5	5/5**	5/5**	0/5	0/5	0/5	3/5	5/5**
Periportal hepatocytes with intracytoplasmic eosinophilic inclusions	0/5	0/5	4/5*	3/5	4/5*	0/5	0/5	0/5	1/5	3/5

[1] Adjusted to body weight; Statistical significance: *p≤0.05; **p≤0.01

Conclusion:

At the lowest dose, effects already occurred – although mostly without statistical significance. Thus, based upon the data obtained from this study, it was recommended that a suitable low level for further investigation should be below 500 ppm, whilst a suitable high level should be 5000 ppm or above, but not approaching 7500 ppm.

3.12.1.2 Anonymous (1996a)

Reference: Palatability/Preliminary dose range finding study in mice by dietary administration for 4 weeks
Author(s), year: Anonymous 1996a
Report/Doc. number: 70 PXA (TOX2001-283) / TON '3/960337
Guideline(s): OECD 407 (1981)
GLP: Yes
Deviations from OECD 407 (2008): Yes (Pathology: No weight of thymus recorded, no preservation of peripheral nerve (sciatic or tibial); no full histopathology carried out on the preserved organs, only for liver and thyroid; no haematology measurements recorded; no clinical chemistry measurements (with the exception of drug metabolizing liver enzymes) recorded).

Acceptability: Yes (limited information); range-finding test

Material and Methods:

Test material: Pethoxamid; Batch: TB-930727; Purity: 95%.
Test animals: Groups of 16 male and 16 female Crl: CD-1 (ICR) BR mice, 6 weeks of age, 26-38 g (males), 22-29 g (females), from Charles River, UK, Ltd, Margate, Kent, England.
The mice received the test material by dietary administration at concentrations of 0, 100, 500, 3000 and 10000 ppm for a duration of 4 weeks. This was equal to 17, 85, 539 and 1786 mg/kg bw/d for males and 22, 114, 679 and 2206 mg/kg bw/d for females.

Results:

Mortality and clinical findings: Treatment related clinical signs were not observed. No mortalities were recorded.

Body weight, Body weight gain and food consumption: From 3000 ppm, the food consumption was significantly lower in Week 1 (Table 3.12.1-5). This was accompanied in males (at 10000 ppm also in females) by a body weight loss. The body weight gain was reduced in males over the entire dosing period.

Pathological examinations:
Necropsy: No treatment related findings were detected.
Organ weights: The body weight adjusted liver weights were significantly increased, beginning in males from 500 ppm (Table 3.12.1-5). An increase in body weight adjusted kidney weights from this dose onwards was also observed (males: 17, 20, 10%; females: 6, 11, 6% above the mean control values).

Histopathology: Hepatocellular hypertrophy was observed.

Hepatic enzyme activities: In mice as in rats, pethoxamid was found to be acting as a phenobarbitone-type inducer of liver enzymes starting already at the 100 ppm dose level (larger induction of 7-pentoxyresorufin O-dependentylase activity (CYP2B) and smaller induction of other P450-related enzyme activities as well as induction of p-nitrophenol UDP-glucuronyltransferase).

Table 3.12.1-5: Mouse oral 28-day study (data summary)

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**100**	**500**	**3000**	**10000**	**0**	**100**	**500**	**3000**	**10000**
Body weight, term. kill	39	39	38	35	31	27	28	29	27	25
Body weight gain										
Week 0-1 (g/mouse)	2.3	2.5	2.3	-0.2**	-2.5**	0.6	0.3	0.5	0.3	-1.2**
Week 1-4 (g/mouse)	4.4	4.3	4.4	3.4*	1.8**	1.1	2.4*	2.8*	2.7*	1.6*
Week 0-4 (g/mouse)	6.7	6.8	6.7	3.2**	-0.8**	1.7	2.8	3.2	3.0	0.4
Food consumption										
Week 1 (g/mouse)	40	42	39	32**	28**	37	38	37	32**	28**
Week 2-4 (g/mouse)	125	134	129	127	118	131	125	132	130	116**
Organ weights										
Absolute liver weight (g)	2,06	2,18 +6%	2,21 +7%	2,20 +7%	2,32 +13%	1,46	1,50 +3%	1,62 +11%	1,69 +16%	1,77 +21%
Adjusted liver weight (g)[1]	1.85	1.98 +7%	2.10** +14%	2.35** +27%	2.69** +45%	1.47	1.47 +0%	1.53 +4%	1.71** +16%	1.87** +27%
Hepatocellular hypertrophy										
-Centrilobular	1/16	0/16	4/16	5/16	7/16*	0/16	0/16	0/16	0/16	0/16
-Generalised	0/16	0/16	2/16	5/16*	9/16**	0/16	0/16	0/16	0/16	0/16
-Periportal	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	14/16**	16/16**

[1] Body weight adjusted; Statistical significance: *p≤0.05; **p≤0.01

Conclusion:

The NOAEL is 100 ppm corresponding to 17 mg/kg bw/d based on liver weight increase and liver histopathology findings at the dose level of 500 ppm (85 mg(kg bw/d).

At 100 ppm, phenobarbitone-type liver enzyme induction was already observed, but this was not considered as an adverse effect on its own.

3.12.1.3 Anonymous (1996b)

Reference: Maximum Tolerated Dose and Four Week Constant Dose Study in Dogs
Author(s), year: Anonymous, 1996b
Report/Doc. number: 72 PXA (TOX2001-284) / TON '2/960653
Guideline(s): No OECD guideline for 28 day non-rodents available, based on OECD 407
GLP: Yes
Deviations from OECD 407: Yes (No concurrent controls; Microscopic examinations were limited to liver and thyroid; the number of animals was anyhow too low for any other information but estimation of approximate dose for further testing).

Acceptability: Yes (limited information); range-finding test

Deviations from study protocol: Hepatic drug metabolizing enzymes were tested. The food ration offered to the pair of dogs receiving 400 mg/kg bw/day was modified on occasion in an attempt to stimulate appetite and to improve general condition.

Material and Methods:
Test material: (1): Pethoxamid; Batch:TB 930727; Purity: 96.0%.
(2): Pethoxamid; Batch: TB 951005; Purity 95.1%.
Test animals: Four male and 4 female pure bred beagle dogs, 19-24 weeks old, 6.5-10.9 kg of weight, from Consort Limited, Harewood Park, Harewood End, Herefordshire, HR2 8 TS.
Maximum tolerated dose (MTD) phase: A pair of beagle dogs was given a series of escalating doses of pethoxamid (25, 50, 100, 200, 400, 800, 1000 and 1600 mg/kg bw/d), orally by capsule. The dose level was increased every 3 to 4 days. Based on the results of the MTD phase, three dose levels were chosen to dose further pairs of dogs for 28 days.
Repeat dose phase: Initially, 1 male and 1 female animal received 50, 200 or 800 mg/kg bw/d. However, the pair receiving the highest dosing level showed adverse signs and dosing was suspended after 7 days. Following a period of 7 days off dose, this pair was dosed at 400 mg/kg bw/d for 28 days. Control data were recruited from historical data.

Results:
MTD phase:
In view of the findings (inappetance) seen, it was considered that dose levels of 50, 200 and 800 mg/kg bw/d were suitable for the 28-day study.
Repeat dose phase:
Clinical findings:
No mortalities occurred. The clinical findings comprehended liquid/mucoid faeces, vomiting, salivation, subdued behaviour and tucked up appearance (Table 3.12.1-6). These symptoms were dose related.
Body weight, body weight gain and food consumption:
At 200 mg/kg bw/d, a minor effect on body weight in the female dog was recorded (Table 3.12.1-6). At 400 mg/kg bw/d, both animals recorded overall weight gain but it was necessary to manipulate the diet because of the immediate decrease in appetite. According to the marked reduction in food consumption, a marked loss of body weight was recorded at 800 mg/kg bw/d.
Haematology:
At the high dose (800/400mg/kg bw/d), a slight decrease in red blood cell parameters and increases in reticulocytes and platelets were observed. The blood smear showed slight hypochromasia and/or macrocytosis from Day 20.
Clinical chemistry:
At 800/400 mg/kg bw/d, a dose related decrease in cholesterol levels was noted. In this dose group, the values for most of the measured parameters of hepatic drug metabolism (microsomal protein- and cytochrome P450 concentration, 7-ethyoxyresorufin O-deethylase, 7-pentoxyreso-rufin O-depentylase, lauric acid 11-hyroxylase, lauric acid 12-hydroxylase, p-nitrophenol UDP-glucuronyltransferase) were below the range of historical data. This decrease is obviously related to the hepatotoxic effect of pethoxamid.
Pathological examinations:
Necropsy: Accompanying the increased absolute and relative liver weights in the female dog, an enlarged liver was found at 800/400 mg/kg bw/d.
Organ weights: The liver weight was above the normal historical upper limit of 4% of the terminal body weight.
Histopathology: A minimal centrilobular hepatocyte hypertrophy was observed; at 200 mg/kg bw/d in both sexes and at 800/400 mg/kg bw/d in the female only.

Table 3.12.1-6: Overview of 28-day toxicity in dogs treated orally with pethoxamid

Sex	**Male**					**Female**				
Dose (mg/kg bw/day)	**0**	**50**	**200**	**800**	**400**	**0**	**50**	**200**	**800**	**400**
Clinical signs										
Vomiting	HD		+	+	+	HD		+	+	+
Liquid/mucoid faeces	HD	+	+	+	+	HD	+	+	+	+
Subdued behaviour	HD			+	+	HD		+	+	+
Tucked up appearance	HD			+	+	HD		+	+	+
Salivation	HD				+	HD				
Body weight gain										
Day 1-29 (g/dog)	HD	0.7	0.6	–	–	HD	0.7	-0.2	–	–
Day 1-8 (g/dog)	HD	–	–	-1.8	–	HD	–	–	-1.6	–
Day 8-14 (g/dog)	HD	–	–	0.7	–	HD	–	–	1.1	–
Day 15-43 (g/dog)	HD	–	–	–	0.2	HD	–	–	–	1.7
Food consumption										
Day 1-28 (g/dog)	HD	400	369	–	–	HD	400	378	–	–
Day 1-7 (g/dog)	HD	–	–	160	–	HD	–	–	151	–
Day 15-42 (g/dog)	HD	–	–	–	[1]	HD	–	–	–	[1]
Haematology[2]										
PCV (%)	HD	40.2	44.1	40.1	31.9	HD	44.0	43.1	39.5	35.9
Hb (g/dl)	HD	12.7	14.1	13.1	9.7	HD	14.0	13.6	12.7	11.0
RBC (x10^{12}/l)	HD	5.86	6.32	6.09	4.12	HD	6.11	5.89	5.38	4.72
Reticulocytes (%)	HD	0.5	0.5	<0.1	3.2	HD	1.2	1.1	0.1	3.8
Platelets (10^9/l	HD	290	427	385	592	HD	341	473	431	566
Hypochromasia	HD				+	HD				+
Macrocytosis	HD				+	HD				+
Clinical chemistry										
Cholesterol (mg/dl)	HD	165	144		86	HD	134	101	102	
Organ weights										
Absolute liver weight (g)	HD	383.9	384.7	393.8		HD	334.6	358.3	492.4	
Relative liver weight (%)[3]	HD	3.49	4.01	3.82		HD	3.64	4.03	5.02	
Macroscopic pathology										
Enlarged liver	No treatment-related findings					HD	0/1	0/1	1/1	
Liver histopathology										
-Min. centrilob. hypertrophy	HD	0/1	1/1	0/1		HD	0/1	1/1	1/1	

HD: Historical data used; [1] Range of 100 to 550 g food/dog for male and 150 to 550 g food/dog for female due to loss of appetite and measures to improve palatability; [2] Animals treated with 800/400 mg/kg bw/d were measured at Day 10 for 800 mg/kg bw/d and Day 42 for 400 mg/kg bw/d; [3] Relation to body weight; +: present; -: not calculated for this time interval; PCV: Packed cell volume; Hb: hemoglobin; RBC: Red blood cell count.

Conclusion:
The findings in this study indicate that dosages of 400 mg/kg bw/d and above are not suitable for further investigations on dogs. No induction of drug metabolizing enzymes - as in rats and mice - was found in dogs.

3.12.1.4 Anonymous (1996)

Reference: Toxicity to rats by dietary administration for 13 weeks
Author(s), year: Anonymous, 1996
Report/Doc. number: 61 PXA (TOX2001-285) / TKS '24/951565
Guideline(s): OECD 408 (1981)
GLP: Yes
Deviations from OECD 408 (1998): Yes (Pathology: no weight of thymus recorded; Histology: no full histopathology of accessory sex organs, no histopathology of skin and peripheral nerve)
Acceptability: Yes

Deviations study protocol: In addition, levels of drug metabolizing enzymes were measured.

Material and Methods:
Test material: Pethoxamid, Batch TB-930727; Purity: 95.2%.
Test animals: Groups of 10 male and 10 female Crl:CD BR rats, six weeks old, 179 g to 224 g (males), 135 g to 171 g (females) of weight, from Charles River U.K., Ltd., Margate, Kent.
The rats received the test compound by dietary administration at concentrations of 0, 100, 500, 2500 and 5000 ppm for a duration of 13 weeks. This was equal to 7.5, 36.2, 196 and 388 mg/kg bw/d for males and 8.0, 41.6, 207 and 426 mg/kg bw/d for females.

Results:
Mortality, clinical and ophthalmologic findings:
No deaths occurred. No treatment related clinical and ophthalmoscopic findings were detectable.

Body weight, body weight gain, food- and water consumption:
The absolute body weights were dose related but not statistically significantly lowered. However, the body weight gains were statistically significantly lowered in males from 500 ppm. At 5000 ppm, even a body weight loss occurred. The food- and water consumption were dose related reduced, partly attaining statistical significance.

Table 3.12.1-7: Rat oral 90-day study (Body weight, body weight gain, food- and water consumption)

Sex	Male					Female				
Dose (ppm)	**0**	**100**	**500**	**2500**	**5000**	**0**	**100**	**500**	**2500**	**5000**
Body w., term. kill (g)	543	521	496	457	438	292	301	288	272	255
Body weight gain										
Week 0-1 (g)	60	58	50*	28**	-9**	23	25	23	18	3**
Week 1-13 (g)	278	260	246	228*	240*	118	125	113	102	104
Week 0-13 (g)	338	317	296*	256**	232**	141	150	136	120*	106**
Food consumption										
Week 1 (g/rat)	222	214	191	170*	113**	132	129	133	128	100*
Week 1-13 (g/rat)	2729	2622	2457	2470	2312*	1830	1773	1786	1707	1645
Water consumption										
Week 12 (g/rat)[1]	254	249	219	203*	214*	203	192	181	177	170

[1] Only measured in Week 12; Statistical significance: * p≤0.05, ** p≤0.01

Haematology:
From 2500 ppm, both sexes showed lower mean platelet values, which was statistically significant for males.

Clinical chemistry:
From 2500 ppm, the cholesterol values were statistically significantly increased. At 5000 ppm, the total protein values were significantly higher and the glucose values lower (significant in males only) as the control values. Values for parameters of drug metabolism in the liver altogether increased from 2500 ppm; especially the 7-pentoxyresorufin O-dependentylase activity in females. This indicates pethoxamid acting as a phenobarbitone-type inducer of drug metabolizing enzymes in rats.

Table 3.12.1-8: Rat oral 90-day study: Haematology and clinical chemistry

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**100**	**500**	**2500**	**5000**	**0**	**100**	**500**	**2500**	**5000**
Haematology										
Mean platelet value ($x10^3/mm^3$)	794	752	773	695*	706*	772	722	728	681	681
Clinical chemistry										
Cholesterol (mg/dl)	59	65	67	86**	123**	76	77	85	104**	133**
Total mean protein (g/dl)	6.6	6.7	6.6	6.9	7.3**	7.1	7.1	7.1	7.4	7.6*
Mean glucose (mg/dl)	117	116	112	111	102**	123	120	125	115	109
Liver, parameters of drug metabolism										
Cytochrome P450[1] (nmoles/g liver)	1	0.9	1.1	1.7**	2.1**	1	1.0	1.3	1.6**	2.3
7-Ethoxyresorufin O-deethylase[1] (nmol/min/g liver)	1	1.2	2.2**	4.0**	5.6**	1	1.2	1.8*	2.8**	3.2
7-Pentoxyresorufin O-depentylase[1] (nmol/min/g liver)	1	0.9	2.1**	7.8**	12.1**	1	1.0	12.4**	276**	791.6**
Lauric acid 11-hydroxylase (nmol/min/g liver)	1	1.0	1.1	1.6**	1.8**	1	1.1	1.3*	1.7**	1.9*
Lauric acid 12-hydroxylase (nmol/min/g liver)	1	1.2	1.0	1.2	1.3	1	1.2	1.3*	1.5*	1.2*
p-Nitrophenol UDP-glucuronyltransferase[1] (µmoles/hr/g liver)	1	0.9	1.3	3.5**	5.8**	1	1.0	1.5*	3.0**	8.1**

[1] Statistical analysis performed on logarithmically transformed data; Statistical significance: *p≤0.05; **p≤0.01.

Pathological examinations:
Necroscopy: No treatment-related findings were observed.
Organ weights: The body weight adjusted liver weights were significantly increased in both sexes from 2500 ppm, and the body weight adjusted thyroid weight in females at 5000 ppm (Table 3.12.1-9).
Histopathology: The single incidences of fat deposition among rats up to 500 ppm were within the background control range. From 2500 ppm, treatment-related changes in the liver comprehended periportal hepatocytes with margination of cytoplasm, fat deposition and concentric intracytoplasmic inclusions. At 5000 ppm, the hepatocytes were generally minimally enlarged in some animals.
In the thyroids, follicular cell hypertrophy was significantly increased in males (from 2500 ppm) and females (5000 ppm).

Table 3.12.1-9: Rat oral 90-day study: Findings on liver and thyroid (organ weights and histological findings)

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**100**	**500**	**2500**	**5000**	**0**	**100**	**500**	**2500**	**5000**
Liver										
Abs. Liver weight (g)	21,3	20,1 -6%	20,6 -3%	22,9 +8%	24,8 +16%	10,6	11,0 +4%	10,6 +0%	12,2 +15%	13,7 +29%
Adj. liver weight (g)[1]	19.1	18.8 -2%	20.3 +6%	24.3** +27%	27.1** +42%	10.3	10.4 +1%	10.4 +1%	12.5** +21%	14.6** +42%
Periportal hepatocyte margination of cytoplasm	0/10	0/10	0/10	9/10**	10/10**	0/10	0/10	0/10	1/10	10/10**
Occasional concentric intracytoplasmic inclus.	0/10	0/10	0/10	3/10	7/10**	0/10	0/10	0/10	0/10	2/10
Minimally generalised hepatocyte enlargement	0/10	0/10	0/10	0/10	4/10*	0/10	0/10	0/10	0/10	4/10*
Fat deposition in periportal hepatocytes	0/10	0/10	2/10	5/10*	6/10**	1/10	3/10	4/10	1/10	1/10
Thyroid										
Abs. thyroid weight (mg)	21.7	22.8 +5%	20.1 -7%	25.4 +17%	24.9 +15%	14.4	17.1 +19%	17.3 +20%	16.7 +16%	17.8 +24%
Adj. thyroid weight (mg)[1]	[2]					14.0	16.3 +16%	17.1 +22%	17.0 +21%	18.8** +34%
Follic. cell hypertrophy	2/10	0/10	0/10	7/10*	9/10**	0/10	0/10	0/10	2/10	4/10*
Sparse colloid	1/10	0/10	0/10	3/10	7/10**	0/10	0/10	0/10	0/10	2/10

[1] Body weight adjusted; [2] Not adjusted as not appropriate; Statistical significance: *p≤0.05; **p≤0.01

Conclusion:
The NOAEL (NOEL) in this study was at 100 ppm corresponding to 7.5 mg/kg bw/d based on decreased body weight gain and food intake. The liver and thyroid findings at higher doses indicate an effect on the liver-thyroid axis known for phenobarbitone-type inducers of drug metabolizing enzymes in the liver.

3.12.1.5 Anonymous (1998)

Reference: Toxicity to mice by dietary administration for 13 weeks
Author(s), year: Anonymous, 1998
Report/Doc. number: 71 PXA (TOX2001-286) / TON '5/971279
Guideline(s): OECD 408 (1981)
GLP: Yes
Deviations from OECD 408 (1981): Yes (Haematology: no haematocrit measured; no organ weights of uterus and thymus recorded; - no histopathological examinations of aorta, gall bladder, skin recorded; not all accessory sex organs preserved; - no organ weights of uterus and thymus recorded; - no histopathological examinations of aorta, gall bladder, skin recorded; not all accessory sex organs preserved).
Acceptability: Yes (limited information)

Supplementary Evaluation of 13-week mice study:

Reference: Toxicity to mice by dietary administration for 13 weeks – Electron Microscopy Report
Author(s), year: Anonymous, 1997
Report/Doc. number: 1288 PXA (TOX2001-286) / TON '5/970411
Guideline(s): Not applicable
GLP: Yes
Deviations: Not applicable
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306; Purity: 95.0%.
Test animals: 10 male and 10 female Crl:CD-1 (ICR) BR mice
Based on the results in the 28-day study (Report no. TON 3/960337), the mice received the test material by dietary administration at concentrations of 0, 50, 400, 3000 and 10000 ppm for a duration of 13 weeks in order to select appropriate dosages for the carcinogenicity study. This was equal to 9.1, 70.5, 610 and 2354 mg/kg bw/d for males and 12.0, 93, 724 and 2492 mg/kg bw/d for females.

Results:
Mortality, clinical and ophthalmologic findings:
There were three deaths which were not treatment-related. One mouse from the control group was found dead during Week 13 due to urogenital tract lesions. Two mice (one male and one female from 3000 ppm) were found dead during Week 13 and 14 due to an anaesthetic accident.
No treatment-related clinical effects and no ophthalmologic effects were observed.

Body weight, body weight gain and food consumption:
No treatment-related effects on food consumption were recorded.
The mice gained less weight than the control animals from 3000 ppm and lost weight at 10000 ppm.

Table 3.12.1-10: Mouse oral 90-day study: Body weight and body weight gain

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Body weight, term. kill	39	42	43	37	29	30	32	29	28	26
Body weight gain										
Week 0-1 (g)	2.3	2.8	2.3	0.7**	-2.7**	1.2	1.4	1.2	0.3	-1.1**
Week 1-12 (g)	7.7	11.3	9.4	8.6	2.5**	6.6	7.2	5.2	4.3	2.8**
Week 0-12 (g)	9.9	14.1	11.7	9.2	-0.2**	7.9	8.6	6.4	4.6*	1.6**

Statistical significance: *p≤0.05; **p≤0.01

Haematology:
At the highest dose, both sexes showed lower mean packed cell volume, hemoglobin and red blood cell values, with males also showing higher mean corpuscular volume values and lower mean corpuscular hemoglobin concentration values. Males showed statistically significant lower mean total white blood cell counts, associated mainly with lower lymphocyte counts.

Table 3.12.1-11: Mouse oral 90-day study: Haematology

Sex	Male					Female				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
PCV (%)	41.7	43.8	42.7	42.8	38.2*	43.4	44.8	44.4	42.6	40.5**
Hb (g/dl)	13.7	14.4	14.0	14.0	12.2**	14.2	14.7	14.7	14.0	13.1**
RBC (1012/l)	8.80	9.05	9.08	8.60	7.71**	8.78	9.09	9.11	8.83	8.12**
MCV (fl)	47.4	48.4	47.1	49.8**	49.7**	49.5	49.3	48.7	48.3	49.9
MCHC (g/dl)	32.9	32.8	32.8	32.5	31.8**	32.8	32.7	33.1	33.0	32.3
WBC (109/l)	5.54	5.74	6.45	5.67	3.68*	3.16	3.31	3.82	3.80	3.43
Lymphocyte counts (10^9/l)	4.14	4.26	5.48	4.61	2.43*	2.60	2.64	3.32	3.30	2.97

PCV: Packed cell volume; RBC: Red blood cell count; MCV: Mean corpuscular volumes; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell count; Statistical significance: *p≤0.05; **p≤0.01

Clinical chemistry and urinalysis:

The main findings at the two high doses were reduced protein values and increased cholesterol values. The electrolyte values varied slightly from control values in males. Higher alkaline phosphate values were noted for a few individual males at 10000 ppm, but the group mean value was not statistically different from the control data and no histopathological findings could be associated with this effect.

In females, at the two high doses, marked ketonuria was found. There was no histopathological correlate to this finding and it was considered to be of unlikely toxicological importance. Lower urinary protein values were noted for both sexes, achieving statistical significance in females.

Table 3.12.1-12: Mouse oral 90-day study: Clinical chemistry and urinalysis

Sex	Male					Female				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Clinical chemistry										
Total protein (g/dl)	5.4	5.4	5.3	5.2	4.8**	5.4	5.3	5.2	5.4	5.1*
Albumin (g/dl)	2.9	2.8	2.9	2.6*	2.6**	3.2	3.1	3.1	3.0**	2.8**
Globulin (g/dl)	2.6	2.6	2.4	2.5	2.3*	2.2	2.2	2.1	2.4*	2.3*
K (mEq/l)	5.1	4.7	5.5	5.2	4.3*	4.5	4.5	4.5	4.6	4.6
Ca (mEq/l)	5.1	5.0	5.0	4.9*	4.7**	5.0	5.0	4.9	5.0	4.9*
P (mEq/l)	3.9	3.6	3.6	4.1	4.5*	4.0	3.9	3.6	4.1	4.1
Cl (mEq/l)	111	110	111	114**	115**	113	113	113	113	114
Cholesterol (mg/dl)	124	150	139	165**	165**	88	99	109	153**	183**
ALP (mU/ml)	90	65	93	61	140	117	105	108	97	88
Urinalysis										
Ketonuria (samples)	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	3/5	5/5
Protein (mg/dl)	668	1102	870	1018	298	199	201	133	101	49**

K: Potassium; Ca: Calcium; P: Phosphorus; Cl: Choride; ALP: Alkaline Phosphatase; Statistical significance: *p≤0.05; **p≤0.01

Pathological examinations:

Necroscopy: At 10000 ppm, a reduction in adipose tissue was noted in the majority of male mice compared to control mice (Table 3.12.1-13).
Organ weights: The body weight adjusted liver weights were dose related increased, attaining statistical significance at the two high doses. In females, the body weight adjusted thyroid weights were statistically significantly increased, too. A decrease of the absolute spleen weight occurred in both sexes at 10000 ppm.

Table 3.12.1-13: Mouse oral 90-day study: Absolute and relative[1] organ weights, necroscopy findings

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Organ weights										
Abs.liver wt.(g)	1,87	1,99 +6%	2,09 +12%	2,25 +20%	2,27 +21%	1,55	1,66 +7%	1,45 -6%	1,84 +19%	2,24 +45%
Adj. liver wt. (g)	1.82	1.88 +3%	1.98 +9%	2.29** +26%	2.51** +38%	1.55	1.55 +0%	1.46 -6%	1.89** +22%	2.29** +48%
Abs. thyroid wt.(mg)	4,5	5,1 +13%	5,0 +11%	4,6 +2%	4,7 +4%	4,0	4,2 +5%	4,0 +0%	4,7 +18%	4,8 +20%
Adj. thyroid wt.(mg)	[2]	[2]	[2]	[2]	[2]	4.0	4.0 +0%	4.0 +0%	4.8* +20%	4.9** +23%
Abs. spleen wt. (g)	0.123	0.120	0.122	0.124	0.082** -33%	0.142	0.147 +4%	0.107 -25%	0.128 -10%	0.104* -27%
Adj. spleen wt. (g) [2]										
Necroscopy findings										
Adipose tissue reduced	0/9	0/10	1/10	2/9	6/10	4/10	1/10	2/10	4/9	4/10

[1] Body weight adjusted; [2] Not adjusted as not appropriate; Statistical significance: *p≤0.05; **p≤0.01

Histopathology: Generally, from 3000 ppm, hepatocyte hypertrophy was seen. The light microscopic finding was confirmed by electron microscopy and was concomitant to the higher cholesterol values and relative liver weights. The findings in the spleen at 10000 ppm (increased incidence of hemosiderosis, decreased degree of extramedullary hemopoiesis, reduced cellularity of the marginal zone of the white pulp) were consistent with the slight anaemia. In males, the incidence and degree of involution/atrophy of the thymus were increased. In the small intestine, swelling and cytoplasmic rarefaction of villous epithelial cells were seen at high doses.

Table 3.12.1-14: Mouse oral 90-day study (Histopathology)

Sex	**Male**					**Female**				
Dose (ppm)	**0**	**50**	**400**	**3000**	**10000**	**0**	**50**	**400**	**3000**	**10000**
Liver										
Hepatocyte hypertrophy										
- generalised	0/9	0/10	0/10	1/9	2/10	0/10	0/10	0/10	0/10	0/10
- centrilobular midzonal	0/9	0/10	0/10	1/9	8/10**	0/10	0/10	0/10	0/10	0/10
- centrilobular	0/9	0/10	0/10	3/9	0/10	0/10	0/10	0/10	0/10	0/10
- periportal	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	9/9**	10/10**
Spleen										
Reduced cellularity of the white pulp – marginal zone										
- Total	1/9	3/10	3/10	6/9	9/10**	1/10	1/10	1/10	2/9	2/10
- Slight	1/9	3/10	2/10	5/9	1/10	1/10	0/10	1/10	1/9	0/10
- Moderate	0/9	0/10	1/10	1/9	8/10*	0/10	1/10	0/10	1/9	2/10
Extramedullary hemopoiesis										
- Total	9/9	10/10	10/10	9/9	10/10	10/10	10/10	10/10	9/9	10/10
- Minimal	0/9	0/10	0/10	0/9	4/10*	0/10	0/10	0/10	0/9	2/10
- Slight	4/9	6/10	7/10	3/9	4/10	5/10	6/10	8/10	5/9	8/10
- Moderate	5/9	4/10	3/10	6/9	2/10	5/10	3/10	2/10	3/9	0/10
- Marked	0/9	0/10	0/10	0/9	0/10	0/10	1/10	0/10	1/9	0/10
Hemosiderosis										
- Total	3/9	5/10	4/10	4/9	10/10**	7/10	9/10	9/10	8/9	9/10
- Minimal	1/9	3/10	2/10	2/9	2/10	2/10	2/10	2/10	3/9	0/10
- Slight	2/9	2/10	2/10	2/9	8/10*	5/10	6/10	5/10	4/9	5/10
- Moderate	0/9	0/10	0/10	0/9	0/10	0/10	1/10	2/10	1/9	4/10*
Thymus										
Involution/atrophy										
- Total	5/9	4/10	6/9	7/8	9/9	8/10	0/10	0/10	0/10	5/10
- Minimal	4/9	1/10	5/9	4/8	3/9	5/10	0/10	0/10	0/10	4/10
- Slight	0/9	3/10	0/9	2/8	4/9	3/10	0/10	0/10	0/10	1/10
- Moderate	1/9	0/10	1/9	1/8	2/9	0/10	0/10	0/10	0/10	0/10
Duodenum										
Villous epithelial cells swollen with cytoplasmic rarefaction	0/9	0/10	0/10	6/9**	10/10**	0/10	0/10	0/10	3/9	9/10**
Jejunum										
Villous epithelial cells swollen with cytoplasmic rarefaction	0/9	0/10	0/10	0/9	4/10	0/10	0/10	0/10	0/9	4/10*

Statistical significance: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$

Conclusion:
The NOAEL (NOEL) was at 400 ppm (70.5 mg/kg bw/d) based on decreased body weight gain, increased cholesterol, increased organ weights of liver and thyroid and hepatocyte hypertrophy swelling.

3.12.1.6 Anonymous (1997b)

Reference: Toxicity to Dogs by Repeated Oral Administration for 13 Weeks
Author(s), year: Anonymous, 1997
Report/Doc. number: 73 PXA (TOX2001-288) / TON '4/970936
Guideline(s): OECD 409 (1981)
GLP: Yes
Deviations from OECD 409 (1998): Yes (Haematology: no haematocrit measurements; Clinical chemistry: no ornithine decarboxylase measurements).
Acceptability: Yes

Material and Methods:
Test material: Pethoxamid; Batch: TB-960306: Purity: 95.0%.
Test animals: Four male and four female pure bred beagle dogs, 18-15 weeks old, 6.4-9.7 kg of weight, from Huntington Life Sciences supplied by Interfauna UK Limited.
Based on a preliminary study (Report no. TON 2/960653), the dogs were administered pethoxamid by oral capsule at dosages of 0, 8, 50 and 300 mg/kg bw/d for 13 weeks. Due to the decline in the clinical condition of the animals receiving 300 mg/kg bw/d, treatment stopped on Day 4 of Week 2 and following a recovery period of 4 days, dosing was at 200 mg/kg bw/d for the remainder of the study.

Results:
Mortality, clinical and ophthalmologic findings:
There were no treatment-related mortalities.
From 8 mg/kg bw/d, a slightly higher - but dose-related - incidence of post-dosing liquid faeces was noted. This is seen commonly in control dogs, too. Although this finding is considered to be of minor importance, it is associated with treatment. Additional symptoms at the middle and high dose were salivation and vomiting. No treatment-related ophthalmologic effects were evident.

Body weight, body weight gain and food consumption:
At the middle dose, lower weight gain and at the high dose, weight loss (all animals) occurred in Week 1. According to the significant lower food intake at the high dose, the treatment resulted in significant lower mean body weight gains throughout the whole study.

Haematology:
At 300/200 mg/kg bw/d, at both measure times in Week 6 and in Week 12, all animals showed higher platelet and reticulocyte values (significant only in Week 12) and a significantly lower haemoglobin concentration. In Week 12, macrocytosis and hypochromasia were evident in males and in both sexes lower red blood cell packed cell volume counts. The haematological findings were indicating a slight anaemia.

Clinical chemistry and urinalysis:
At the high dose in both Weeks 6 and 12, statistically lower mean albumin and ALT values were evident for both sexes and lower protein levels in Week 6. Significantly lower phosphorus values were noted for males in Weeks 6 and 12 and for females in Week 6 only. This is considered likely to be associated with the clinical conditions of the dogs and therefore not a direct effect of treatment.
At the high dose, abnormal urine colouration and in individual males higher urinary protein levels were noted in Weeks 6 and 12. However, no histopathological findings were found in the kidneys.

Pathological examinations:
Necroscopy: No treatment-related effects were evident.
Organ weights: At the high dose, the following changes in organ weights compared to the control values were observed for both sexes: a lower absolute heart (23%), lung (ca. 20%) and spleen weight (for males 24%, for females 35%), a higher absolute (ca 15%) and body weight adjusted (22%) adrenal weight as well as a higher body weight adjusted liver (19%) and pancreas weight (for males 24%, for females 59%). For males only were observed: a lower absolute brain (7%), prostate (61%) and gonad weight (4%) as well as a higher body weight adjusted kidney weight (39%). The lower organ weights reflect the weight loss at this dose.
Histopathology: The histopathological findings which occurred in other doses than the high dose are summarized in the table below. The vacuolation of the cortical tubules at 8 mg/kg bw/d was only of trace nature. At any dose, this lesion had no inflammatory component and there was no evidence of a progressive degeneration. Because vacuolation is a normal physiological finding in the dog, primarily in the female, the increased incidence of this finding was considered of no toxicological significance. The involution of the thymus which was found in the majority of the animals at the high dose is commonly encountered in animals which show poor condition and/or a dramatic effect on body weight gain. Only at the high dose, both sexes showed glycogen depletion in the liver (8/8) and myeloid atrophy in the bone marrow (7/8). Males showed reduced lymphoid cellularity in the lymph nodes (2/4), immaturity of prostate (3/4) and testes (2/4) with an absence of spermatozoa in the epididymides (2/4) and diffuse vacuolation of the zonae fasciculata and reticularis in adrenals (4/4). These effects were considered to be related to the poor clinical condition of the dogs.

Table 3.2.1-15: Overview of 90-day toxicity in dogs treated orally with pethoxamid

Sex	**Male**				**Female**			
Dose (mg/kg bw/d)	**0**	**8**	**50**	**300/200**	**0**	**8**	**50**	**300/200**
Clinical signs								
Liquid faeces	1	6	19	22	0	6	17	23
Salivation	0	0	2	14	0	0	0	20
Vomiting	0	0	0	1	0	1	0	7
Body weight, term. kill	11.7	11.5	11.2	8.8	11.2	11.1	11.2	9.3
Body weight gain								
Week 0-1 (kg)	0.4	0.4	0.2	-0.3**	0.4	0.4	0.2	-0.8**
Week 0-13 (kg)	3.7	3.4	2.7	0.9**	3.2	3.0	3.1	1.3**
Food consumption								
Week 1 (g/dog/week)	2675	2535	2580	2265**	2685	2585	2618	1440**
Week 3-13 (g/dog/week)	2782	2780	2690	2603	2746	2758	2753	2516**
Haematology (Week 12)								
Platelets (10^9/l)	342	368	322	457**	355	311	394	488*
Reticulocytes (%)	0.2	0.4	0.5	0.9*	0.2	0.5	0.3	1.1*
MCHC (g/dl)	32.5	32.8	32.8	31.4*	32.6	32.6	32.7	32.1
Hb (g/dl)	12.9	12.5	12.2	11.0*	14.1	12.6	13.2	12.3*
PCV (%)	39.6	38.1	37.1	35.2	43.3	38.5	40.3	38.3*
RBC (10^{12}/l)	5.49	5.41	5.17	4.63*	6.08	5.39*	5.57*	5.21**
Urinalysis (Week 12)								
Abnormal urine colouration	0/4	0/4	0/4	2/4	0/4	0/4	1/4	2/4
Higher protein levels	0/4	0/4	0/4	2/4	0/4	0/4	0/4	0/4
Organ weights								
Abs. liver weight	395.6	415.5 +5%	417.1 +5%	355.6 -10%	364.1	368.7 +1%	384.3 +6%	369.9 +2%
Adj. liver weight[1)]	361.6	388.1 +7%	403.9 +12%	430.2 +19%	348.2	357.4 +3%	368.4 +6%	413.0 +19%
Abs. thyroid weight[2)]	0.70	0.84	0.92	0.75	0.73	0.74	0.78	0.66
Abs. heart weight	82.4	83.4	82.7	63.0	85.6	81.6	77.3	66.0
Adj. heart weight[1)]	76.9	78.9	80.5	75.2	83.0	79.7	74.7	73.1
Abs. lung weight	106.7	103.9	100.2	83.6	103.1	100.3	94.3	88.3
Adj. lung weight[1)]	101.6	99.8	98.2	94.8	100.2	98.1	92.5	94.8
Abs. spleen weight	71.1	76.3	74.3	54.0	63.3	70.0	71.3	71.2
Adj. spleen weight[1)]	88.8	95.9	77.8	57.2*	2)			
Abs. adrenal weight	1.22	1.19	1.17	1.42*	1.24	1.20	1.22	1.36

Adj.adrenal weight[1)]	2)				1.21	1.18	1.19	1.44*
Abs. pancreas weight	28.2	27.5	25.4	24.5	23.2	27.5	25.9	26.6
Adj. pancreas weight[1)]	25.1	25.0	24.2	31.2	20.8	25.8	23.6	33.0
Abs. brain weight	82.6	78.6	79.4	76.7**	75.2	75.4	77.0	74.4
Adj. brain weight[1)]	2)				74.3	74.8	76.1	76.7
Abs. prostate weight[2)]	3.04	2.79	5.37	1.18	-	-	-	-
Abs. testes+epid. weight[2)]	20.55	17.81	19.95	13.22**	-	-	-	-
Abs. kidney weight	47.6	51.4	50.8	54.6	47.6	48.8	51.2	50.7
Adj.kidney weight[1)]	44.4	48.9	49.6	61.6*	[2)]			
Histopathology								
Kidney								
Vacuolation cort. tubules	2/4	4/4	4/4	4/4	2/4	4/4	3/4	4/4
Thymus								
– Involution (Total)	0/4	0/4	1/4	3/4	0/4	0/4	2/4	3/4
Spleen								
– Minimal hemosiderosis	1/4	0/4	1/4	2/4	1/4	0/4	1/4	3/4

MCHC: Mean corpuscular hemoglobin concentration; Hb: Hemoglobin; PCV: Packed cell volume; RBC: Red blood cell count; Statistical significance: $^{*}p\leq0.05$; $^{**}p\leq0.01$
[1] Body weight adjusted; [2] Relative organ weight not adjusted as not appropriate

Conclusion:
The alteration of the dosage level from 300 mg/kg bw/d to 200 mg/kg bw/d was not considered to have unduly affected the assessment/interpretation of the data obtained. The effects indicative of anaemia, the lower organ weights and the histopathological findings on adrenal, prostate and gonad at this dose range were characteristic for the poor condition of the dogs.
The NOAEL was set at 8 mg/kg bw/d; based on post dose liquid faeces together with a decreased body weight gain in males at the higher dose of 50 mg/kg bw/d.

3.12.1.7 Anonymous (1999)

Reference: A 12-Month Chronic Toxicity Study of TKC-94 Administered Orally to Beagles
Author(s), year: Anonymous. 1999
Report/Doc. number: 74 PXA (TOX2001-289)/ SBL '98-08
Guideline(s): OECD 452 (1981)
GLP: Yes
Deviations from OECD 4452 (2009): No
Acceptability: Yes

Material and Methods:

Test material: Pethoxamid, Batch number: TB-960306, Purity: 95.0%.

Test animals: Four groups of four male and four female beagle dogs from Shin Nippon Biomedical Laboratories, Ltd., 7.2 to 10.0 kg (male), 5.9 to 8.4 kg (females).

The dogs were administered pethoxamid by oral capsule at dosages of 0, 2, 20 and 150 mg/kg bw/d for 12 months.

Results:

Mortality, clinical and ophthalmologic findings:
At 150 mg/kg bw/d, one male and one female were sacrificed due to moribundity on Day 322 (week 47) and 268 (week 38) respectively, preceded by loss of appetite, bloody stool, salivation, dehydration signs, bradypnea, hypothermia, pale oral mucosa, pale conjunctiva, emaciation, decrease in spontaneous activity, prone and lateral position.
At 20 mg/kg bw/d and for the surviving animals at 150 mg/kg bw/d, increases in the frequency of soft stool and diarrhoea were observed throuout the study period. No treatment-related ophthalmologic effects were observed.

Body weight, body weight gain and food consumption:
At the high dose, the body weights were decreased (Table 3.12.1-16, sacrificed animals excluded). The final body weights of the sacrificed animals were 52 (male) and 64% (female) of their maximum values. Concerning the food consumption, no treatment-related effects were recorded for the surviving animals.

Table 3.12.1-16: 1-year oral dog study: Mortality, clinical signs and terminal body weight

Sex	**Male**				**Female**			
Dose (mg/kg bw/day)	0	2	20	150	0	2	20	150
Mortality	0/4	0/4	0/4	1/4	0/4	0/4	0/4	1/4
Clinical signs[1]								
Soft stool	+	+	++	+++	+	+	++	+++
Diarrhoea	–	–	+	++	–	–	+	++
Vomiting	+	+	+	++	+	+	+	++
Bodyweight[1]								
Week 54 (kg)	14.18	13.08	13.05	13.00	12.83	12.33	12.85	10.83*

[1] Sacrificed animals not included;
Frequency and severity of occurrence:- Normal, + Slight, ++ Moderate, +++ Severe;
Statistical significance: *$p<0.05$

Haematology:
Many haematological changes were observed for the two sacrificed animals, but these were considered to be related to a nutritional disorder caused by anorexia.
For the surviving animals of this dose group, a slight hypochromasia was noted for 2 males and 1 female at Week 39, and all 3 males and 2 females at Week 53.

Clinical chemistry and urinalysis:
For the sacrificed animals, high values of alkaline phosphatase, AST (male, week 39: 673% of pre-dosing value), ALT (male, week 39: 1439% of pre-dosing value), γ-glutamyl transferase, total bilirubin, total cholesterol, triglyceride and/or blood urea nitrogen were noted. These changes were considered to be related to the disorders of the gastrointestinal tract, kidney and/or liver observed on the histopathology examination. Activity increases in alkaline phosphatase were seen from 20 mg/kg bw/day at different occasions (Table 3.12.1-17).

Urinalysis:
The decreases in urinary excretion of potassium and chloride at the high dose were due to the loss of electrolytes caused by diarrhoea.

Table 3.12.1-17: 1-year oral dog study: Clinical chemistry and urinalysis

Sex	**Male**				**Female**			
Dose (mg/kg bw/day)	**0**	**2**	**20**	**150**	**0**	**2**	**20**	**150**
Clinical chemistry								
ALP (IU/l) Week 26	100.8	110.5	136.0	184.8	93.5	102.0	145.3	218.5*
Week 39	100.8	110.5	151.5	222.3*	100.0	106.5	161.8	218.3*1
Week 53	107.8	114.8	147.0	176.3[1]	104.3	112.0	165.0	261.3*1
Urinalysis								
Potassium (mEq) Week 13	43.0	43.5	41.0	23.5*	41.3	37.8	40.8	32.3
Week 26	37.3	50.3	29.8	13.0	43.3	38.5	45.8	17.8*
Chloride (mEq) Week 13	30.5	31.8	34.3	9.5**	28.0	26.3	34.3	24.5
Week 26	17.0	24.5	20.3	10.0	23.0	24.0	26.8	6.0*
Week 39	19.3	32.0**	38.8**	8.0*	25.3	26.0	20.3	8.7[1]
Week 53	18.0	27.0	23.8	13.0[1]	26.0	18.3	26.0	17.3[1]

[1] Surviving animals;
ALP: Alkaline Phosphatase;
Statistical significance: *p<0.05; **p<0.01

Pathological examinations:
Necroscopy: For the unscheduled sacrificed animals atrophy of the spleen, pancreas, thymus and bilateral testes, enlargement of the bilateral adrenals and accentuated lobular pattern in the liver were observed.
No abnormal changes were observed for the surviving animals.

Organ weights: Compared to the control values, the organ weight increases in females were seen at the mid and high dose for the liver (absolute 44 and 41%, relative 43 and 67% respectively) and for the kidneys (absolute 18 and 34%, relative 18 and 59% respectively; not statistically significant at the mid dose). In males, a significant increase of the liver weights (absolute 28%, relative 38%) was only seen at the high dose.
For the sacrificed animals a high absolute weight of the bilateral adrenals, and low absolute weights of the spleen, testes, bilateral epididymis, heart, lung and unilateral submandibular gland for the male and low absolute weights of the spleen, thymus, heart and bilateral submandibular glands for the female were noted.

Histopathology: The main findings considered substance related concerned the gastrointestinal tract. For the unscheduled sacrificed animals were found: Vacuolation, atrophy, and mononuclear cell infiltration in the muscle layer of the small and large intestines; in the stomach atrophy of the chief cells in the fundus and in the mucosal epithelium in the pylorus, and only in the male swelling of the parietal cells in the fundus; erosion and inflammatory cell infiltration of the mucosa in the oesophagus.
For the kidney, vacuolation and regeneration of the tubular epithelium were noted for both animals, and necrosis and black-blue pigment in the tubular epithelium, debris and hemorrhage in the tubule were noted for the male. The vacuolation in the tubular epithelium was severe. Focal necrosis and foamy change in the hepatocytes, and brown pigment in the hepatocytes and Kupffer cells of the liver were noted for the male. Further changes were considered to be related to the nutritional disorder caused by anorexia.
From the surviving dogs of the high dose group, two males showed very slight to slight findings in the large intestines and one of these dogs additionally findings in stomach and small intestine.

Table 3.12.1-18: 1-year oral dog study: Organ Weights and Histopathology[1,2]

Sex	**Male**				**Female**			
Dose (mg/kg bw/day)	**0**	**2**	**20**	**150**	**0**	**2**	**20**	**150**
Organ weights								
Abs. liver weight (g)	372	338	353	476** +28%	273	290 +6%	392* +44%	386* +41%
Rel. liver weight (g/kg)	26.5	26.0	27.4	36.6** +38%	21.3	23.5 +10%	30.4** +43%	35.7** +67%
Abs. kidney weight (g)	58	54	51	64 +10%	46	47 +2%	55 +18%	62** +34%
Rel. kidney weight (g/kg)	4.2	4.2	3.9	5.0 +19%	3.6	3.8 +6%	4.3 +18%	5.8** +59%
Stomach (pylorus)								
Mucosa, micronecrosis	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Mucosa, epithel. hyperplasia	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Vacuolation and mononuclear cell infiltration in muscle layer	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Small intestine								
Vacuolation and mononuclear cell infiltration in muscle layer	0/4	0/4	0/4	1/3	0/4	0/4	0/4	0/3
Large intestine								
Vacuolation and mononuclear cell infiltration in muscle layer	0/4	0/4	0/4	2/3	0/4	0/4	0/4	0/3

[1] Sacrificed animals were not included in this table;
[2] All findings very slight to slight;
Statistical significance: *p<0.05; **p<0.01.

Conclusion:

In this study, the target organ identified was the gastrointestinal tract. The NOAEL in this study was at 2 mg/kg bw/d based on increased absolute and relative liver weight in females and slightly increased frequency of diarrhoea.

3.12.1.8 Anonymous (2000a)

See section 3.9.1

3.12.1.9 Anonymous (2000b)

See section 3.9.1

3.12.1.10 Anonymous (2014d)

Reference: A 28-day dermal toxicity study of pethoxamid in rats

Author(s), year: Anonymous, (2014d)
Report/Doc. number: 1216 PXA / Charles Rivers, Testing Facility Study Number 20039159
Guideline(s): OECD 410 (1981)
GLP: Yes
Deviations from OECD 410 (1981): No
Acceptability: Yes

EXECUTIVE SUMMARY

Rats (Crl:CD (SD)) were administered pethoxamid or the control substance once daily for a 6-hour exposure period for 28 consecutive days at dose levels of 0, 100, 300 or 1000 mg/kg bw/day. The following parameters and end points were evaluated in this study: viability, clinical signs, dermal scoring, body weights, body weight changes, food consumption, ophthalmic examinations, clinical pathology evaluations, necropsy examinations, organ weights and histopathological evaluations.

Dermal exposure to pethoxamid at dose levels as high as 1000 mg/kg bw/day for 28 consecutive days did not result in any adverse clinical signs, differences in body weights, feed consumption values, any of the variables evaluated during the detailed clinical observations or the ophthalmologic examination performed at the end of the exposure period for both male and female rats. There was a statistically significant increase in the number of male rats at 1000 mg/kg bw/day observed with slight erythema and flaking compared with the control group. Slight erythema and flaking were observed in females at all exposure levels (including controls). No test substance-related or adverse changes were observed in haematology or clinical chemistry. At necropsy, there were no test substance-related adverse gross lesions noted. Both absolute and relative liver weights were increased in the high dose males by 9%, reaching statistical significance only for the relative liver weights. As these findings were not accompanied by clinical chemistry changes or histopathological findings, they were not considered adverse.

All of the microscopic alterations apparent during the histopathological evaluation of the study were considered to represent common background lesions that were not relevant to dermal exposure to pethoxamid. Decreased numbers of anagen-phase follicles with some associated minimal to mild hyperkeratosis were noted within sections of treated skin from the male and female rats in the 1000 mg/kg bw/day dose group, but these findings were considered to most likely be due to treatment-related localized irritation and/or increased grooming behaviour.

In conclusion, repeated dermal exposure to pethoxamid resulted in some localized skin irritation (at the site of exposure) in male rats at 1000 mg/kg bw/day and in female rats at all dose levels (including controls). The no-observed-adverse-effect-level (NOAEL) for systemic toxicity produced by dermal exposure to pethoxamid for 28 days is considered to be 1000 mg/kg bw/day for male and female rats.

MATERIALS AND METHODS

Materials:

Test material:	Pethoxamid technical
Lot/batch number:	P1351-JaK-T2-23-6
Purity:	95.80% (w/w) (dose calculation was not adjusted to take account of purity)
Stability of test item:	06 January 2014 (stored at ambient temperature) *NB: stable during the conduct of the study*
Storage conditions:	At room temperature, protected from light

Study Design:

The test material was applied to the shaved back of male and female Crl:CD(SD) rats (10/sex/group) at dose levels of 0, 100, 300 or 1000 mg/kg bw/day for a 6-hour exposure period for 28 days. The test material was applied neat and was covered with a semi-occlusive wrap. After 6 hours, the wrap was removed and the site was washed.

RESULTS AND DISCUSSION

Mortality: No mortalities occurred.

Clinical signs: No treatment-related findings were observed.

Skin reactions: 1000 mg/kg bw/day: A statistically significant increase in the number of male rats with grade 1 erythema and flaking was observed.
Body weight gain: No treatment-related findings were observed.

Food consumption: No treatment-related findings were observed.

Haematology: No treatment-related findings were observed.

Clinical Chemistry: No treatment-related findings were observed.

Ophthalmology: No treatment-related findings were observed.

Organ Weights: 1000 mg/kg bw/day: An increase in absolute and relative liver weight was observed in males, being statistically significant only for the relative liver weight. This finding was not accompanied by clinical chemistry findings or liver histopathology; therefore it was not considered adverse.

Macroscopic findings: No treatment-related findings were observed.

Microscopic findings: No treatment-related findings were observed.

Table 3.12.1-19: Summary of findings from 28-day dermal toxicity study in rats

Parameter	**Males**			
	Dose Level (mg/kg bw/day)			
	0	**100**	**300**	**1000**
Mortality	0	0	0	0
Body Weight (g)				
Day 1	302.3	302.7	302.9	306.3
Day 28	407.7	412.1	406.3	410.8
Body Weight Gain (g)				
Days 1 to 28	105.4	109.4	103.4	104.5
Food Consumption				
Absolute Food Consumption (g/day)				
Days 1 to 28	27.2	26.9	27.3	26.6
Relative Food Consumption (g/kg/day)				
Day 1 to 28	74.8	73.8	75.4	73.4
Skin findings				
Residue[1]	0/0[2]	52/9**	51/10**	55/8**
Erythema, grade 1	8/3	0/0	3/3	21/7*
Flaking, grade 1	6/2	0/0	0/0	21/6**

Back: Scab(s)	0/0	0/0	0/0	45/4**
Ophthalmology findings	No treatment-related effects			
Haematology	No treatment-related effects			
Clinical chemistry	No treatment-related effects			
Organ weights (g)				
Liver (absolute)	10.91	11.08 (+ 2%)	11.12 (+ 2%)	11.9 (+ 9%)
Liver (relative)	2.842	2.851 (+ 0%)	2.898 (+ 2%)	3.100* (+ 9%)
Necropsy findings	No treatment-related findings			
Microscopic findings	No treatment-related findings			
Parameter	Females			
	Dose Level (mg/kg bw/day)			
	0	100	300	1000
Mortality	0	0	0	0
Body Weight (g)				
Day 1	226.9	226.9	226.5	228.6
Day 28	257.3	259.8	257.0	265.6
Body Weight Gain (g)				
Days 1 to 28	30.4	32.9	30.5	37.0
Food Consumption				
Absolute Food Consumption (g/day)				
Days 1 to 28	19.0	18.6	18.4	19.2
Relative Food Consumption (g/kg/day)				
Days 1 to 28	78.2	75.6	75.9	77.6
Skin findings				
Residue[1]	0/0[2]	109/9**	179/10**	164/10**
Erythema, grade 1	63/8	16/5	5/3	14/4
Erythema, grade 2	12/2	5/1	0/0	0/0
Flaking, grade 1	56/7	14/2**	4/1**	20/5
Flaking, grade 2	2/1	0/0	0/0	0/0
Back: Scab(s)	47/5	13/3	11/2	57/7
Ophthalmology findings	No treatment-related effects			
Haematology	No treatment-related effects			
Clinical chemistry	No treatment-related effects			
Organ weights	No treatment-related effects			
Necropsy findings	No treatment-related findings			
Microscopic findings	No treatment-related findings			

[1] Presumed to be test material residue that couldn't be washed off.
[2] Number of observations/number of animals. *p<0.05; **p<0.01.

CONCLUSION

In conclusion, repeated dermal exposure to pethoxamid resulted in some localized skin irritation (at the site of exposure) in male rats at 1000 mg/kg bw/day and in female rats at all dose levels (including controls). The no-observed-adverse-effect-level (NOAEL) for systemic toxicity produced by dermal exposure to pethoxamid for 28 days is considered to be 1000 mg/kg bw/day for male and female rats.

3.12.2 Human data

No relevant studies.

3.12.3 Other data

No relevant studies.

3.13 Aspiration hazard

3.13.1 Animal data

No relevant studies.

3.13.2 Human data

No relevant studies.

3.13.3 Other data

No relevant studies.

4 ENVIRONMENTAL HAZARDS

4.1 Degradation

4.1.1 Ready biodegradability

4.1.1.1 144 PXA (1999)

Reference: TKC-94 Assessment of ready biodegradability: modified sturm test
Author(s), year: Anonymous, 1999
Report/Doc. number: TON 044/984510, 144 PXA
Guideline(s): OECD Test Guideline 301, 1992; US EPA OPPTS 835.3110, 1998
GLP: Yes
Deviations: No
Acceptability: Yes

Materials:

Lot/batch number:	TB-9960306-C
Purity:	94.8 %

The study was conducted to a current test method (OECD Test Guideline 301, 1992) and no major study deficiencies were identified. The data in 144 PXA (1999) indicated that pethoxamid is not readily biodegradable. Therefore, re-analysis was not considered necessary and the following EU endpoint is proposed: Pethoxamid is not readily biodegradable.

4.1.2 BOD_5/COD

No relevant studies.

4.1.3 Hydrolysis

4.1.3.1 42 PXA (1999)

Reference: TKC-94 Hydrolysis under Laboratory Conditions
Author(s), year: Anonymous, 1999
Report/Doc. number: TON 023/983760, WAS2001-56, 42 PXA
Guideline(s): US EPA, N, 161-1, 1982; SETAC, 1995
GLP: Yes
Deviations: No
Acceptability: Yes

Materials:

Lot/batch number:	TP-960418A
Purity:	99.9 %

Although the study was not conducted to the current test method (OECD Test Guideline 111, 2004), no major study deficiencies were identified. Data in 42 PXA (1999) indicate that pethoxamid is stable to hydrolysis at all pH values investigated. Therefore, re-analysis was not considered necessary and the following EU endpoints are proposed: Pethoxamid is stable under conditions of abiotic hydrolysis at pH 4, 7 and 9 (buffer solutions, 50 °C and sterile conditions).

4.1.4 Water, water-sediment and soil degradation studies

4.1.4.1 1443 PXA (2015)

Reference: Aerobic Mineralization of [^{14}C]Pethoxamid in Surface Water
Author(s), year: Anonymous, 2015
Report/Doc. number: 2517W-1, 1443 PXA
Guideline(s): OECD Test Guideline 309, 2004
GLP: Yes
Deviations: No
Acceptability: Yes

Executive Summary:

An aerobic mineralisation study was conducted with [phenyl-^{14}C]pethoxamid using aerobic surface water (pelagic test). Pethoxamid was applied at two concentrations, 102.3 µg / L (high dose) and 10.3 µg / L (low dose) and the samples were incubated in the dark under aerobic conditions at 20 °C. In addition, reference and sterile control samples were incubated to confirm the microbial activity of the test water and examine possible abiotic degradation, respectively. The test was performed in flow-through systems allowing humidified air to pass over the sample headspace and through traps to collect volatile organic components (foam bung) and $^{14}CO_2$ (NaOH). Sample aliquots were taken at eight time

points throughout the study and analysed by LSC. The distribution of radioactivity between pethoxamid and its degradates was determined in the high dose samples (only) by HPLC. Mass balance averaged 95.2 % AR and 94.9 % AR for the high and low dose samples, respectively. At the end of the study $^{14}CO_2$ averaged 5.7 % AR and 4.9 % AR for the high and low dose samples, respectively, demonstrating that mineralisation of pethoxamid was not dose dependent. Pethoxamid degraded moderately under the conditions of the test and represented an average of 57.3 % AR at the end of the study. The main metabolite observed was MET-30 (a cysteine conjugate), which represented an average of 19.6 % AR at the end of the incubation period. The degradation rate of pethoxamid was determined with the Hockey Stick (HS) model and the half-life was 138 days.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid
Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide
Lot/Batch #: CFQ41685
Specific Activity: 29 mCi/mmol
Radiochemical purity: 96.3 %
2. Test systems: One US natural surface water characterised by AGVISE laboratories

Table 4.1.4-1: Physical and chemical properties of the water used

Water	pH	Hardness (mg $CaCO_3$ / L)	Conductivity (mmhos / cm)	Dissolved Solids (ppm)	Turbidity (NTU)	Ca (ppm)	Mg (ppm)	Na (ppm)
Lake Tuckahoe	7.5	68	0.21	56	1.06	16	6.4	7.5

Study Design:

1. Experimental conditions

The aerobic mineralisation of [^{14}C]pethoxamid was studied in lake water (pelagic test). Pethoxamid was applied at the rate of 102.3 µg / L in three aqueous samples (high dose samples) and 10.3 µg / L in three aqueous samples (low dose samples). Two additional samples were dosed with 11.3 µg / L [^{14}C]benzoic acid as positive controls. One final sample was prepared using lake water sterilised by autoclave, dosed at a rate of 20.5 µg / L as an abiotic control. The water was dispensed into amber glass flasks and traps (polyurethane foam bungs and sodium hydroxide) were attached to the outlet of each treated flask (except the abiotic controls) to trap volatiles. Solutions of the test substance in acetonitrile were prepared, diluted and dispensed into each unit. The organic solvent added was equivalent to 0.05 % (high concentration) or 0.005 % (low concentration) by volume. Following treatment, each flask was incubated at 20 °C and stirred continuously throughout the course of the study. Humidified air was pulled over the samples and through the traps. The kinetic modelling followed the guidance of FOCUS kinetics employing the software tool for kinetic evaluation KinGUI (v.2).

2. Sampling

Sub-samples from each test system were removed at each sampling interval through a sampling port. Sampling for the high and low dose samples was conducted at zero time and the following intervals; 7, 13, 21, 28, 67 and 102 days. Additional water samples were also removed and analysed for entrained CO_2 by acidification. [^{14}C]benzoic acid dosed vessels were sampled after 13 and 21 days of incubation. Sterile vessels were sampled after 13 days incubation. At 49, 67 and 102 day sampling occasions, the dissolved oxygen (DO) of the water and the pH of the high and low dose samples were recorded. NaOH traps were analysed at each sampling occasion. Foam bung traps were analysed at the end of the incubation.

3. Description of analytical procedures

Aliquots of aqueous samples (and NaOH traps) were quantified by LSC. Foam bung traps were extracted with acetonitrile prior to sampling for LSC. High dose samples (only) were further analysed by HPLC by direct injection with co-chromatography with reference standard solutions. Peak assignments for parent and degradates were based on co-elution with reference standards. Metabolite confirmation was performed by LC/MS analysis of selected samples.

Results and Discussion:

Results of the [^{14}C]benzoic acid dosed vessels at 13 and 21 days show that the system was viable during the conduct of the study. Dissolved oxygen measurements indicate that the system remained aerobic throughout the study period (6.31 – 7.84 ppm). pH measurements ranged from 7.31 to 8.54 and averaged 7.74. The stability and homogeneity of the test substance under conditions of administration were confirmed by LSC and HPLC analysis. Total mass balances for the high dose and low dose samples averaged 95.2 % AR and 94.9 % AR, respectively. Mass balance for the sterile vessel was 97.5 % AR. Volatile compounds accounted for < 6.3 % AR for all samples tested. Results of acidified samples showed < 2 % AR in the system was attributable to dissolved CO_2. The distribution of radioactivity between pethoxamid and its degradates was determined in the high dose samples only. Pethoxamid represented an average of 57.3 % AR at the end of the test. An unknown metabolite (U-1) was observed in the course of the study (max. 19.6 % AR at the end of the test). LC/MS/MS analysis of a representative aqueous sample identified the metabolite as MET-30 (a cysteine conjugate of pethoxamid), and its identity was confirmed by co-chromatography with an authentic MET-30 reference standard by HPLC. Another minor unknown metabolite (U-2) was observed at a maximum of 4.2 % AR after 102 days. At the end of the study $^{14}CO_2$ averaged 5.7 % AR and 4.9 % AR for the high and low dose samples, respectively, demonstrating that mineralisation of pethoxamid was not dose dependent.

Table 4.1.4-2: Mass balance of [^{14}C]pethoxamid expressed as % AR for low dose samples

% of AR	Incubation Time (days)							
	0	**7**	**13**	**21**	**28**	**49**	**67**	**102**
Water	96.1	96.7	94.7	93.0	91.1	89.3	90.6	89.1
NaOH	n.a.	0.4	1.0	1.8	2.4	3.6	4.1	4.9
Foam Plug	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.0
Total	96.1	97.0	95.8	94.8	93.5	92.9	94.7	94.0

Table 4.1.4-3: Mass balance of [^{14}C]pethoxamid expressed as % AR for high dose samples

% of AR	Incubation Time (days)							
	0	**7**	**13**	**21**	**28**	**49**	**67**	**102**
Water	95.2	96.0	95.6	92.0	92.3	92.1	89.1	89.5
NaOH	n.a.	0.3	1.2	1.9	2.4	3.7	4.5	5.7
Foam Plug	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.0
Total	95.2	96.4	96.8	93.9	94.7	95.8	93.6	95.2

Table 4.1.4-4: Product balance of [^{14}C]pethoxamid expressed as % AR for high dose samples (metabolites > 5 % shaded in grey)

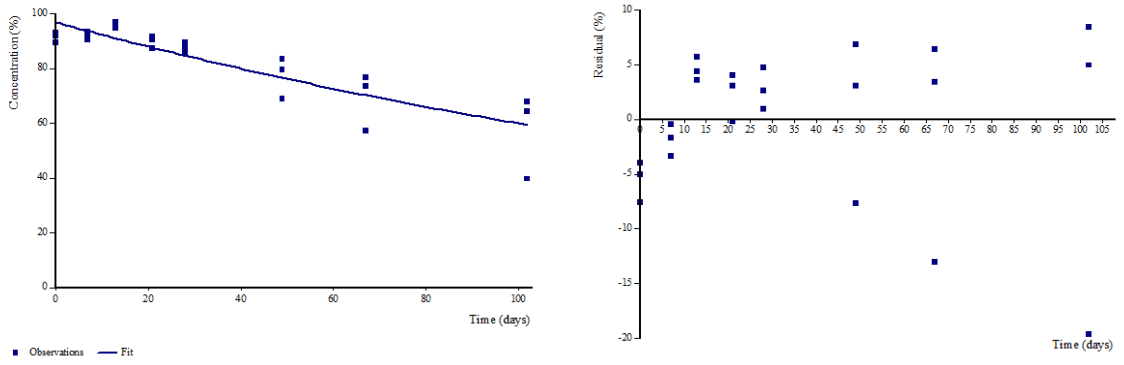
% of AR	Incubation Time (days)							
	0	**7**	**13**	**21**	**28**	**49**	**67**	**102**
Pethoxamid	91.3	91.8	95.6	89.9	87.4	77.3	69.1	57.3
MET-30	0.0	0.0	0.0	0.9	2.9	8.8	11.9	19.6

U-2	0.0	0.0	0.0	0.0	0.0	2.6	2.5	4.2
Others[a]	3.8	4.2	0.0	1.2	2.0	3.4	5.6	8.4
CO_2	NA	0.3	1.2	1.9	2.4	3.7	4.5	5.7

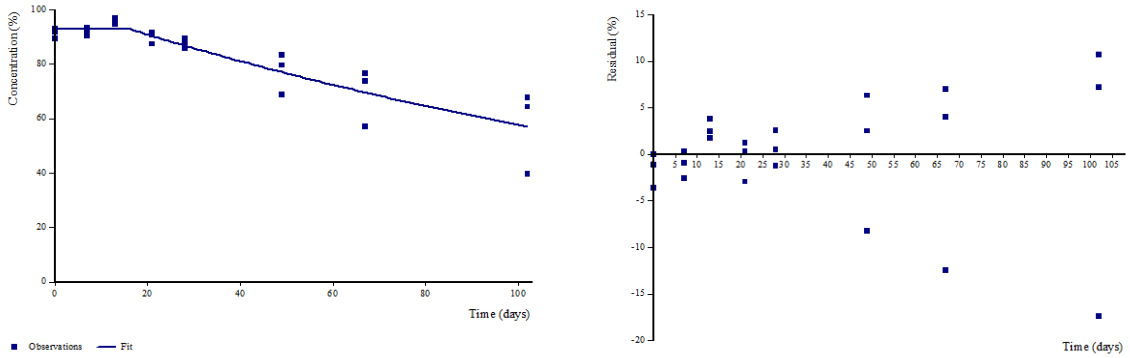
[a] individual peaks represent < 5 % AR

The *DT50* and *DT90* of pethoxamid in aerobic surface water (high dose samples only) were calculated using the Hockey-Stick (HS) kinetic model and based on FOCUS guidance. The HS model represented the best fit for the data.

Figure 4.1.4-1: Kinetic fit of pethoxamid to residues (% AR) measured in aerobic water (high dose samples)



Aerobic water, Lake Tuckahoe – SFO



Aerobic water, Lake Tuckahoe – HS

Table 4.1.4-5: Summary of pethoxamid dissipation kinetics in aerobic water (high dose samples, persistence triggering endpoints in bold)

Test medium	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DT50* / *DT90* (d)
Aerobic water, Lake Tuckahoe	SFO	k (d^{-1})	0.0048	0.0006	2.9	< 0.001	144 / 480
	HS	k_1 (d^{-1})	0.0[a]	-	1.2	-	-
		k_2 (d^{-1})	0.0057	0.0008		< 0.001	-
		t_{split} (d)	16.1	6.5		nd	-
		Overall	-	-		-	**138 / 423**

[a] Fixed to 0

Conclusions:

Pethoxamid degraded moderately in aerobic surface water to one main metabolite, MET-30 (a cysteine conjugate). MET-30 represented a maximum of 19.6 % AR after 102 days. Production of $^{14}CO_2$ in high

and low dose samples averaged 5.7 % and 4.9 % AR, respectively, demonstrating that the mineralisation of pethoxamid was not dose dependent.

Comments (RMS AT):

Kinetic fitting was redone by the applicant in accordance with pertinent FOCUS guidance using CAKE 3.1. Only this reassessment is shown in the study evaluation above. The kinetic re-assessment is considered acceptable. The applicant claims to use results from the HS fit (lower χ^2 error) as a persistent endpoint whereas the more simple SFO fit (*DT50* = 144 days) is considered equally appropriate by the RMS AT.

4.1.4.2 1444 PXA (2015)

Reference: Aerobic aquatic metabolism of [^{14}C]pethoxamid
Author(s), year: Anonymous, 2015
Report/Doc. number: 2518W-1, 1444 PXA
Guideline(s): OECD Guideline 308, 2002
GLP: Yes
Deviations: No
Acceptability: Yes
Status: New submission

Executive Summary:

An aerobic aquatic metabolism study was conducted with [phenyl-U-^{14}C]pethoxamid using two water/sediment systems; Golden Lake (GL) and Goose River (GR). Pethoxamid was applied to the water layers at the rate of 0.12 µg/mL (GL) or 0.11 µg/mL (GR) and the samples were incubated in the dark under aerobic conditions at 20 °C for up to 102 days. The test was performed in amber bottles, each connected to a set of traps. Humidified air was gently bubbled through the samples and then through the traps to collect volatile organic components (ethylene glycol) and $^{14}CO_2$ (NaOH). The dissolved oxygen, pH and redox potential of the test systems were measured throughout the study. At each time point the water layers were decanted and the sediments were extracted three times with acetonitrile:water (4:1, v/v). Selected sediment samples were also subject to harsh microwave extraction with the same solvent. Samples were analysed by LSC and the distribution of radioactivity between pethoxamid and its degradates was determined by HPLC and, in selected samples, by 2-D TLC. Mass balances averaged 98.1 % AR (GL) and 99.0 % AR (GR). Pethoxamid degraded quickly throughout the study in both water/sediment systems tested. MET-6 was the major metabolite observed, reaching a maximum of 9.4 % AR at 14 days (GL). MET-2, MET-3, MET-22 and MET-42 were also observed, but represented less than 5 % AR throughout the study. An unknown metabolite was detected in both test systems at a maximum of 8.8 % AR (GL). The unknown metabolite was identified by high-resolution accurate mass LC-MS and named MET-104. The rate of degradation of pethoxamid in the total system was determined using SFO kinetics, which represented the best fit to the data. DT50 and DT90 values were 7.0 days and 23.1 days, respectively, for the GL test system, and 13.0 days and 43.1 days, respectively, for the GR test system.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid

Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide
Lot/Batch #: CFQ41685
Specific Activity: 29 mCi/mmol
Radiochemical purity: 100 %

Reference standards: Pethoxamid, MET-1, MET-2, MET-3, MET-4, MET-5, MET-6, MET-22, MET-31, MET-42, MET-46 (all unlabelled)

2. Test systems: Two US water/sediment systems characterised by AGVISE laboratories.

Table 4.1.4-6: Physical and chemical properties of the water/sediment systems used

Sediment									
System	pH (H_2O)	OC (%)	Sand (%)	Silt (%)	Clay (%)	CEC (mEq / 100 g)	Biomass (mg C / 100 g sediment)	Sediment texture USDA	Bulk density (g / cm^3)
Golden Lake	8.2	0.8	88	8	4	9.5	Start: 329 End: 218	Sand	1.19
Goose River	7.8	3.1	32	38	30	19.3	Start: 389 End: 249	Clay Loam	0.97

Water								
System	pH	Hardness (mg $CaCO_3$ / L)	Conductivity (mmhos / cm)	Dissolved Solids (ppm)	Turbidity (NTU)	Ca (ppm)	Mg (ppm)	Na (ppm)
Golden Lake	8.7	679	1.43	1170	10.5	110	97	97
Goose River	8.1	897	1.74	1496	16.0	192	100	117

CEC = Cation exchange capacity, OC = Organic carbon

Study Design:

1. Experimental conditions

The aerobic aquatic metabolism of [^{14}C]pethoxamid was studied in two water sediment systems. Pethoxamid was applied at the target rate of 0.12 µg/mL, equivalent to 1200 g a.s./ha assuming a water depth of 1 m, and samples were incubated under aerobic conditions at 20 °C for up to 102 days. The sediment and water were dispensed (approximately 3:1 volume ratio) into amber bottles and the samples were allowed to acclimatise under study conditions for 10 days prior to treatment. Ethylene glycol and NaOH solutions were attached to each treated flask to trap volatiles. Solutions of the test substance in acetonitrile were prepared, diluted and dispensed into each unit. Following treatment, humidified air was gently bubbled through the samples and then through the traps to collect volatile organic components and $^{14}CO_2$ throughout the course of the study. The sediment biomass was also monitored over the period of the study by the substrate induced respiration (SIR) method. The kinetic modelling followed the guidance of FOCUS kinetics employing the software tool for kinetic evaluation KinGUI (v. 2)

2. Sampling

Duplicate samples of each test system were removed at each sampling interval. Sampling was conducted at time 0 and after 3, 7, 14, 31, 60 and 102 days of incubation. At each sampling occasion, the dissolved oxygen (DO) of the water and the pH and redox potential (ORP) of the water and sediment were recorded. Water and sediment layers were separated prior to analysis. Sample processing began immediately following sampling.

3. Description of analytical procedures

Aliquots of water layers were analysed by LSC. Sediment extraction was performed by shaking with 100 mL of acetonitrile:water (4:1, v/v) followed by centrifugation (three times). The extracts were combined before sampling for LSC analysis. Water and sediment extracts were further analysed by HPLC analysis (where appropriate) with co-chromatography with reference standard standards solutions. Peak assignments for parents and degradates were based on co-elution with reference standard, and identities confirmed by 2-D TLC in selected samples. $BaCl_2$ precipitation was used for CO_2 confirmation for selected trapping samples. Selected post-extracted sediment samples were subjected to microwave accelerated extraction and analysed by LSC. Residual sediment samples were homogenised and combusted for analysis by LSC. Post-extracted samples from 102 days were selected for humic acid/fulvic acid partitioning.

Results and Discussion:

Results of the biomass at the start and the end of the study show that both sediments were viable during the conduct of the study. Dissolved oxygen and redox potential measurements indicate that the systems remained aerobic throughout the study period. The stability of the test substances under conditions of administration was confirmed by HPLC analysis. Total mass balances for the Golden Lake (GL) and Goose River (GR) systems averaged 98.1 % AR and 99.0 % AR, respectively. Bound residues in the GL system increased during the study period to a maximum of 63 % AR at 60 days and decreased to an average of 51.7 % AR by 102 days. In the GR system the bound residues increased to a maximum of 64.9 % AR at 101 days. Pethoxamid in the total water/sediment system represented an average of 0.9 % AR (GL) and 1.0 % AR (GR) at the end of the study. The major metabolite observed was MET-6, which reached a maximum of 9.4 % AR (GL) and 8.0 % AR (GR) at 14 days and 31 days, respectively. MET-2 was present at a maximum of 3.0 %AR (GL) and 4.5 % AR (GR) at 60 days and 31 days, respectively. MET-3, MET-22 and MET-42 were also detected throughout the study period, but these metabolites averaged less than 3.3 % AR. The second major metabolite observed was an unknown which did not co-elute with any of the supplied reference standards (Unknown 1). Unknown 1 represented a maximum of 8.8 % AR (GL) and 4.2 % AR at 31 days.

A sample containing pethoxamid, MET-6 and Unknown 1 was prepared by fraction collecting multiple HPLC injections of a sediment extract. The sample was analysed by high-resolution accurate mass LC/MS which allowed structural elucidation of the metabolite. The structure has been named MET-104, and its identity was confirmed by a small scale chemical synthesis.

Table 4.1.4-7: Product balance of [^{14}C]pethoxamid expressed as % AR for Golden Lake system (mean of two replicates, metabolites > 5 % shaded in grey)

Substance	Compartment	Incubation Time (days)						
		0	**3**	**7**	**14**	**31**	**60**	**102**
Pethoxamid	Water	95.1	86.9	39.1	17.2	1.5	0.3	0.2
	Sediment	4.1	5.3	11.5	3.6	2.3	0.6	0.7
	Total system	99.2	92.2	50.6	20.8	3.8	0.9	0.9
MET-6	Water	0.0	0.0	1.8	3.8	0.4	0.5	0.6
	Sediment	0.0	1.6	6.2	5.7	6.2	3.4	3.8
	Total system	0.0	1.6	8.0	9.4	6.6	3.9	4.4
MET-104	Water	0.0	0.0	0.0	0.0	0.0	0.3	0.0
	Sediment	0.0	0.0	0.6	6.2	8.8	0.4	0.8
	Total system	0.0	0.0	0.6	6.2	8.8	0.7	0.8

MET-2	Water	0.0	0.0	0.0	0.0	0.6	0.2	0.2
	Sediment	0.0	0.0	0.0	0.8	0.0	2.8	1.7
	Total system	0.0	0.0	0.0	0.8	0.6	3.0	1.9
MET-3	Water	0.0	0.0	0.0	0.0	0.0	0.3	0.2
	Sediment	0.0	0.0	0.0	0.0	0.0	0.5	0.2
	Total system	0.0	0.0	0.0	0.0	0.0	0.8	0.4
MET-22	Water	0.0	0.0	0.0	0.0	2.3	0.6	0.8
	Sediment	0.0	0.0	0.0	0.0	0.0	1.7	1.5
	Total system	0.0	0.0	0.0	0.0	2.3	2.3	2.3
MET-42	Water	0.0	0.0	0.0	0.0	0.0	1.3	2.0
	Sediment	0.0	0.0	0.0	0.0	0.0	0.6	0.2
	Total system	0.0	0.0	0.0	0.0	0.0	1.9	2.2
Others*	Water	0.0	0.0	0.0	0.0	4.6	3.4	3.4
	Sediment	0.0	0.0	0.0	1.3	0.0	4.0	4.7
	Total system	0.0	0.0	0.0	1.3	4.6	7.4	8.1
CO_2 trapped in water layers	Water	na	na	na	na	1.2	0.5	0.6
Bound Residues	Sediment	0.0	6.3	45.0	51.3	59.7	63.0	57.1
Volatile Organics	Total system	na	0.0	0.0	0.0	0.0	0.0	0.0
CO_2	Total system	na	0.3	1.2	3.5	9.9	12.1	16.3

* Individual peaks represent < 4.5% AR (total system)

Table 4.1.4-8: Product balance of [^{14}C]pethoxamid expressed as % AR for Goose River system (mean of two replicates, metabolites > 5 % shaded in grey)

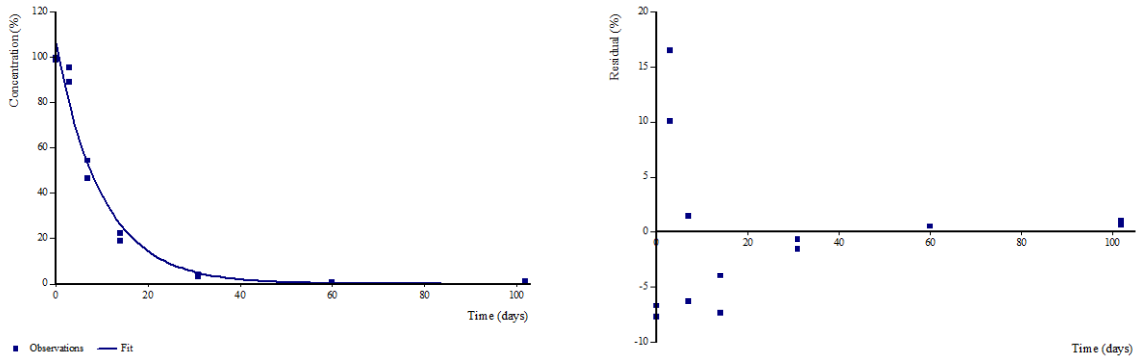
Substance	**Compartment**	**Incubation Time (days)**						
		0	**3**	**7**	**14**	**31**	**60**	**102**
Pethoxamid	Water	96.2	67.6	76.9	29.7	12.1	1.0	0.2
	Sediment	4.9	14.6	8.9	12.4	5.9	1.8	0.8
	Total system	101.1	82.2	85.8	42.1	18.0	2.8	1.0
MET-6	Water	0.0	0.0	0.0	0.0	1.8	0.8	0.3
	Sediment	0.0	1.9	1.3	4.1	6.2	5.3	3.0
	Total system	0.0	1.9	1.3	4.1	8.0	6.1	3.3
MET-104	Water	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sediment	0.0	0.0	0.0	3.5	4.2	0.5	0.7
	Total system	0.0	0.0	0.0	3.5	4.2	0.5	0.7
MET-2	Water	0.0	0.0	0.0	0.0	3.6	0.5	0.3
	Sediment	0.0	0.0	0.0	0.0	0.9	2.3	0.9
	Total system	0.0	0.0	0.0	0.0	4.5	2.8	1.2
MET-3	Water	0.0	0.0	0.0	0.0	0.0	0.3	0.3

	Sediment	0.0	0.0	0.0	0.0	0.0	0.2	0.4
	Total system	0.0	0.0	0.0	0.0	0.0	0.5	0.7
MET-22	Water	0.0	0.0	0.0	0.0	1.2	1.1	0.5
	Sediment	0.0	0.0	0.0	0.0	1.1	2.2	1.4
	Total system	0.0	0.0	0.0	0.0	2.3	3.3	1.9
MET-42	Water	0.0	0.0	0.0	0.0	1.1	0.8	1.2
	Sediment	0.0	0.0	0.0	0.0	0.0	1.1	1.0
	Total system	0.0	0.0	0.0	0.0	1.1	1.9	2.2
Others*	Water	0.0	0.0	0.0	0.0	4.1	4.1	2.3
	Sediment	0.0	0.3	0.0	0.0	0.0	4.1	7.1
	Total system	0.0	0.3	0.0	0.0	4.1	8.2	9.4
CO_2 trapped in water layers	Water	NA	NA	NA	NA	3.3	0.8	0.3
Bound Residues	Sediment	0.1	13.5	11.1	50.1	50.4	56.8	64.9
Volatile Organics	Total system	NA	0.0	0.0	0.0	0.0	0.0	0.0
CO_2	Total system	NA	0.5	0.7	2.7	5.0	11.4	13.4

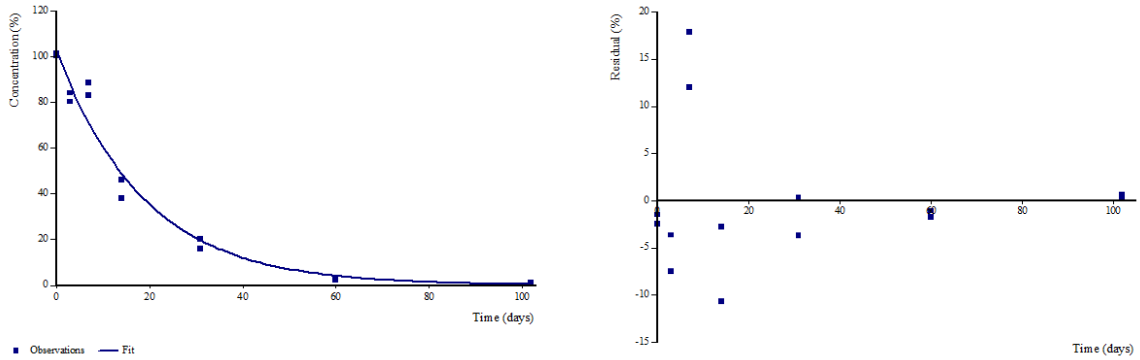
* Individual peaks represent < 3.1% AR (total system)

The *DT50* and *DT90* of pethoxamid in the total water/sediment system were calculated using the SFO and FOMC kinetic models and based on FOCUS guidance. The SFO model represented the best fit for the data.

Figure 4.1.4-2: Kinetic fit (all SFO) of pethoxamid to residues (% AR) measured in two water/sediment systems (<u>total system</u>)

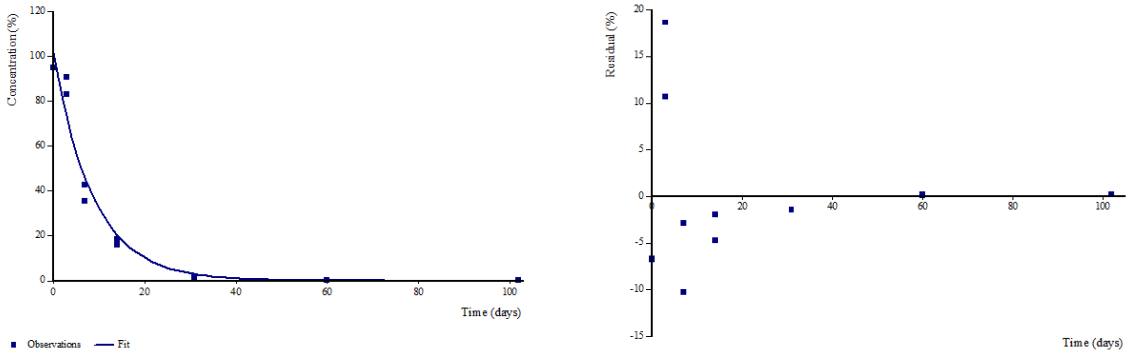


Golden Lake – Total system – SFO

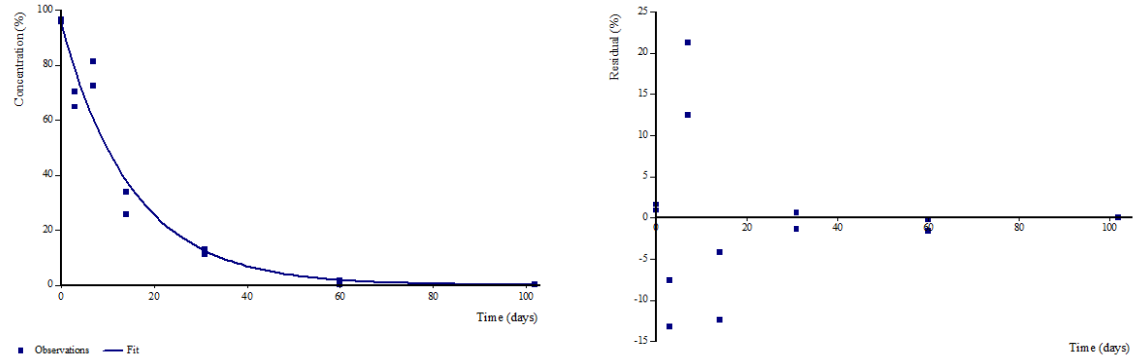


Goose River – Total system – SFO

Figure 4.1.4-3: Kinetic fit (all SFO) of pethoxamid to residues (% AR) measured in two water/sediment systems (<u>water phase</u>)



Golden Lake – Water phase – SFO



Goose River – Water phase – SFO

Table 4.1.4-9: Summary of pethoxamid degradation/dissipation kinetics in two water/sediment systems (all SFO kinetics)

Test medium	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DT50 / DT90* (d)
Golden Lake – Total system	SFO	*k* (d^{-1})	0.100	0.010	12.9	< 0.001	7.0 / 23.1
Goose River – Total system	SFO	*k* (d^{-1})	0.053	0.006	11.1	< 0.001	13.0 / 43.1
Golden Lake – Water phase	SFO	*k* (d^{-1})	0.115	0.013	15.6	< 0.001	6.0 / 20.1
Goose River – Water phase	SFO	*k* (d^{-1})	0.065	0.010	16.0	< 0.001	10.6 / 35.2

Conclusions:

Pethoxamid degraded quickly in aerobic water/sediment systems to two main metabolites, MET-6 and MET-104, MET-6 representing a maximum of 9.4 % AR at 14 days and MET-104 representing a maximum of 8.8 % AR at 31 days. Using SFO kinetics, the *DT50* values for pethoxamid in the total water/sediment systems were between 7.0 and 13.0 days. *DT90* values were between 23.1 and 43.1 days.

4.1.4.3 134 PXA (2000)

Reference: (^{14}C)-TKC-94: Soil Metabolism and Degradation
Author(s), year: Anonymous, 2000
Report/Doc. number: CLE 1465/2-D2142, 134 PXA
Guideline(s): SETAC, 1995; JMAFF, 1985
GLP: Yes
Deviations: No major deviations
Acceptability: Yes

The rate of degradation of ^{14}C-pethoxamid (TKC-94) was studied in four UK soils (PT 102, PT 103, PT 070 and SK 961089) under aerobic conditions in darkness at 45 % of the soils maximum water holding capacity at ca. 20 ºC, in PT 102 also at ca. 10 ºC. Separate samples (50 g dry weight) of the test soils (2 mm sieved) were acclimatised for 7 to 12 days under test conditions prior to test substance application. Soils were treated with a single application of ^{14}C-pethoxamid in acetonitrile at a nominal rate of 0.06 mg/50 g dry weight soil. ^{14}C-pethoxamid was radiolabelled uniformly in the phenyl ring. To determine the microbial biomass of the soils at the end of the incubation period, control soil samples were treated with non-radiolabelled test compound and incubated under the same conditions as for the labelled soil samples.

The water content of the test soils was maintained at 45 % of their maximum water holding capacity during the incubation period. Samples were taken at 0, 1, 2, 3, 6, 10, 30, 59, 90 and 120 days after application.

Soil samples were extracted with acetonitrile and aqueous acetonitrile, combined, concentrated and analysed by TLC and HPLC. Unextractable bound residues were air-dried, combusted in oxygen and quantified by LSC. Radiolabelled volatile degradation products were trapped and quantified. The soil microbial biomass was determined prior to application, and in control vessels sampled at the end of the incubation period.

Table 4.1.4-10: Characteristics of the test soil

Parameter	**PT 102**	**PT 103**	**PT 070**	**SK 961089**
Clay (< 2 µm)	9.6	11.5	11.4	28.1
Silt (2 – 63 µm)	44.5	12.4	62.8	42.8
Sand (63 µm – 2 mm)	46.0	76.1	25.9	29.1
Textural class (UK)	Sandy silt loam	Sandy loam	Sandy silt loam	Clay loam
Textural class (BBA)	Silty loam sand	Loamy sand	Sandy loam silt	Clay loam
Textural class (USDA)[a]	Loam	Sandy loam	Silt loam	Clay loam
Soil pH (1:2.5 v/v in water)	7.2	5.3	6.6	7.8
Soil pH (KCl)	6.8	4.6	5.9	7.2
Organic carbon (%)	1.7	1.0	1.9	3.5
Organic matter[b] (%)	2.9	1.7	3.3	6.0
Cation exchange capacity (mEq/100g)	14.9	10.0	17.0	38.2
MWHC (% w/w dry soil)	58.4	45.6	63.5	81.1
WHC at pF 2.5 (% w/w dry soil)	17.4	10.5	20.6	31.0
Microbial biomass (µg C per g soil)				
Pre-study	227.3	263.5	250.1	734.3
Post-study	273.2 (20 °C) 368.9 (10 °C)	123.3	141.2	883.2

[a] Estimated USDA texture (refer to comment section)
[b] Calculated as organic carbon content × 1.724

Results:

The mean recovery of applied radioactivity from the soil samples in each treatment group was in the range 85.5 to 98.5 %. The distribution of the applied radioactivity between the extractable residues, non-extractable residues and evolved volatiles was similar for each soil type. Extractable radioactivity decreased in the 20 ºC soils from 96 to 98 % at time zero to ca. 12 to 26 % after 120 days. Unextractable radioactivity increased during the incubation period representing ca. 21 to 35 % of the applied radioactivity after 120 days. This non-extractable radioactivity was found upon further fractionation to be evenly distributed between the humin, humic acids and fulvic acids fractions at the 10 and 90 day sample points for each soil. Volatile radioactivity recovered in the sodium hydroxide trapping solution increased throughout the course of the study and represented 34 to 46 % of the applied radioactivity after 120 days. This was later confirmed to be from mineralised $^{14}CO_2$.

Analysis of the concentrated soil extracts from the 20 ºC incubation indicated that pethoxamid rapidly declined to between 24 and 40 % after 10 days and thereafter to < 2 % after 120 days.

Degradation products formed at 20 °C were at levels < 10 % of the applied radioactivity. The most significant of these products, unk@51'30 (confirmed by LC-MS/MS to be MET-42) and unk@47'58 were present at maximum levels of 9.7 and 9.0 %, respectively, 30 days after application. Other degradation products, MET-2, unk@1'20 and unk@36'20 were present at maximum levels of 3.2, 2.9 and 4.4 % of the applied radioactivity. Other unidentified degradation products were present at low levels.

Degradation of pethoxamid in soil incubated at 10 ºC resulted in the formation of one major degradation product, unk@51'30 (MET-42), which represented a maximum of 11.2 % of the applied radioactivity. At least 4 other degradation products (MET-2, unk@1'20, unk@36'20, and unk@47'58) were present at maximum levels of 2.1, 2.0, 9.0 and 4.9 % of the applied radioactivity.

Table 4.1.4-11: Extraction and recovery of radioactivity from soil PT 102 (20 °C and 10 °C) after application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates)

DAT	20 °C					10 °C				
	Extracted	Not extracted	Volatiles		Total	Extracted	Not extracted	Volatiles		Total
			Organic	$^{14}CO_2$				Organic	$^{14}CO_2$	
0	97.60	0.10	n.a	n.a	97.70	98.02	0.08	n.a	n.a	98.10
1	93.72	3.42	n.d	1.36	98.50	95.46	1.73	n.d	0.81	97.99
2	89.50	5.75	n.d	2.33	97.58	93.59	2.88	n.d	1.36	97.82
3	84.02	9.25	0.02	3.41	96.69	91.17	3.91	n.d	1.56	96.63
6	72.34	16.67	0.08	6.87	95.95	86.98	7.15	n.d	2.48	96.61
10	62.52	21.81	0.10	10.99	95.41	81.12	11.26	0.02	4.55	96.94
30	43.00	30.60	0.09	20.09	93.77	60.74	23.01	n.d	11.97	95.72
59	36.52	31.68	0.07	25.42	93.69	47.19	27.83	n.d	19.59	94.61
90	29.23	32.43	0.06	31.59	93.30	41.93	29.50	0.02	21.60	93.05
120	20.60	35.14	0.07	34.21	90.02	38.31	32.06	n.d	24.99	95.36

n.a – not applicable, n.d – not detected

Table 4.1.4-12: Extraction and recovery of radioactivity from soil PT 103 and PT 070 after application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates)

DAT	PT 103					PT 070				
	Extracted	Not extracted	Volatiles		Total	Extracted	Not extracted	Volatiles		Total
			Organic	$^{14}CO_2$				Organic	$^{14}CO_2$	
0	96.86	n.d	n.a	n.a	96.86	96.34	n.d	n.a	n.a	96.34
1	92.18	2.67	n.d	0.63	95.47	93.06	2.32	n.d	0.17	95.54
2	87.07	5.65	0.01	2.47	95.19	88.33	4.82	n.d	1.66	94.80
3	83.20	8.93	n.d	3.68	95.80	87.58	7.58	n.d	1.23	96.38
6	72.84	15.22	n.d	7.44	95.50	77.28	13.15	n.d	5.11	95.54
10	59.91	22.45	n.d	11.48	93.83	63.69	22.70	n.d	8.61	94.99
30	41.34	28.30	n.d	22.95	92.58	40.53	31.88	n.d	18.15	90.56
59	34.29	25.70	n.d	30.61	90.59	30.91	31.65	n.d	28.90	91.47
90	29.59	24.84	n.d	35.88	90.31	24.06	32.85	n.d	35.96	92.87
120	26.01	20.65	0.01	38.85	85.51	20.22	31.58	n.d	35.96	87.75

n.a – not applicable, n.d – not detected

Table 4.1.4-13: Extraction and recovery of radioactivity from soil SK 961089 after application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates)

DAT	SK 961089				
	Extracted	Not extracted	Volatiles		Total
			Organic	$^{14}CO_2$	
0	97.71	0.05	n.a	n.a	97.76
1	90.48	4.19	n.d	0.72	95.38
2	87.00	7.34	n.d	1.55	95.88
3	82.69	9.23	n.d	1.93	93.85
6	74.15	15.15	n.d	2.39	91.68
10	55.29	26.71	n.d	4.48	86.47
30	31.25	33.74	0.17	27.08	92.22
59	20.00	34.81	n.d	37.57	92.38
90	13.66	34.67	n.d	42.55	90.88
120	11.80	33.29	n.d	46.20	91.28

n.a – not applicable, n.d – not detected

Table 4.1.4-14: HPLC profile of extractable radioactivity from soil PT 102 maintained at 20 ºC following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	92.39	nd	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	5.21	97.60
1	87.33	0.60	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.92	1.34	3.87	93.72
2	77.79	0.78	n.d	n.d	n.d	0.84	n.d	n.d	n.d	3.76	1.91	6.32	89.50
3	65.92	0.79	n.d	n.d	n.d	2.41	n.d	n.d	n.d	9.52	3.19	5.38	84.02
6	50.39	1.42	n.d	n.d	n.d	0.38	3.68	5.37	n.d	7.66	2.94	3.42	72.34
10	24.23	1.67	n.d	n.d	n.d	1.35	5.56	8.58	1.99	14.70	3.74	4.45	62.52
30	3.83	2.20	n.d	0.40	n.d	2.36	5.45	9.67	4.41	12.12	3.70	2.56	43.00
59	2.39	2.13	n.d	n.d	n.d	1.86	4.83	8.07	2.89	10.85	2.96	3.49	36.52
90	1.72	2.05	n.d	n.d	n.d	2.19	3.30	6.07	2.52	9.75	2.33	1.63	29.23
120	0.46	1.32	n.d	0.66	n.d	2.30	1.47	3.84	3.10	4.95	1.79	2.48	20.60

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-15: HPLC profile of extractable radioactivity from soil PT 102 maintained at 10 ºC following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	92.82	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	5.20	98.02
1	91.72	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	3.74	95.46
2	83.70	0.44	n.d	n.d	n.d	0.60	n.d	n.d	n.d	3.03	1.66	5.82	93.59
3	78.65	0.77	n.d	n.d	n.d	0.74	n.d	n.d	n.d	6.07	2.41	4.94	91.17
6	72.73	0.45	n.d	n.d	n.d	1.08	n.d	n.d	n.d	7.43	3.91	5.30	86.98
10	59.42	0.90	n.d	n.d	n.d	n.d	2.37	5.91	1.10	8.89	2.43	2.52	81.12
30	21.95	1.08	n.d	1.17	n.d	1.34	4.86	11.17	4.64	12.02	4.81	2.51	60.74
59	6.71	2.08	n.d	0.42	n.d	1.64	3.49	8.90	8.14	12.71	3.92	3.10	47.19
90	3.71	2.02	n.d	n.d	n.d	1.52	3.72	9.29	2.34	16.69	6.05	2.64	41.93
120	1.94	1.78	n.d	n.d	n.d	2.03	2.61	7.86	9.02	9.96	3.46	3.11	38.31

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-16: HPLC profile of extractable radioactivity from soil PT 103 following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	95.01	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.85	96.86
1	89.87	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	2.30	92.18
2	77.81	2.07	n.d	1.25	0.79	0.36	n.d	n.d	n.d	2.38	1.18	2.41	87.07
3	69.79	2.02	n.d	n.d	n.d	0.38	n.d	n.d	n.d	7.63	1.96	3.38	83.20
6	48.75	2.40	n.d	n.d	n.d	2.13	n.d	n.d	n.d	15.39	3.95	4.15	72.84
10	30.31	1.49	n.d	n.d	n.d	0.66	7.14	2.49	n.d	15.65	3.43	2.18	59.91
30	4.52	2.18	1.05	0.99	n.d	1.48	9.01	4.04	n.d	15.86	3.37	3.58	41.34
59	2.33	2.35	0.31	1.25	n.d	1.53	6.07	2.73	n.d	15.74	3.64	2.00	34.29
90	1.45	2.00	0.33	n.d	n.d	1.21	5.99	1.98	n.d	14.79	2.14	1.84	29.59
120	1.09	1.91	0.36	n.d	n.d	1.19	5.76	1.93	n.d	12.58	2.30	1.18	26.01

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-17: HPLC profile of extractable radioactivity from soil PT 070 following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
7	95.54	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.80	96.34
1	91.71	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.35	93.06
2	81.10	1.15	n.d	0.81	n.d	0.87	n.d	n.d	n.d	2.21	1.29	2.17	88.33
3	74.37	1.42	n.d	n.d	n.d	0.78	n.d	n.d	n.d	5.34	2.08	5.66	87.58
6	59.31	1.54	n.d	n.d	n.d	2.74	n.d	n.d	n.d	10.55	3.05	3.12	77.28
10	40.18	0.62	n.d	n.d	n.d	0.36	5.85	3.41	0.38	8.84	2.86	4.06	63.69
30	5.80	1.99	n.d	n.d	n.d	1.21	7.93	5.37	3.56	11.93	3.87	2.75	40.53
59	2.92	2.26	n.d	n.d	n.d	1.41	3.94	5.12	2.57	10.94	2.72	1.74	30.91
90	2.07	1.44	n.d	n.d	n.d	1.27	2.65	2.94	3.22	8.68	1.64	1.80	24.06
120	1.29	1.58	n.d	n.d	n.d	1.70	1.62	2.80	3.22	7.10	1.33	0.89	20.22

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Table 4.1.4-18: HPLC profile of extractable radioactivity from soil SK 961089 following application of ^{14}C-pethoxamid (% applied radioactivity, mean of two replicates, metabolites above 5 % shaded in grey)

DAT	Peth-oxamid	MET-2	MET-3	MET-27	MET-13	unk@1'20	unk@47'58 [MET-101][a]	unk@51'30 MET-42	unk@36'20 [MET-100][b]	Other unknowns	Max. 'other unknowns'	Unresolved Background	Total
0	96.55	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.16	97.71
1	86.57	1.01	n.d	n.d	n.d	0.27	n.d	n.d	n.d	2.07	1.24	0.54	90.48
2	77.83	0.87	n.d	0.47	n.d	0.59	n.d	n.d	n.d	6.14	2.78	1.10	87.00
3	65.23	1.53	n.d	n.d	n.d	0.38	n.d	n.d	n.d	11.41	4.04	4.12	82.69
6	48.79	1.52	n.d	n.d	n.d	2.24	n.d	n.d	n.d	18.09	6.22	3.51	74.15
10	25.84	1.27	n.d	n.d	n.d	0.39	4.43	6.73	0.86	10.24	2.83	5.53	55.29
30	4.78	3.19	n.d	0.14	n.d	1.62	3.31	5.18	1.90	10.10	2.55	1.02	31.25
59	3.72	3.24	n.d	n.d	n.d	2.14	1.76	3.38	0.39	3.42	0.92	1.95	20.00
90	2.75	2.48	n.d	n.d	n.d	2.73	1.01	1.53	n.d	2.30	0.70	0.86	13.66
120	1.47	2.07	n.d	n.d	n.d	2.87	0.91	1.54	n.d	2.27	0.70	0.67	11.80

[a] Renamed and new structure proposed for renewal (refer to comments)
[b] Renamed for renewal (refer to comments)
'Max. other unknowns' - the largest single component in 'other unknowns'
n.d - not detected

Summary:

In soil under aerobic conditions, pethoxamid is initially metabolised via glutathione conjugation with subsequent loss of glycine and glutamic acid to form an intermediate cysteine conjugate, followed by formation of a thiol via beta lyase cleavage – all of which are transitory. Subsequent oxidation gives MET-101 or a sulfonic acid, MET-42. MET-42 is then degraded to MET-100.

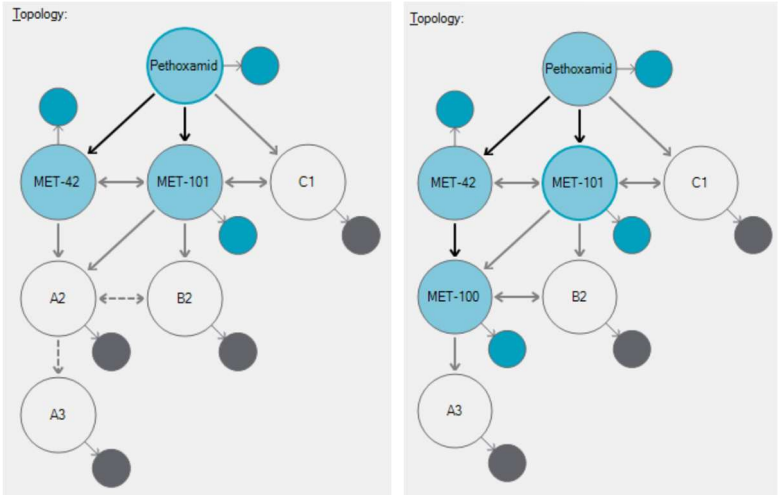
Similar degradation pathways have been documented for compounds containing the chloroacetyl group.

4.1.4.4 1475 PXA (2015)

Reference: Kinetic Analysis of Pethoxamid and its Metabolites in Aerobic Soil Degradation Studies **Author(s), year**: Anonymous, 2015 **Report/Doc. number**: CHA 100625, 1475 PXA **Guideline(s)**: Not applicable **GLP**: No **Deviations**: Not applicable **Acceptability**: Yes

The rate of degradation of pethoxamid and the rates of formation and degradation of its metabolites MET-42, MET-101 and MET-100 in the laboratory aerobic soil degradation study 134 PXA (2000) have been subject to kinetic analysis based on the recommendations of the current EC guidance document in order to derive trigger and modelling endpoints. Calculations were performed using the kinetic evaluation tool CAKE (v. 3.1). The data were evaluated with the SFO model and two degradation schemes (one for studies conducted at 20 °C and one for the study conducted at 10 °C). The results of the kinetic analysis for trigger and modelling endpoints are summarised in the table below. In this case, trigger and modelling endpoints are the same because the SFO model provided an acceptable fit to all data sets.

Figure 4.1.4-4: Degradation schemes applied (left: 20 °C studies; right: 10 °C study)



The study of 134 PXA (2000) was conducted at a soil water content of 45 % maximum water holding capacity (MWHC). Therefore, the $DegT50_{lab}$ values calculated above require normalisation to reference conditions (20 °C and pF 2) to be appropriate as trigger or modelling endpoints. Normalisation to reference conditions was conducted following the EC guidance document (Generic Guidance for Tier 1 FOCUS Groundwater Assessments (version 2.2). Since field capacity (pF 2) was not measured for the soils used, FOCUS default values of water holding capacity for the corresponding soil type were used for the calculation. No correction was necessary with respect to temperature as the incubation studies were conducted at 20 °C.

Table 4.1.4-19: Correction factors for soil moisture and temperature for normalization of degradation rates to reference conditions (pF 2, 20 °C)

Soil name	Soil type (USDA)[a]	MWHC (% w/w)	Study MC, 45 % MWHC (% w/w)	WHC at pF 2 (% w/w)[b]	Moisture correction factor (-)[c]	Study temperature (°C)	Temperature correction factor (-)[d]
PT 102	Loam	58.4	26.3	25	1.0	20	1.0
PT 103	Sandy loam	45.6	20.5	19	1.0	20	1.0
PT 070	Silt loam	63.5	28.6	26	1.0	20	1.0
SK 961090	Clay loam	81.1	36.5	28	1.0	20	1.0

[a] Estimated; also refer to 134 PXA (2000)
[b] Generic Guidance for Tier 1 FOCUS Groundwater Assessments (version 2.2)
[c] Moisture correction factor is 1.0 if WHC at study conditions is above WHC at pF 2, otherwise moisture correction factor is (WHC at study conditions / WHC at pF2)$^{0.7}$
[d] Temperature correction factor based on Q_{10} value of 2.58 is $e^{0.0948 \times (Tref - Tact)}$, where T_{act} is the temperature at study conditions and T_{ref} is the temperature at reference conditions (20 °C)

Results:

The SFO model provides a visually and statistically acceptable fit to the data for pethoxamid, MET-42 and MET-101 for all soils. It was not possible to derive reliable kinetic endpoints for MET-100 from the data available.

Figure 4.1.4-5: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 102 (20 °C)

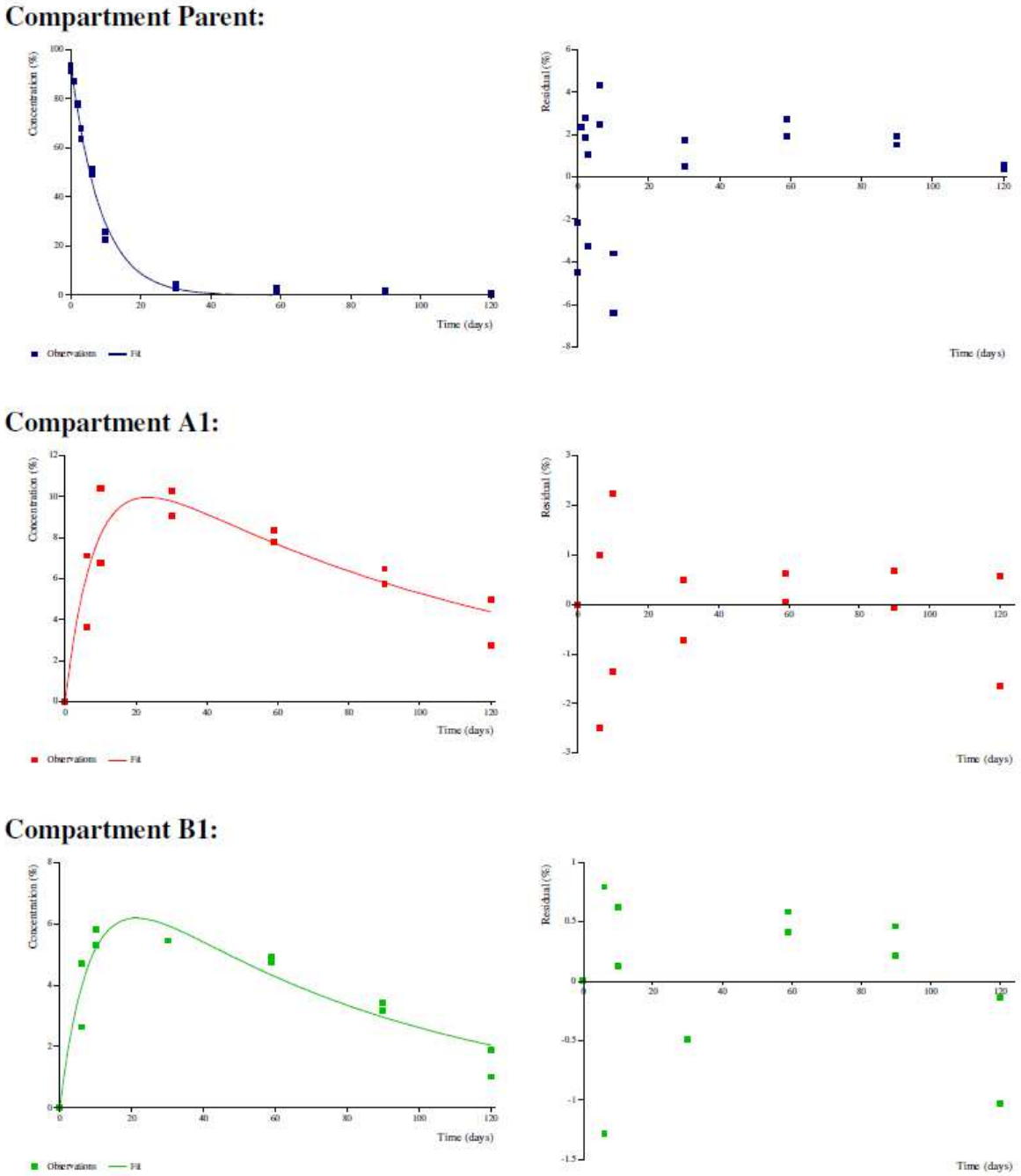
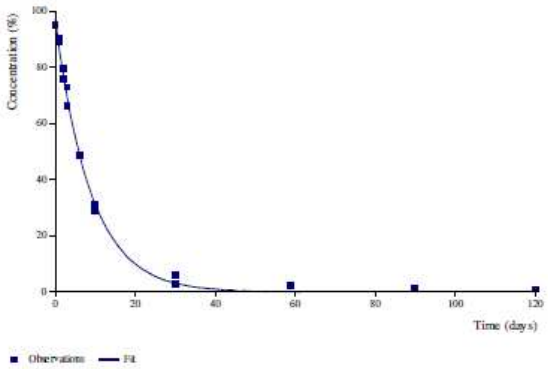
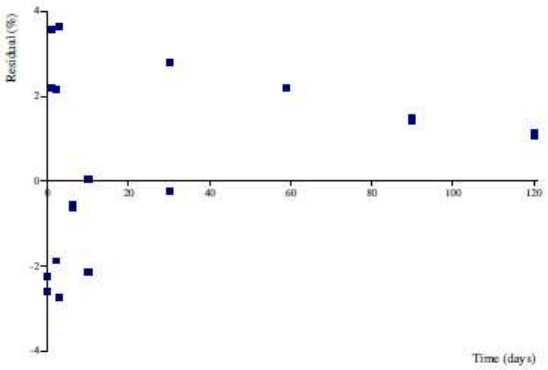


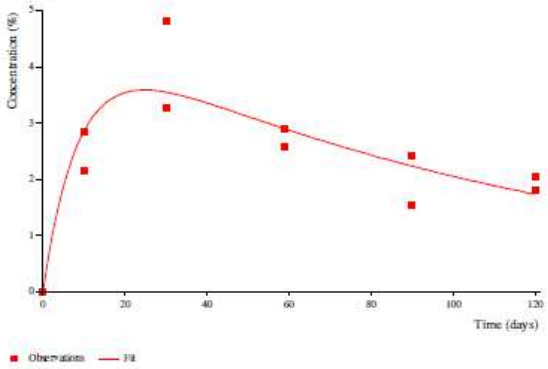
Figure 4.1.4-6: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 103

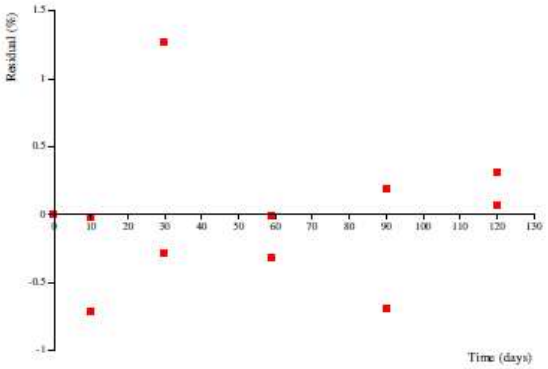
Compartment Parent:



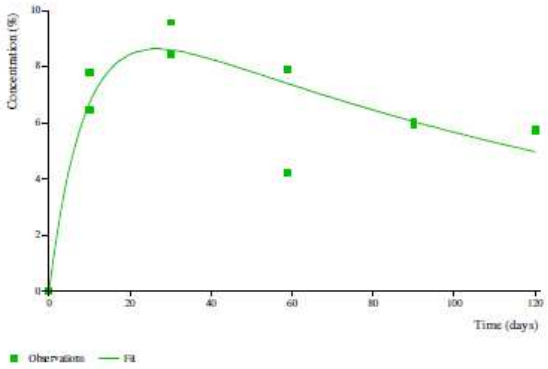


Compartment A1:





Compartment B1:



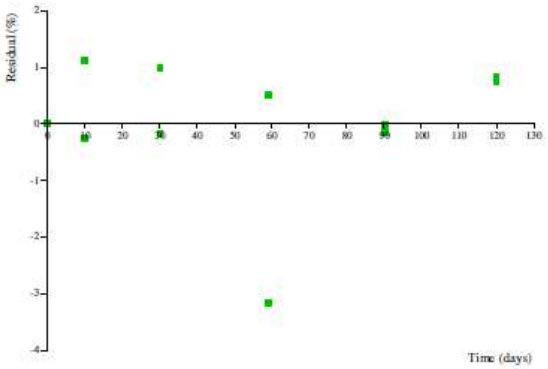


Figure 4.1.4-7: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 070

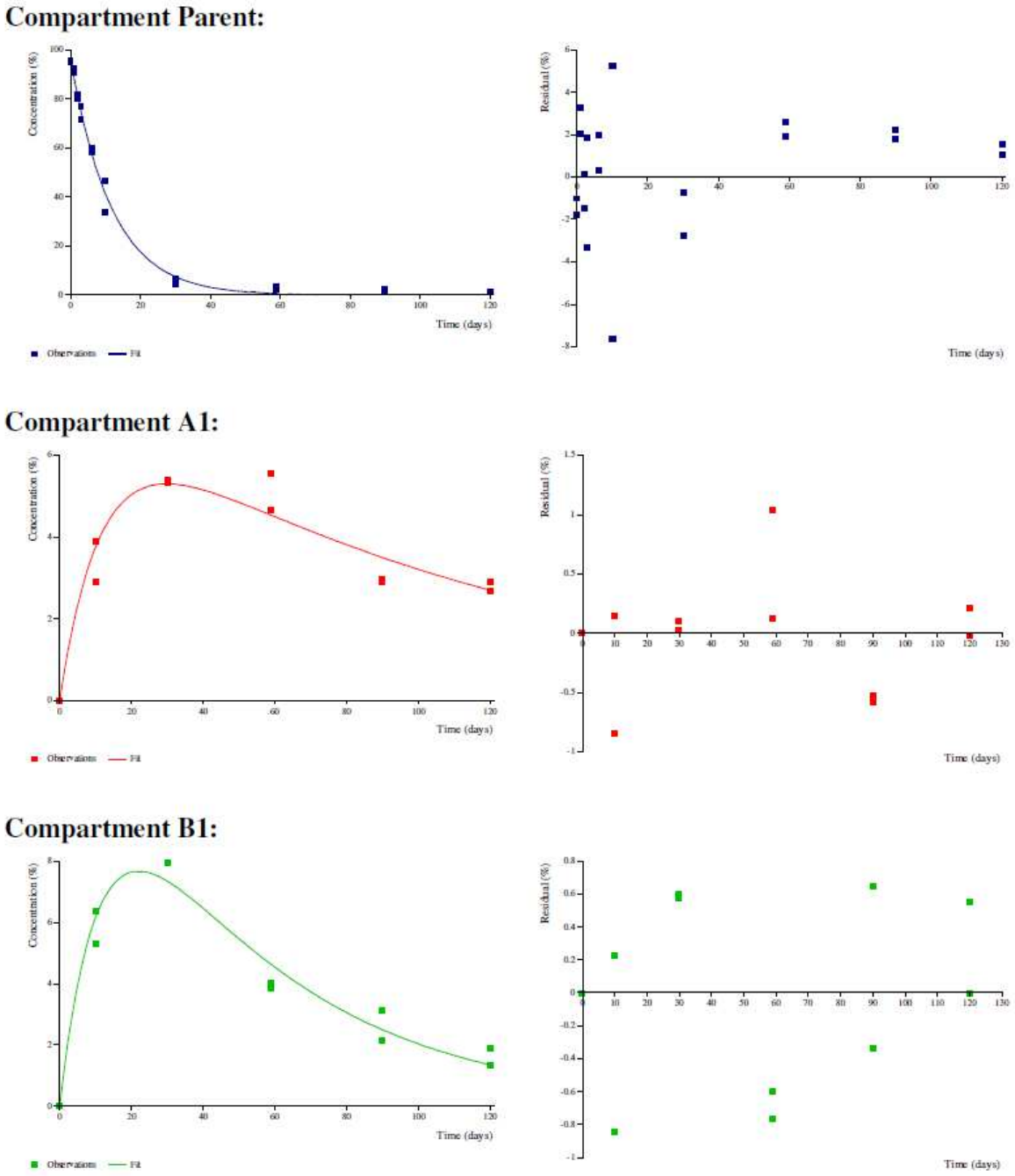
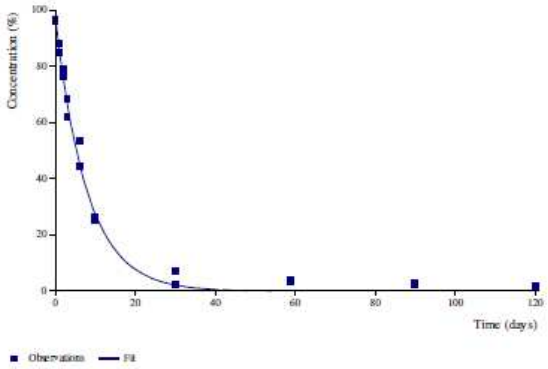
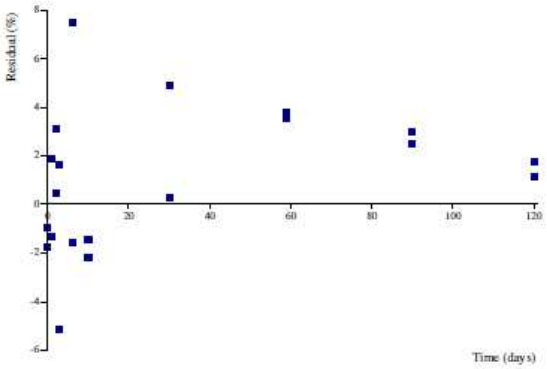


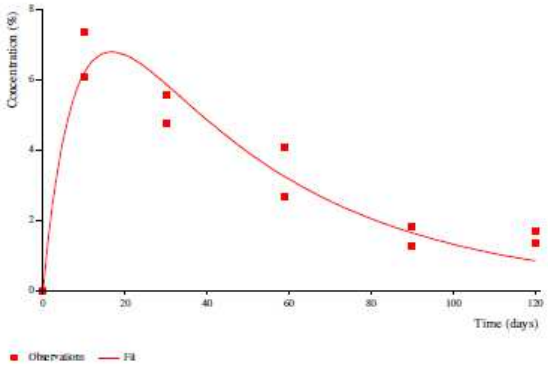
Figure 4.1.4-8: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1) and MET-101 (compartment B1) to residues (% AR) measured in soil SK 961089

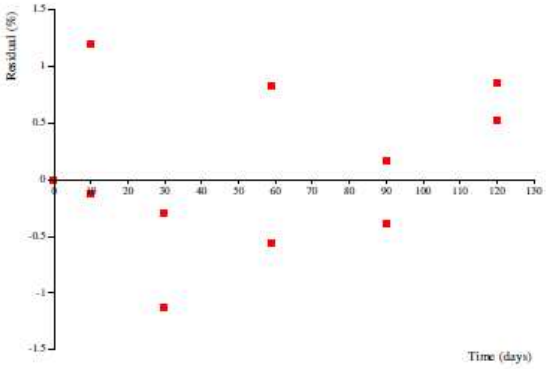
Compartment Parent:



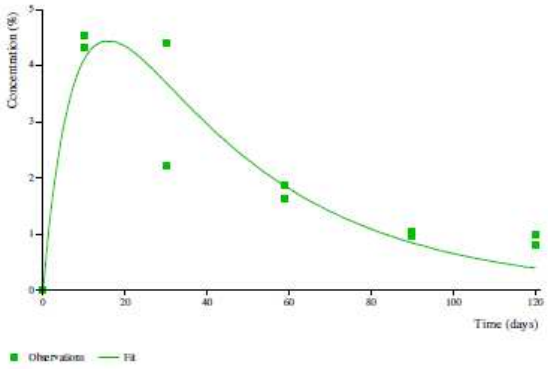


Compartment A1:





Compartment B1:



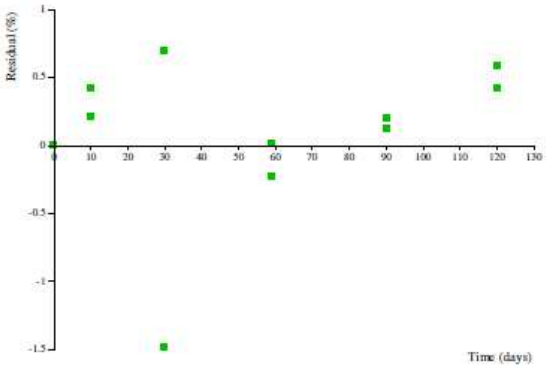
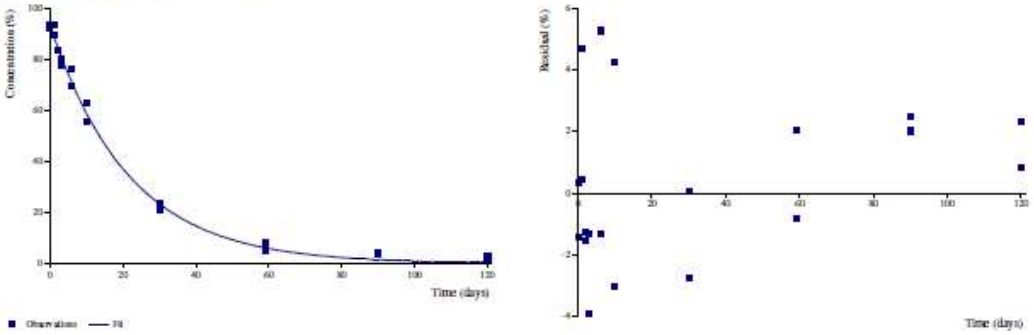
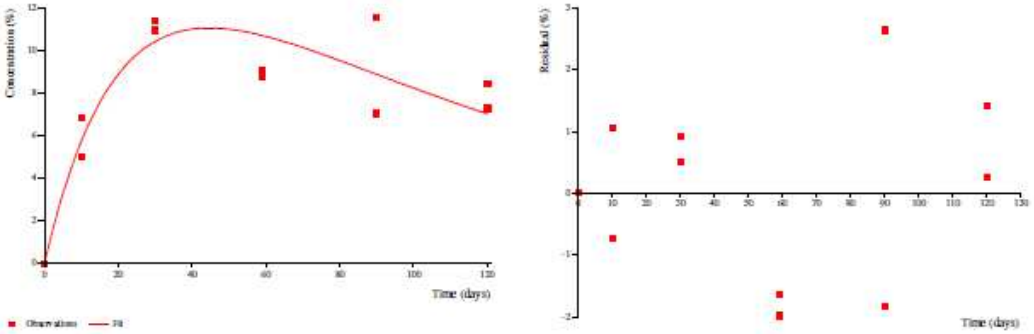


Figure 4.1.4-9: Kinetic fit (all SFO) of pethoxamid (parent), MET-42 (compartment A1), MET-100 (compartment A2) and MET-101 (compartment B1) to residues (% AR) measured in soil PT 102 (10 °C)

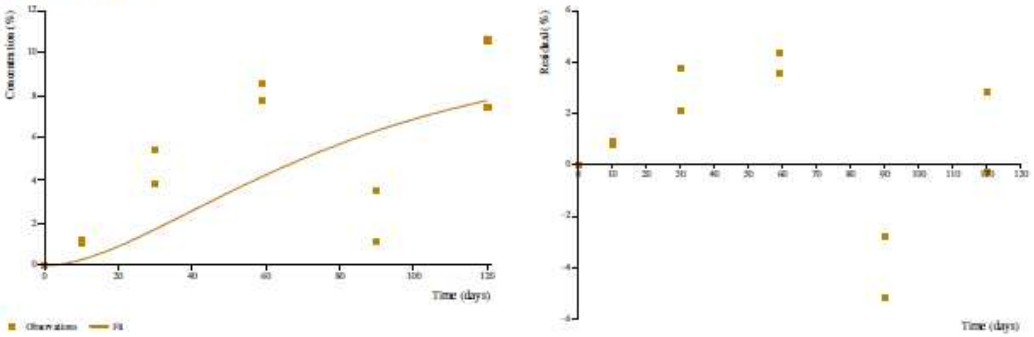
Compartment Parent:



Compartment A1:



Compartment A2:



Compartment B1:

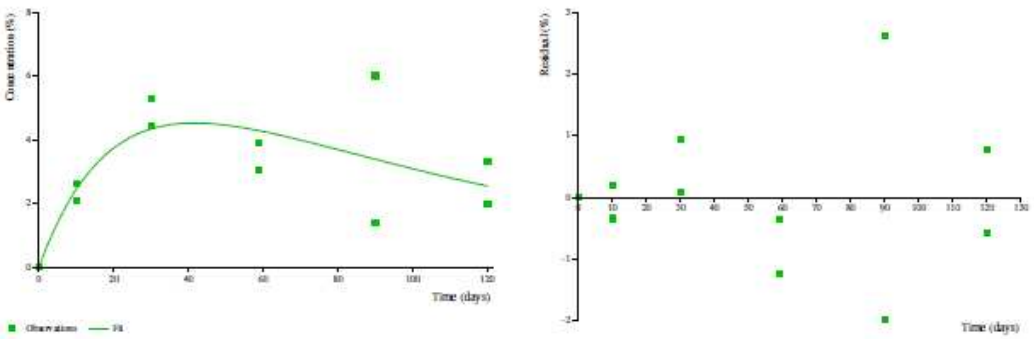


Table 4.1.4-20: Summary of pethoxamid, MET-42 and MET-101 degradation kinetics in aerobic soils at laboratory and reference conditions (20 °C and pF 2) conducted at 20 °C (all SFO kinetics)

Soil name	Compound	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90 (d)*	*DegT50* (d) 20 °C, pF2
PT 102	Pethoxamid	SFO	*k* (d^{-1})	0.119	0.006	5.2	< 0.001	5.9 / 19.4	5.9
	MET-42	SFO	*k* (d^{-1})	0.00935	0.00170	5.3	< 0.001	74.1 / 246	74.1
			ff (-)	0.13	0.01		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0123	0.0017	8.6	< 0.001	56.2 / 187	56.2
			ff (-)	0.08	0.01		nd	-	-
PT 103	Pethoxamid	SFO	*k* (d^{-1})	0.113	0.004	3.1	< 0.001	6.1 / 20.3	6.1
	MET-42	SFO	*k* (d^{-1})	0.00845	0.00206	9.6	< 0.001	82.0 / 272	82.0
			ff (-)	0.05	0.005		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.00654	0.00170	8.1	< 0.001	106 / 352	106
			ff (-)	0.11	0.01		nd	-	-
PT 070	Pethoxamid	SFO	*k* (d^{-1})	0.0852	0.0042	2.9	< 0.001	8.1 / 27.0	8.1
	MET-42	SFO	*k* (d^{-1})	0.00868	0.00135	8.0	< 0.001	79.9 / 265	79.9
			ff (-)	0.07	0.01		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0205	0.0020	8.1	< 0.001	33.8 / 112	33.8
			ff (-)	0.12	0.01		nd	-	-
SK 961089	Pethoxamid	SFO	*k* (d^{-1})	0.127	0.007	4.4	< 0.001	5.5 / 18.2	5.5
	MET-42	SFO	*k* (d^{-1})	0.0220	0.0029	11.1	< 0.001	31.5 / 105	31.5
			ff (-)	0.10	0.01		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0254	0.0044	11.6	< 0.001	27.3 / 90.8	27.3
			ff (-)	0.07	0.01		nd	-	-

Table 4.1.4-21: Summary of pethoxamid, MET-42, MET-101 and MET-100 degradation kinetics in one aerobic soil at laboratory and reference conditions (20 °C and pF 2) conducted at 10 °C (all SFO kinetics)

Soil name	Compound	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90 (d)*	*DegT50* (d) 20 °C, pF2
PT 102	Pethoxamid	SFO	*k* (d^{-1})	0.0462	0.0022	2.7	< 0.001	15.0 / 49.8	-
	MET-42	SFO	*k* (d^{-1})	0.00862	0.00209	9.0	< 0.001	80.4 / 267	-
			ff (-)	0.17	0.02		nd	-	-
	MET-100	SFO	*k* (d^{-1})	0.00292	0.0165	46.2	0.430	238 / 790	-
			ff (-)	1.00	0.83		nd	-	-
	MET-101	SFO	*k* (d^{-1})	0.0104	0.0043	10.6	0.011	66.6 / 221	-
			ff (-)	0.07	0.02		nd	-	-

4.1.4.5 138 PXA (2000)

Reference: (^{14}C)-TKC-94: Metabolism in Flooded Soil **Author(s), year**: Anonymous, 2000 **Report/Doc. number**: CLE 1465/3-D2142, 138 PXA **Guideline(s)**: SETAC, 1995; US EPA Subdivision N, Section 162-3,1982; JMAFF, 1985 **GLP**: Yes **Deviations**: None **Acceptability**: Yes

Test system:

The anaerobic route and rate of degradation of pethoxamid in a flooded UK sandy silt loam soil maintained at 20 °C, in darkness, under anaerobic conditions was studied over an incubation period of 365 days. Prepared individual flooded soil systems were incubated under study conditions for 32 days prior to test substance application in order to establish anaerobic conditions. ^{14}C-pethoxamid (uniform phenyl labelling) was applied to the surface water overlying the soil samples at a rate of 0.061 mg / 50 g soil, equivalent to a field application rate of 1.22 kg ai/ha. To allow determination of the soil microbial biomass at the end of the incubation period, control samples were treated with non-radiolabelled pethoxamid at the same rate and incubated under the same conditions as for the main study samples.

Duplicate treated samples were taken for analysis at predetermined intervals. Sampled water and soil were separated; the water acidified and partitioned with ethyl acetate, the soil was extracted with acetonitrile. Quantification of metabolites in water and soil was by HPLC. Selected samples were analysed by LC/MS to obtained further information on the chemical structures of significant metabolites. Radiolabelled volatile degradation products were trapped and quantified. Extracted soils were air-dried and combusted prior to radioassay. The unextracted soil residues from 30 and 59 day samples were Soxhlet extracted then fractionated into humin, humic acids, and fulvic acids.

Table 4.1.4-22: Soil characteristics

Parameter	Test soil
Particle size distribution (%) USDA	
Clay (< 2 µm)	10.0
Silt (2 – 50 µm)	43.2
Sand (50 – 2 mm)	46.8
Particle size distribution (%) UK, BBA	
Clay (< 2 µm)	9.6
Silt (2 – 63 µm)	44.5
Sand (63 – 2 mm)	46.0
Textural class (USDA)	Loam
Textural class (UK)	Sandy silt loam
Textural class (BBA)	Silty loam sand
Soil pH (1:2.5 v/v in water)	7.2
Soil pH (1:2.5 KCl)	6.8
Organic carbon (%)	1.7
Organic matter (%)	2.9
Cation exchange capacity (CEC, mEq / 100 g)	14.9
Water holding capacity at pF 0, MWHC (% w/w dry soil)	58.4
Water holding capacity at pF 2.5 (% w/w dry soil)	17.4

Microbial biomass (µg C / g)		
	Pre-study	227.3
	Post-study	324.7

Findings:

The overall recovery of radioactivity in all samples was in the range from 91 to 98 %. Over the incubation period, the quantity of radioactivity in the surface water decreased to 12 % AR. The quantity of extractable radioactivity from the soil increased to 28 % AR after 14 days and thereafter represented 19 % AR after 365 days. The level of unextractable radioactivity represented 61 % AR after 365 days. Volatile radioactivity represented 5 % AR after 365 days.

Pethoxamid was degraded in the test system representing < 1 % AR in the surface water after 30 days and 1 % AR in the soil extracts after 59 days.

Pethoxamid degraded to MET-22, 5 principal unknown components (1 – 8 % AR) and numerous minor components. MET-22 accounted for up to 8.8 % AR and Unk 1 (the largest unknown) accounted for up to 8.1 % AR from the total system.

The unextractable soil residues from the 30 and 59 day sampling intervals were Soxhlet extracted and then fractionated into humin, humic acids and fulvic acids. There was an approximately even distribution of radioactivity across the Soxhlet extract and three residue fractions. The activity in the Soxhlet extract comprised of Unk 3 (32 % of extract, *ca* 3 %AR) and other minor components. The organic phase phase after partition of the fulvic acid fractions comprised of one unknown component. The aqueous phase after partition of the humic acid fraction consisted of at least three poorly resolved components.

LC/MS analysis confirmed the presence of pethoxamid and MET-22 in selected samples. A structure was proposed for the unknown product Unk 1.

Table 4.1.4-23: Extraction and recovery of radioactivity from anaerobic soil after application of ^{14}C-pethoxamid (% of applied radioactivity, mean of two replicates)

DAT	Surface water	Soil extracts	Not Extracted	Volatiles		Total
				Organic	NaOH	Recovery
0	97.09	0.76	0.03	n.a	n.a	97.88
1	90.86	4.85	0.35	n.d	n.d	96.06
3	84.69	10.36	1.00	n.d	n.d	96.05
7	62.28	28.32	5.87	n.d	0.01	96.48
14	46.02	27.67	22.30	n.d	0.05	96.04
30	22.92	21.82	49.91	n.d	0.17	94.82
59	12.91	19.54	60.98	n.d	0.45	93.88
120	12.67	17.44	64.75	n.d	1.60	96.46
181	11.76	18.66	60.82	n.d	2.70	93.94
269	12.23	15.11	60.91	n.d	3.39	91.64
302[a]	13.36	13.86	60.31	n.d	3.80	91.33
330[a]	15.77	15.68	56.84	n.d	4.16	92.45
365	11.62	17.24	60.48	n.d	4.63	93.97

[a] Individual vessels
n.s – no sample, n.d – not detected

Table 4.1.4-24: Proportions of radioactive components in anaerobic soil and overlying surface water (total system) after application of ^{14}C-pethoxamid (% of applied radioactivity, mean of two replicates, metabolite > 5 % shaded in grey)

DAT	Pethoxamid	MET-22	Unk 1 [MET-46]	Unk 2	Unk 3	Unk 4	Unk 5	Other unknowns	Max 'other unknowns'	Unresolved Background	Total
0	93.67	n.d	0.38	n.d	n.d	n.d	n.d	0.36	016	0.34	94.76
1	94.06	n.d	0.47	n.d	n.d	n.d	n.d	0.51	0.29	0.94	95.98
3	92.98	n.d	0.23	0.13	n.d	n.d	n.d	0.08	0.08	0.97	94.41
7	84.85	0.69	1.02	0.59	0.16	0.06	n.d	0.14	0.09	0.24	87.76
14	52.24	3.19	5.51	1.25	1.06	0.46	0.08	3.90	0.98	0.61	68.32
30	10.62	7.17	8.10	1.68	1.00	0.97	n.d	8.25	1.50	0.32	38.11
59	1.48	6.67	4.77	3.44	1.40	1.48	0.50	8.90	1.68	0.37	29.01
120	0.56	7.53	6.02	3.95	1.49	1.24	0.72	5.82	1.07	0.23	27.57
181	0.43	8.77	4.11	3.32	1.15	2.28	1.94	4.41	1.09	0.19	26.61
269	0.31	7.26	5.79	3.96	0.68	1.68	1.22	3.87	1.16	0.08	24.88
302*	0.38	7.84	3.62	2.61	1.45	2.55	1.77	4.38	1.25	0.12	24.71
330*	0.50	8.08	6.79	5.01	0.93	2.46	1.51	4.19	1.31	0.31	29.78
365	0.19	6.78	7.23	3.98	1.35	1.30	1.01	5.21	1.36	0.33	27.40

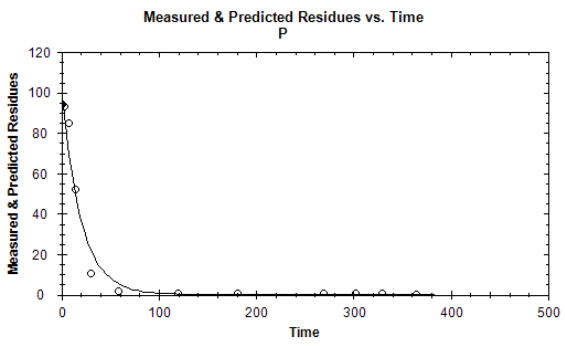
n.d – not detected
Max other unknowns – the largest single component found amongst 'Other unknowns'
Mean values of duplicate samples except where indicated '*'

Summary:

Pethoxamid was metabolised in soil under anaerobic conditions. Radioactive residues were incorporated into the soil matrix and evenly distributed between the humin, humic acid and fulvic acid fractions. The main degradation products were identified as MET-22 and Unk1 (later confirmed as MET-46) and represented a maximum of 8.8 and 8.1 % AR, respectively. Other minor degradates represented < 5 % AR.

Comments (RMS AT):

- Soil *DegT50* of pethoxamid under anaerobic conditions was recalculated by the RMS AT following pertinent FOCUS guidance using KinGUI 2:



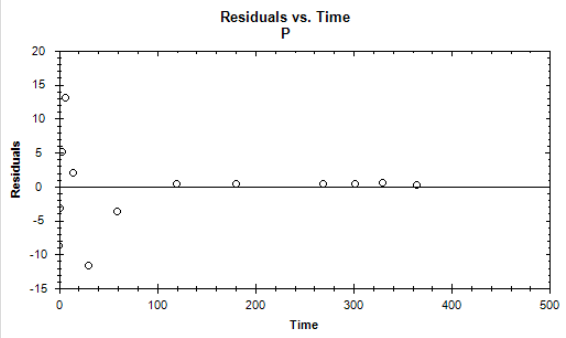


Figure 4.1.4-10: Kinetic fit (SFO) of pethoxamid to residues (% AR) measured in anaerobic soil – RMS AT assessment.

Table 4.1.4-25: Summary of pethoxamid degradation kinetics in anaerobic soil – RMS AT assessment

Soil	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90* (d)
UK sandy silt loam	SFO	k (d^{-1})	0.0507	0.0059	14.9	< 0.001	13.7 / 45.4

DegT50 of pethoxamid in soil under anaerobic conditions is considered to be 13.7 days.

4.1.4.6 135 PXA (2000)

Reference: Terrestrial Field Dissipation Study with TKC-94 EC60 Applied to Bare Soil in Spain and France in 1998 and 1999
Author(s), year: Anonymous, 2000
Report/Doc. number: TON 021/002214, 135 PXA
Guideline(s): SETAC, 1995; OEPP/EPPO Bulletin 23, 27-49, 1993; BBA Guidelines, Part IV, 4-1, 1986
GLP: Yes
Deviations: No major deviations
Acceptability: Yes

Test system:

Three terrestrial field dissipation trials were performed to establish the decline of residues of pethoxamid and its metabolite MET-42 resulting from a single application to bare soil at the maximum labelled rate of TKC-94 EC60 (ASU 95620 H, batch no. 9802, content: 599 g/l). A fourth trial (TON/021-02) was terminated 1998, because residues of pethoxamid were found in all the horizons within the soil cores at the 0 DAT event, which was thought to be due to the sandy soil type. When the soil cores were taken, a small gap was evident between

the soil core and the inside of the acetate tube. This allowed the sandy soil to run freely up and down the core, thus contaminating the whole sample.

Trials TON/021-01 (sandy clay loam, Spain), TON/021-03 (silty sandy loam, France) and TON/021-04 (sandy clay, Spain) were sprayed in May 1998, June 1998 and April 1999 respectively. The soil types of each trial were representative of crop production areas for the intended use of TKC-94 EC60. Details of the soil characteristics and microbial biomass of each trial site are given below.

Table 4.1.4-26: Characteristics of the soils from TKC-94 dissipation sites

Parameter	**TON/021-01 (Spain)**	**TON/021-03 (France)**	**TON/021-04 (Spain)**
pH (KCl)	7.5	6.9	7.3
Organic carbon (%)	0.8	0.8	0.8
Water holding capacity (at 0.001 bar suction (pF0) % w/w of dry soil)	57.8	59.5	49.7
Cation exchange capacity (mEq/100g)	19.1	14.8	16.3
Clay (%)	37	24	31
Silt (%)	23	46	16
Sand (%)	40	30	53
Classification (BBA)	Sandy clay loam	Silty sandy loam	Sandy clay
Microbial biomass (mg C / 100 g soil) (-1 DAT) (1)	10.88	3.06	204.13
Microbial biomass (mg C / 100 g soil) (120 DAT) (1)	7.00	5.02	5.40

(1)Mean value for the treated plots

The trial design at each site consisted of a non-treated plot (Plot 1) and two replicated treated plots (Plots 2 and 3). TKC-94 EC60 (emulsifiable concentrate at a nominal concentration of 600 g/l, actual concentration 599 g/l) was applied once at a nominal rate of 2 l/ha (1200 g ai/ha) by research backpack boom sprayer. The nominal application volume was 250 l/ha. The treatment rate was verified by the analysis of spray target cards placed on the test site prior to treatment. Adequate herbicides were applied to each plot for the duration of the study to maintain bare soil conditions.

Soil cores were taken at predetermined intervals and maintained frozen until analysed. Each sample consisted of twenty soil cores, collected using hydraulic soil coring equipment with 5 cm (diameter) × 50 cm (length) acetate tubes. Prior to analysis, the soil cores were cut into 0-10 and 10-20 cm horizons. For trial TON/021-01, as pethoxamid residues were found in the 10-20 cm horizons for sample points 0, 3, 7, 16, 26, 60 and 90 days after treatment, the 20-30 cm horizons for these time points were also analysed. Soil samples for microbial biomass determination were collected prior to treatment and nominally at 120 days after treatment.

Each soil sample (20 cores) was bulked according to horizon and sieved to remove any debris. Soil was extracted by shaking with aqueous acetone and an aliquot of the resulting extract reduced to dryness by rotary film evaporation. Quantification was performed by liquid chromatography using mass spectroscopy. Control soil samples were fortified with either pethoxamid or MET-42 to validate the analytical method. The LOD and LOQ were set at 2 ng/g and 10 ng/g respectively in soil for both compounds.

Findings:

Analysis of the deposition cards from the treated plots during application indicated a favourable comparison with the target application rate of 3.8 mg/5 deposition cards. Values ranged from 2.8 mg/5 deposition cards to 3.8 mg/5 deposition cards with a mean of 3.3 mg/5 deposition cards. This corresponded to 74-100 % (mean 86 %) of the actual application rate as determined by timed spray passes.

Procedural recoveries for pethoxamid and MET-42 in soil all fell within the acceptable range of 70 – 110 %. No residues of either pethoxamid or MET-42 were detected in any soil horizons taken from the untreated plot of any of the three trials.

Trial TON/021-01: A mean pethoxamid residue of 308 ng/g was detected in the soil horizon (0 – 30 cm) from the sample taken at the time of treatment. Thereafter the level of pethoxamid residues declined to less than the limit of detection (2 ng/g) in soil sampled 360 days after treatment (DAT). The presence of pethoxamid residues in the lower horizons up to 90 DAT was considered to be due to contamination occurring during the coring procedure. Mean residues of MET-42 reached a maximum of 30 ng/g in the 0 – 10 cm horizon of soils sampled 185 DAT and thereafter declined to less than the limit of detection in soils 360 DAT. No residues of MET-42 were at quantifiable levels in the 10–20 cm horizon of soils from this trial.

Trial TON/021-03: A mean pethoxamid residue of 746 ng/g was detected in the upper horizon (0 – 10 cm) of soils sampled at the time of treatment and thereafter declined to less than the limit of quantification in soils sampled 179 DAT. No residues of pethoxamid were detected in the lower horizon (10 – 20 cm) of soils in this trial. Mean residues of MET-42 represented a maximum of 37 ng/g in the 0 – 10 cm horizon of soils sampled 96 DAT declining to less than the limit of quantification in the corresponding horizons of soils sampled 122 DAT. No residues of MET-42 were detected in the 10 – 20 cm horizons of soils from this trial.

Trial TON/021-04: A mean pethoxamid residue of 831 ng/g in the upper horizon of soils sampled at the time of treatment declined to less than the limit of quantification in soils sampled 183 DAT. The presence of pethoxamid residues at detectable levels in the lower soil horizons was considered to be due to contamination during sampling. Mean residues of MET-42 represented a maximum of 33 ng/g in the 0 – 10 cm horizon of soils sampled 59 DAT, declining to 19 ng/g in the corresponding horizon of soils sampled 183 DAT. No residues of MET-42 were detected in the 10 – 20 cm horizon of soils from this trial.

Table 4.1.4-27: Summary TON/021-01 (Spain) findings

Plot number	**Soil sample**	**Amount of TKC-94 (ng/g) (0 – 30 cm)**	**Amount of MET-42 (ng/g) (0 – 10 cm)**
Plot 2+3	-4 DAT	ND	ND
Plot 2+3	0 DAT	308	NQ
Plot 2+3	3 DAT	180	NQ
Plot 2+3	7 DAT	123	NQ
Plot 2+3	16 DAT	82	NQ
Plot 2+3	26 DAT	96	NQ
Plot 2+3	60 DAT	86	NQ
Plot 2+3	90 DAT	93	12
Plot 2+3	122 DAT	20	25
Plot 2+3	185 DAT	8	30
Plot 2+3	360 DAT	0	NQ

DAT = days after treatment
Plot 2 + 3 = replicated treated plots
ND = not detected (< 2 ng/g)
NQ = not quantifiable (< 10 ng/g)

Table 4.1.4-28: Summary TON/021-03 (France) findings

Plot number	**Soil sample**	**Amount of TKC-94 (ng/g) (0 – 10 cm)**	**Amount of MET-42 (ng/g) (0 – 10 cm)**
Plot 2+3	-1 DAT	ND	ND
Plot 2+3	0 DAT	746	NQ
Plot 2+3	3 DAT	669	14

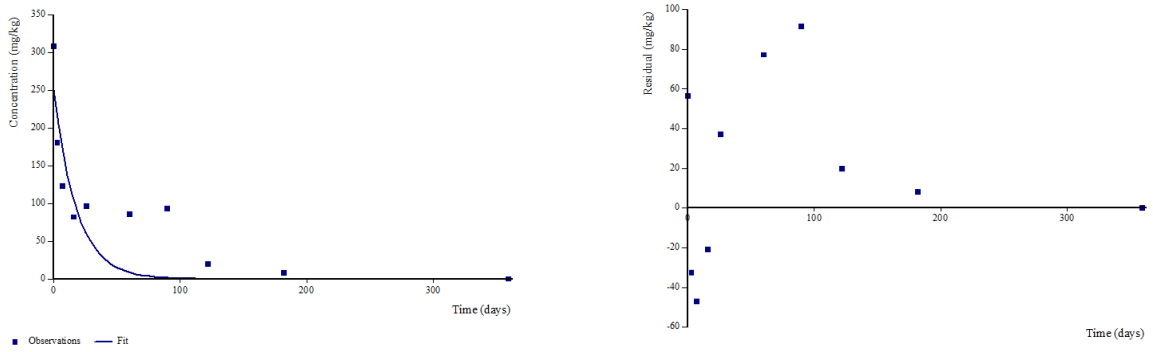
Plot 2+3	7 DAT	609	14
Plot 2+3	14 DAT	460	21
Plot 2+3	28 DAT	329	23
Plot 2+3	60 DAT	107	27
Plot 2+3	96 DAT	21	37
Plot 2+3	122 DAT	33	NQ
Plot 2+3	179 DAT	NQ	ND
Plot 2+3	364 DAT	ND	NQ

DAT = days after treatment
Plot 2 + 3 = replicated treated plots
ND = not detected (< 2 ng/g)
NQ = not quantifiable (< 10 ng/g)

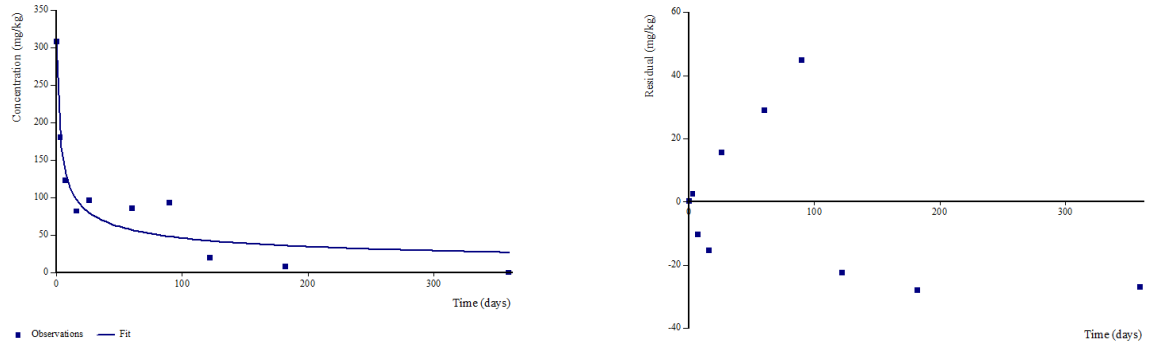
Table 4.1.4-29: Summary TON/021-04 (Spain) findings

Plot number	**Soil sample**	**Amount of TKC-94 (ng/g) (0 – 10 cm)**	**Amount of MET-42 (ng/g) (0 – 10 cm)**
Plot 2+3	-4 DAT	ND	ND
Plot 2+3	0 DAT	831	ND
Plot 2+3	3 DAT	409	ND
Plot 2+3	7 DAT	494	NQ
Plot 2+3	14 DAT	332	16
Plot 2+3	28 DAT	288	21
Plot 2+3	59 DAT	133	33
Plot 2+3	92 DAT	121	24
Plot 2+3	126 DAT	56	28
Plot 2+3	183 DAT	NQ	19

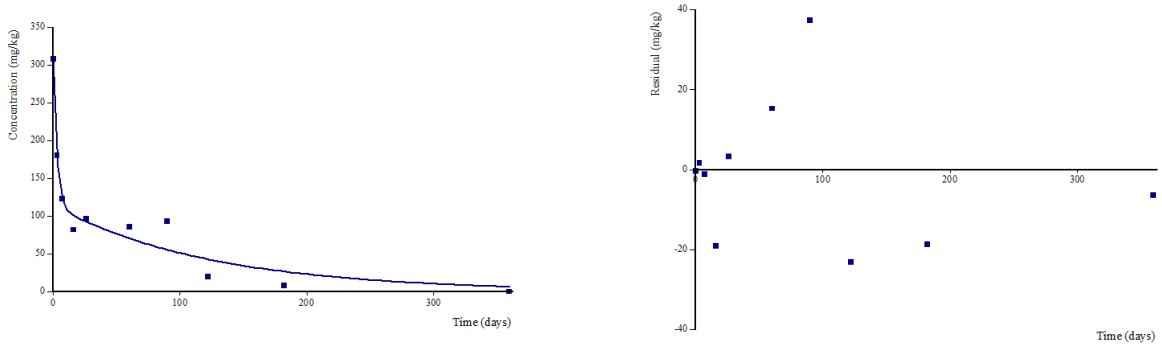
DAT = days after treatment
Plot 2 + 3 = replicated treated plots
ND = not detected (< 2 ng/g)
NQ = not quantifiable (< 10 ng/g)



TON/021-01, Spain – SFO

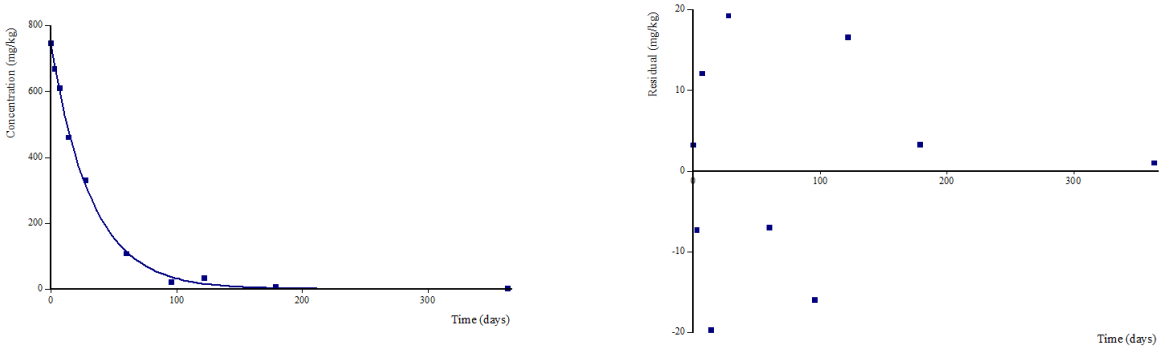


TON/021-01, Spain – FOMC



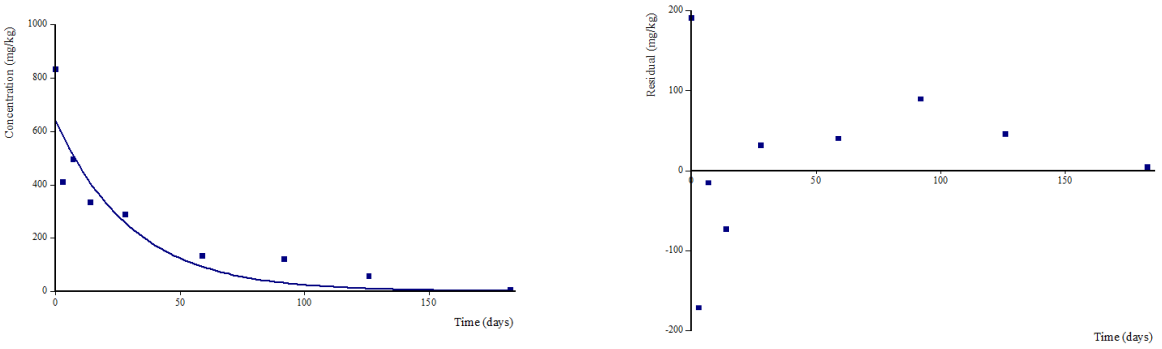
TON/021-01, Spain – DFOP

Figure 4.1.4-11: Kinetic fit (SFO, FOMC and DFOP) of pethoxamid to residues (mg/kg) measured in TON/021-01 (Spain) field trial

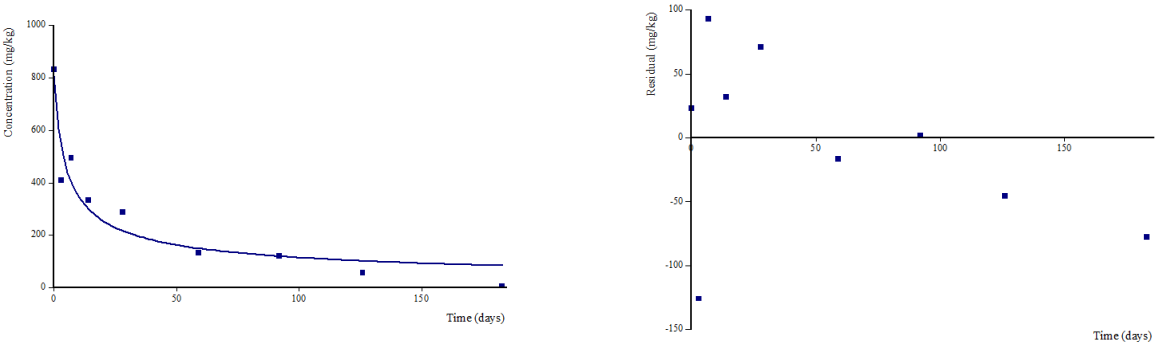


TON/022-03, France – SFO

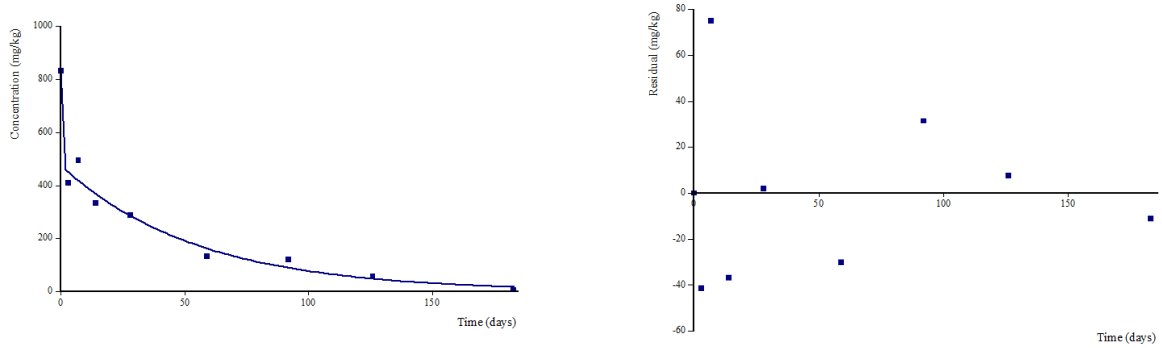
Figure 4.1.4-12: Kinetic fit (SFO only) of pethoxamid to residues (mg/kg) measured in TON/022-03 (France) field trial



TON/021-04, Spain – SFO



TON/021-04, Spain – FOMC



TON/021-04, Spain – DFOP

Figure 4.1.4-13: Kinetic fit (SFO, FOMC and DFOP) of pethoxamid to residues (mg/kg) measured in TON/021-04 (Spain) field trial

Table 4.1.4-30: Summary of pethoxamid dissipation kinetics in three field trials (numbers in bold indicate persistence trigger endpoints)

Field trial	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DisT50 / DisT90* (d)
TON/021-01, Spain	SFO	*k* (d^{-1})	0.056	0.024	38.7	0.026	12.5 / 41.3
	FOMC	*alpha* (-)	0.42	0.12	19.8	nd	-
		beta (-)	1.1	1.0		nd	-
		Overall	-	-		-	4.7 / 264
	DFOP	k_1 (d^{-1})	0.36	0.13	15.3	0.019	2.0 / 6.5
		k_2 (d^{-1})	0.008	0.003		0.022	86.9 / 289
		g (-)	0.63	0.07		nd	-
		Overall	-	-		-	**4.2 / 162**
TON/022-03, France	SFO	*k* (d^{-1})	0.031	0.001	3.4	< 0.001	**22.2 / 73.7**
TON/021-04, Spain	SFO	*k* (d^{-1})	0.033	0.011	26.1	0.012	21.2 / 70.4
	FOMC	*alpha* (-)	0.53	0.19	18.9	nd	-
		beta (-)	2.5	2.3		nd	-
		Overall	-	-		-	6.9 / 195
	DFOP	k_1 (d^{-1})	5.5	nd	10.5	nd	0.1 / 0.4
		k_2 (d^{-1})	0.018	0.003		0.002	38.2 / 127
		g (-)	0.43	0.05		nd	-
		Overall	-	-		-	**7.3 / 96.0**

Comments (RMS AT):

- Kinetic fitting was redone by the applicant in accordance with pertinent FOCUS guidance using CAKE 3.1. Only this reassessment is shown in the study evaluation above. The kinetic re-assessment is considered acceptable.

4.1.5 Photochemical degradation studies

4.1.5.1 1442 PXA (2015)

Reference: Direct aqueous photodegradation of [^{14}C]Pethoxamid
Author(s), year: Anonymous, 2015d
Report/Doc. number: 2516W-1, 1442 PXA
Guideline(s): OECD Test Guideline 316, 2008
GLP: Yes
Deviations: None
Acceptability: Yes

Executive Summary:

An aqueous photolysis study was conducted with pethoxamid using a tiered approach. A Tier 1 theoretical screen indicated that the half-life of pethoxamid could be < 30 days. Therefore, a Tier 2 experimental study was conducted using [phenyl-U-^{14}C] pethoxamid in sterilised pH 7 phosphate buffer at a dose rate of 3.91 µg/mL. Samples were irradiated in quartz tubes under a Xenon lamp, with filters blocking light of wavelengths < 290 nm, at an average intensity of 45.2 W / m^2 (290 - 400 nm). Samples were exposed continuously for up to 16 days (equivalent to approximately 30 solar days at 40 - 50 °N) at 25 °C. Dark control samples were protected from light and incubated at 25 °C. Volatile gases were trapped using ethylene glycol and NaOH traps. PNAP-PYR chemical actinometer samples were used to determine the quantum yield. Samples were removed at 0, 1, 3, 6, 10 and 16 days, quantified by LSC and analysed by HPLC and LC-MS. Total recoveries averaged 98.8 % AR for light exposed samples and 100.7 % AR for dark control samples. Pethoxamid was degraded moderately fast in light exposed samples. Two main degradates were observed: PD-1 (max. 31.6 % AR) and PD-3 (max. 21.5 % AR). The photo-degradates were identified by co-chromatography and/or LC/MS analysis as benzoic acid and MET-102, respectively. MET-2 was also present at up to 3.3 % AR in light exposed samples. The *DT50* value calculated for pethoxamid was 7.7 days, equivalent to 13.9 days of summer sunlight (based on OECD values for 40 – 50 °N). The quantum yield of pethoxamid was determined to be 2.85 × 10^{-1}. Pethoxamid was stable in the dark control samples.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid
Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide
Lot/Batch #: CFQ41685
Specific Activity: 29 mCi / mmol
Radiochemical purity: 100 %

2. Test systems: Sterilised 0.05 M pH 7 phosphate buffer

Study Design:

1. Experimental conditions
Samples were dosed with [phenyl-U-^{14}C]pethoxamid at a rate of 3.91 µg/mL with acetonitrile co-solvent (< 1 % of total sample by volume). Samples were exposed to artificial light with a Suntest CPS+ unit equipped with a Xenon arc lamp with UV filter to block radiation < 290 nm for up to 16 days of continuous irradiation. Irradiated samples were contained in sterilised quartz glass tubes and maintained in a water bath maintained at 25 °C. Corresponding dark control samples were placed in an incubator maintained at 25 °C. Samples were connected to a series of liquid traps (ethylene glycol and NaOH) for volatile compounds. A chemical actinometer solution of p–nitroacetophenone and pyridine (PNAP-PYR) was prepared, added to sterile water and incubated concurrently with samples containing pethoxamid to determine the quantum yield. The kinetic modelling followed the guidance of FOCUS kinetics employing the software tool for kinetic evaluation KinGUI (v. 2).

2. Sampling
Duplicate samples were removed at each sampling interval. Sampling was conducted at zero time and the following intervals: 1, 3, 6, 10 and 15 days (samples were also removed after 16 days irradiation for comparison with actinometer samples).

3. Description of analytical procedures
Sample processing began immediately following sampling. Sample sterility was assessed at the start and end of exposure by plating representative aliquots in Trypticase Soy Agar (TSA) plates. Test solutions were quantified by LSC and analysed by HPLC and LC-MS. The pH of the samples was measured at each sampling.

Results and Discussion:

Stability and homogeneity of the dosing solution was demonstrated by HPLC analysis. Sterility was confirmed from inspection of incubated agar plates. Sample pH was stable throughout the study period (6.97 and 6.88 in light and dark samples, respectively). Total mass balances for the definitive experimental study averaged 98.8 % AR and 100.7 % AR in light and dark samples, respectively. The majority of the radioactivity was recovered in the incubated aqueous samples. Pethoxamid degraded moderately fast in aqueous pH 7 buffer when exposed to artificial sunlight, and averaged 20.3 % AR after 16 days of continuous light exposure. Two major unknown photo-degradates which did not co-elute with any of the supplied reference standards were observed; unknown PD-1 (max. 31.6 % AR after 16 days) and unknown PD-3 (max. 21.5 % AR after 6 days). MET-2 was also present at up to 3.3 % AR in light exposed samples. Several minor degradates were also observed in light exposed samples, but no single degradate was present as > 4.2 % AR. Pethoxamid was stable in dark control samples.

The structure of unknown PD-3 was identified by LC-MS/MS. This structure has been named MET-102, and its identity was corroborated with simulated spectrum analysis. The structure of unknown PD-1 was identified by high resolution LC-MS as benzoic acid. The identity of this metabolite was confirmed by co-chromatography with an authentic analytical reference standard and simulated spectrum analysis.

Table 4.1.5-1: Balance of [^{14}C]pethoxamid expressed as % AR for light exposed samples (metabolites > 5 % shaded in grey)

% of AR	Incubation Time (days)					
	0	1	3	6	10	16
Pethoxamid	100.1	93.8	81.9	53.5	48.1	20.3
Benzoic acid (PD-1)	0.0	0.0	3.8	14.5	17.3	31.6
MET-102 (PD-3)	0.0	7.0	13.8	21.5	20.8	15.9
MET-2	0.0	0.0	0.0	2.0	1.5	3.3
Others[a]	0.0	0.0	0.0	5.2	9.3	16.5
Volatile traps (ethylene glycol)	na	0.2	0.8	1.8	2.5	3.3
Volatile traps (CO_2)	na	0.0	0.1	0.4	0.8	2.1

[a] Individual peaks represent < 4.2 % AR

The *DT50* and *DT90* of pethoxamid were calculated using the SFO kinetic model and based on FOCUS guidance. The quantum yield of pethoxamid was calculated by comparison of the percent pethoxamid present in solutions in irradiated samples and degradation of PNAP in irradiated actinometer solutions. The quantum yield of pethoxamid was determined to be 2.85×10^{-1}.

Figure 4.1.5-1: Kinetic fit (SFO) of pethoxamid to residues (% AR) measured under conditions of aquatic photolysis

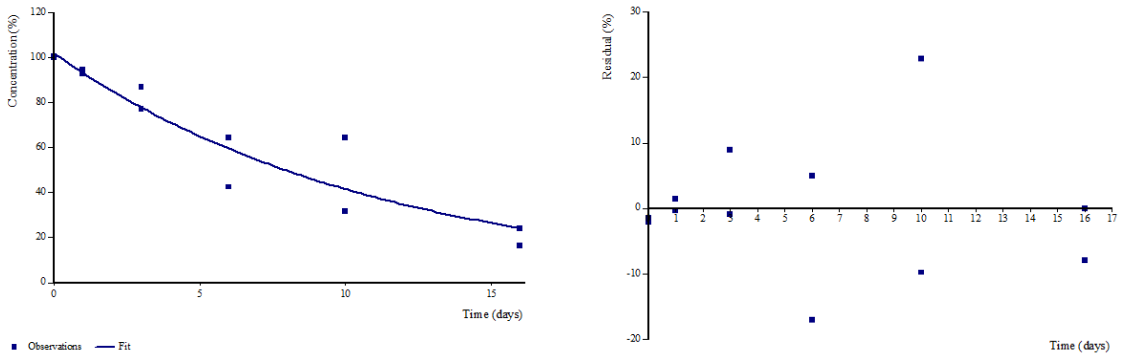


Table 4.1.5-2: Summary of pethoxamid dissipation kinetics under conditions of aquatic photolysis (SFO kinetics)

Test medium	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DT50 / DT90* (d)
pH 7 phosphate buffer, light exposed	SFO	*k* (d^{-1})	0.090	0.013	5.3	< 0.001	7.7 / 25.7

Table 4.1.5-3: Summary on direct photochemical degradation of pethoxamid at laboratory and environmental conditions

Light Exposed	Kinetic model	Suntest exposure days[a]		Sunlight equivalent days[b]	
		DT50 (d)	*DT90* (d)	*DT50* (d)	*DT90* (d)
pH 7 phosphate buffer	SFO	7.7	25.7	13.9	46.2

[a] continuous Suntest irradiation
[b] 40 – 50 °N, summer irradiation, 300 - 400 nm

Comparison of the photo-degradation profile with a previous aqueous photolysis study conducted with pethoxamid (S., 2000), showed that similar profiles were obtained in both studies. A major unknown, designated as AP9 in the previous study, corresponded to unknown PD-3 in this study, while unknown AP2 in the previous study was equivalent to PD-1 in the current study. The structure of unknown degradate PD-3 proposed in the present study agrees with the proposed structure of AP9 proposed in the previous study (S., 2000) and is named MET-102 to avoid confusion. However, the structure proposed for AP2 by S. (2000) was MET-36. LC-MS analysis of PD-1 did not support this. Instead, the unknown was identified as benzoic acid. The identity of PD-1 as benzoic acid was further confirmed by HPLC co-elution of PD-1 with an authentic analytical reference standard of benzoic acid.

Conclusions:

Pethoxamid photodegraded to two main degradates, PD-1 (max. 31.6 % AR) and PD-3 (max. 21.5 % AR). The photo-degradates were identified by co-chromatography and/or LC-MS analysis as benzoic acid and MET-102, respectively. MET-2 was also present at up to 3.3 % AR in light exposed samples. The *DT50* of pethoxamid was 7.7 days, equivalent to 13.9 days of summer sunlight (based on OECD values for 40 – 50 °N). The quantum yield of pethoxamid was determined to be 2.85×10^{-1}. Pethoxamid was stable to hydrolysis in dark control samples.

4.1.5.2 139 PXA (1999)

Reference: TKC-94 Soil Photolysis **Author(s), year**: Anonymous, 1999 **Report/Doc. number**: TON 024/983761, Cheminova A/S Report No.: 139 PXA **Guideline(s)**: US EPA Subdivision N, Section 161-3, 1982 **GLP**: Yes **Deviations**: None **Acceptability**: Yes

Test system:

The photo-degradation of pethoxamid was studied on thin layers (*ca.* 2 mm) of a sandy silt loam/sandy loam soil using phenyl radiolabelled form of the test material. The test material was applied to the soil thin layers at a concentration of 12.5 µg/cm^2. This equates to a field rate of 1.25 kg a.s./ha. The soil thin layers were continuously irradiated using a xenon arc light source for up to 15 days at *ca.* 20 °C. Control soil thin layers were similarly incubated in darkness. Humidified air was passed over irradiated/incubated soil thin-layers and passed into a series of trapping solutions to trap any volatiles evolved. Duplicate irradiated and non-irradiated soil plates were taken for analysis at 2, 4, 7, 10 and 15 days after test substance application. One duplicate set of plates was taken for analysis immediately after test substance application and provided a zero-time sample for both irradiated and non-irradiated experiments. HPLC and TLC investigated the proportions and identities of degradation products in soil extracts. The soil characteristics are shown below.

Table 4.1.5-4: Characteristics of the test soil

Textural classification (USDA) 0.053 – 2 mm (%) 0.002 – 0.053 mm (%) < 0.002 mm (%)[c] Textural classification	 52.8 36.1 11.1 Sandy loam
Organic carbon (%) Cation exchange capacity (mEq / 100 g) pH (1 : 5) in water	2.7 18.6 6.9
pH (1 : 5) in 1 M KCl	6.9
pH (1 : 5) in 0.01 M $CaCl_2$	7.1
Maximum water holding capacity (%) Water holding capacity at 0.33 bar (%)	54.5 18.9

Findings:

The recovery of radioactivity from all soil plates was essentially quantitative (94 – 106 % AR). After 15 days of irradiation pethoxamid accounted for a mean of 67 % AR. The amount of $^{14}CO_2$ evolved during the 15 day study period represented 4 % and < 0.1 % AR for irradiated soil and non-irradiated soil respectively. Apart from pethoxamid, a number of degradation products (n = 10) were formed. In irradiated soil after 15 days, metabolite SP5 individually represented a maximum mean proportion of 4.2 % of the applied radioactivity. No unique photoproducts were formed during the study. After 15 days no individual product represented more than 1 % AR in non-irradiated soil. Non extractable radioactivity after 15 days accounted for 4 – 5 % AR for either irradiated or non-irradiated soils.

Table 4.1.5-5: Extraction and recovery of radioactivity from soil thin layers (% of applied radioactivity)

DAT	Irradiated soil			Dark control soil		
	Soil		Total	Soil		Total

	Extracted	Non extracted	Total	Vola-tiles		Extracted	Non extracted	Total	Vola-tiles	
0	99.9	0.4	100.3	-	100.3	97.8	1.2	99.0	< 0.1	99.0
	105.6	0.4	106.0	-	106.0	98.8	1.3	100.1	< 0.1	100.1
2	94.6	1.8	96.4	0.4	96.8	101.5	3.1	104.6	< 0.1	104.6
	94.1	2.2	96.3	0.4	96.7	93.6	2.2	95.8	< 0.1	95.8
4	94.4	2.9	97.3	1.0	98.3	95.0	3.5	98.5	< 0.1	98.5
	95.1	2.1	97.2	1.0	98.2	96.7	3.2	99.9	< 0.1	99.9
7	88.9	4.0	92.9	2.1	95.0	95.6	3.0	98.6	< 0.1	98.6
	91.5	3.3	94.8	2.1	96.9	97.6	2.8	100.4	< 0.1	100.4
10	91.7	3.0	94.7	2.9	97.6	91.8	4.8	96.6	< 0.1	96.6
	88.3	3.2	91.5	2.9	94.4	92.2	4.9	97.1	< 0.1	97.1
15	87.1	4.3	91.4	4.2	95.6	97.8	1.2	99.0	< 0.1	99.0
	87.5	4.1	91.6	4.2	95.8	98.8	1.3	100.1	< 0.1	100.1

Table 4.1.5-6: Proportions of radioactive components in soil (% of applied radioactivity, HPLC, metabolites > 5 % shaded in grey)

DAT	Irradiated soil												Dark control soil	
	0		2		4		7		10		15		15	
Polars (SP1)	0.1	0.1	0.7	0.6	0.8	0.8	0.8	0.7	0.8	1.1	1.2	0.8	< 0.1	< 0.1
SP2	-[a]	-[a]	0.6	0.7	1.0	0.7	0.8	1.1	1.1	1.3	1.8	1.6	< 0.1	< 0.1
SP3	-[a]	-[a]	0.3	0.3	0.4	0.5	0.4	0.5	0.6	0.5	0.6	0.4	< 0.1	< 0.1
SP4	0.6	0.6	1.1	1.5	1.7	2.8	2.0	1.4	1.6	1.7	1.7	2.4	0.5	0.6
SP5	0.2	0.4	2.4	2.4	2.5	4.0	2.8	3.8	3.7	4.1	3.9	4.4	0.4	0.4
SP6	0.3	0.3	2.1	2.4	2.6	2.4	2.4	1.8	2.3	2.6	2.7	2.0	0.4	0.5
SP7	0.6	0.3	2.4	2.4	3.6	3.8	2.6	2.7	2.6	2.0	3.0	2.9	0.6	0.4
SP8	0.4	0.4	2.6	2.9	3.1	3.2	2.4	2.7	2.8	2.6	2.4	2.7	0.4	0.3
SP9	0.4	0.4	2.1	2.0	2.0	2.9	2.5	2.2	2.3	1.9	2.0	2.1	0.3	0.5
Pethoxamid	96.4	101.9	79.3	77.9	75.4	71.0	71.1	73.2	72.9	69.2	66.4	67.1	88.5	88.9
SP10	0.6	0.7	0.7	0.7	0.6	2.3	0.5	0.5	0.6	0.5	0.5	0.5	0.6	0.6
Others[b]	0.3	0.3	0.4	0.5	0.6	0.8	0.6	0.5	0.6	0.7	0.9	0.7	0.2	0.2

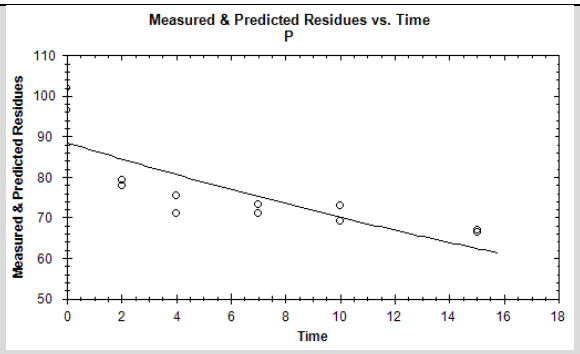
[a] Not apparent : included in 'others'
[b] Radioactivity distributed through regions of the chromatogram other than those specified and which did not contain any radioactive components

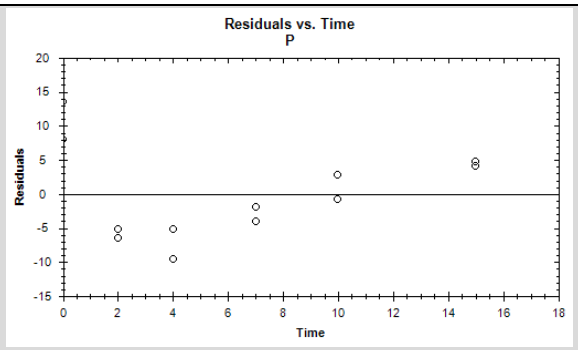
Summary:

Pethoxamid was photodegraded on soil. Degradation of pethoxamid was accelerated in the presence of light. A number of quantitatively minor photo-degradation products (all < 5 % AR) were formed which were also products of aerobic soil metabolism.

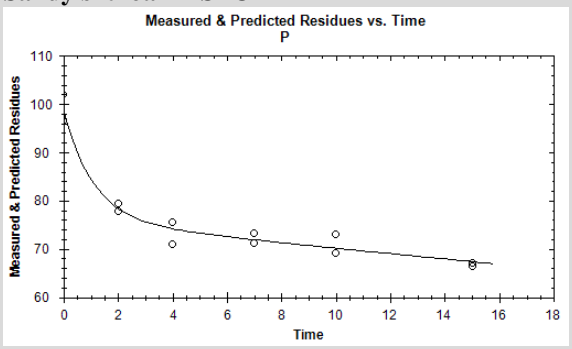
Comments (RMS AT):

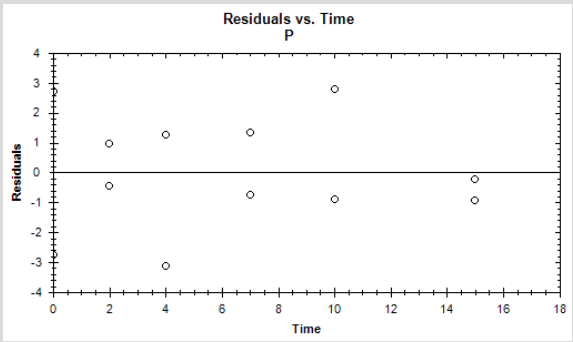
- Dissipation kinetics of pethoxamid under conditions of soil photolysis was recalculated by the RMS AT following pertinent FOCUS guidance using KinGUI 2:





Sandy silt loam - SFO





Sandy silt loam - DFOP

Figure 4.1.5-1: Kinetic fit (SFO above, DFOP below) of pethoxamid to residues (% AR) measured in soil under conditions of soil photolysis – RMS AT assessment

Table 4.1.5-7: Summary of pethoxamid degradation kinetics under conditions of soil photolysis – RMS AT assessment

Soil	Kinetic model	Parameter	Value	σ	χ^2 (%)	Prob. > t	*DegT50 / DegT90* (d)
Sandy silt loam	SFO	k (d^{-1})	0.0233	0.0058	6.4	0.001	29.7 / 98.7
	DFOP	k_1 (d^{-1})	0.937	0.327	0.80	0.011	0.7 / 2.5
		k_2 (d^{-1})	0.00806	0.00321		0.018	86.0 / 286
		g (-)	0.23	0.03		-	-
		Overall	-	-	-	-	**53.0 / 253**

DegT50 of pethoxamid under conditions of soil photolysis is considered to be 53 days (DFOP kinetics).

4.1.6 Environmental fate and other relevant information

4.1.6.1 1423 PXA (2015)

Reference: Pethoxamid: Estimation of Atmospheric Oxidation Rate.
Author(s), year: Anonymous, 2015
Report/Doc. number: CHA 100608, 1423 PXA
Guideline(s): Not applicable
GLP: Not applicable
Deviations: Not applicable
Acceptability: Yes

The atmospheric oxidation rate for pethoxamid was estimated using the Atmospheric Oxidation Program (AOPWIN v 1.92 a module of EPI-Suite™ v 4.11) which is available from the US EPA. AOPWIN estimates the rate constant for the atmospheric, gas-phase reaction between photo-chemically produced hydroxyl radicals and organic chemicals. It also estimates the rate constant for the gas-phase reaction between ozone and olefinic / acetylenic compounds. The rate constants estimated by the program are then used to calculate atmospheric half-lives for organic compounds based upon average atmospheric concentrations of hydroxyl radicals and ozone.

The overall rate constant was calculated as 109.9559 × 10^{-12} cm^3 / molecule × s. From this rate constant the half-life (for a 12 hour day, 1.6 × 10^5 OH radicals / cm^3) was estimated as 1.167 hours. As this is less than 2 days there is considered to be limited potential for long-range transport of pethoxamid.

4.1.6.2 1415 PXA (2015)

Reference: Pethoxamid: Henry's Law Constant.
Author(s), year: Anonymous, 2015f
Report/Doc. number: CHA 100581, 1415 PXA
Guideline(s): Not applicable
GLP: Not applicable
Deviations: Not applicable
Acceptability: Yes

Henry's law constant for pethoxamid calculated from available GLP experimental data at 20 °C was 1.18 × 10^{-3} Pa m^3/mole. Pethoxamid is therefore considered to be moderately volatile at 20 °C.

4.1.6.3 1344 PXA (2014)

Reference: Soil Adsorption/Desorption of [^{14}C]Pethoxamid by the Batch Equilibrium Method.
Author(s), year: Anonymous, 2014
Report/Doc. number: PTRL West Report No. 2515W-1, 1344 PXA
Guideline(s): OECD Test Guideline 106, 2000; US EPA OPPTS Guideline 835.1230, 1998
GLP: Yes
Deviations: None
Acceptability: Yes

Executive Summary:

The adsorption and desorption characteristics of [^{14}C]pethoxamid were determined in four soils which varied in texture, percent organic matter, pH (range 5.3 to 7.4) and cation exchange capacity. Preliminary trials (Tiers 1 and 2) determined the optimal soil to solution ratios for each soil (either, 1:1, 1:2 or 1:5) and the adsorption and desorption equilibration period (24 h) for the definitive study. The definitive study (Tier 3) was conducted with five concentrations of pethoxamid; 3.9, 1.0, 0.4, 0.1, and 0.04 mg/L. The overall mass balance ranged from 88.6 to 105.4 %. Freundlich adsorption coefficients related to organic carbon content (K_{foc}) for the four soils were in the range of 187 to 241 L/kg. Adsorption 1/*n* values for the four soils were in the range 0.87 to 0.91. Pethoxamid was shown to be of medium mobility according to the McCall classification.

Materials:

1. Test material: [Phenyl-U-^{14}C]pethoxamid
Systematic Name: 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenyl-1-propenyl)acetamide
Lot/Batch #: CFQ41685
Specific Activity: 29 mCi/mmol
Radiochemical purity: 98.94 %

2. Test systems: Four US soils characterised by AGVISE laboratories

Table 4.1.6-1: Physical and chemical properties of the soils used

Soil	pH ($CaCl_2$)	OC (%)	Sand USDA (%)	Silt USDA (%)	Clay (%)	CEC (mEq/100 g)	Soil texture USDA	MWHC (%)
ND L	7.4	3.8	28	45	27	25.4	Loam	39.9
CA L	7.0	0.9	38	41	21	10.1	Loam	22.2
IL SiL	5.3	0.6	18	61	21	9.5	Silty loam	23.2
ND SCL	6.4	2.6	62	17	21	17.4	Sandy clay loam	23.7

CEC - Cation exchange capacity, OC - Organic carbon, MWHC - Maximum water holding capacity

Study Design:

1. Experimental conditions

Tier 1 – ratio test:

Following overnight equilibration of the four soil systems with 0.01M $CaCl_2$ (at soil to solution ratios of 1:5, 1:25 and 1:50 for each soil in duplicate), the test item was applied in 0.01M $CaCl_2$ at a rate of 4 mg/L. The teflon tubes were sealed and shaken in the dark at 20 °C for a 24-hour period prior to analysis. Additional soil to solution ratios of 1:1 and 1:2 were also tested for the CA L and IL SiL soils using the same procedure.

Tier 2 – adsorption kinetics:

Following overnight equilibration of the four soil systems with 0.01M $CaCl_2$ at soil to solution ratios of 1:5 (ND L), 1:2 (CA L), 1:1 (IL SiL) and 1:5 (ND SCL), the test item was applied in 0.01M $CaCl_2$ at a rate of 4 mg/L. The teflon tubes were sealed and shaken in the dark at 20 °C for up to a 48-hour period prior to analysis.

Tier 3 – Freundlich isotherms:

Following overnight equilibration of the four soil systems with 0.01M $CaCl_2$ at soil to solution ratios of 1:5 (ND L), 1:2 (CA L), 1:1 (IL SiL) and 1:5 (ND SCL), the test item was applied in 0.01M $CaCl_2$ at five rates; 0.04, 0.1, 0.4, 1.0 and 3.9 mg/L. The teflon tubes were sealed and shaken in the dark at 20 °C for a 24-hour adsorption equilibrium phase prior to analysis. After decanting the adsorption solution, an equivalent amount of fresh 0.01M $CaCl_2$ was added and the tubes were sealed and shaken in the dark at 20 °C for a further 24-hour prior for desorption analysis.

2. Description of analytical procedures

From the Tier 2 test, a time period of 24 hours was found to be sufficient for reaching equilibrium. After adsorption and desorption phases, samples were centrifuged (~2700 x G, 40 min) and triplicate aliquots were taken for all samples and radioassayed by LSC. After the desorption phase, the soil pellets of both replicates of the 3.9 mg/L samples for all soils were extracted with acetonitrile:water (4:1, v/v). At least one replicate of the adsorption and desorption solutions from the 3.9 mg/L samples and one soil extract replicate were analysed by HPLC.

The amount of pethoxamid adsorbed onto soil, the Freundlich adsorption and desorption coefficients (K_f) and the Freundlich adsorption and desorption coefficients on basis of the soil organic carbon content (K_{foc}) were calculated.

Results and Discussion:

For the definitive study (Tier 3) the overall mass balance ranged from 88.6 to 105.4%. Pethoxamid was found to be stable in adsorption, desorption and soil extract solutions.

The amount of test item adsorbed onto soil, the Freundlich adsorption and desorption (K_f) coefficients and the Freundlich adsorption and desorption coefficients on basis of soil organic carbon content (K_{foc}) were calculated for each soil (where possible). The respective adsorption coefficients on the basis of soil organic carbon content

(K_{Foc}) were calculated to be 241 (ND L), 212 (CA L), 187 (IL SiL) and 208 (ND SCLl) L/kg. Corresponding values for 1/*n* were calculated to be 0.90 (ND L), 0.91 (CA L), 0.90 (IL SiL) and 0.874 (ND SCL). No correlation was found between soil adsorption and pH.

Table 4.1.6-2: Adsorption coefficients for pethoxamid in soil

Soil	Soil texture USDA	OC (%)	pH ($CaCl_2$)	Adsorption (L/kg)					
				K_f	K_{foc}	1/*n*	r^2	% adsorbed	K_d × soil/sol. ratio*
ND L	Loam	3.8	7.4	9.15	241	0.898	0.9996	68 - 77	1.7 – 2.7
CA L	Loam	0.9	7.0	1.91	212	0.908	0.9999	52 - 63	0.9 – 1.3
IL SiL	Silty loam	0.6	5.3	1.12	187	0.895	1.000	64 - 74	1.0 – 1.6
ND SCL	Sandy clay loam	2.6	6.4	5.42	208	0.874	0.9999	53 - 68	1.0 – 1.6

* Based on K_d values for individual test concentration (K_d values not shown)

Conclusions:

Adsorption and desorption isotherms of pethoxamid were studied in four US soils (ND L, CA L, IL SiL and ND SCL). Freundlich adsorption coefficients normalised for organic carbon (K_{foc}) were in the range 187 to 241 L/kg. Corresponding values for 1/*n* were in the range 0.87 to 0.91. Pethoxamid was shown to be of medium mobility according to the McCall classification.

4.1.6.4 143 PXA (1999)

Reference: TKC-94 Aged residue soil column leaching.
Author(s), year: Anonymous, 1999
Report/Doc. number: TON 020/984962, 143 PXA
Guideline(s): US EPA Subdivision N, Section 163-1, 1982
GLP: Yes
Deviations: None
Acceptability: Yes

Test system:

The leaching behaviour of the aged soil residues of ^{14}C-pethoxamid (uniform phenyl labelled, radiochemical purity > 97 %) was studied using the column leaching method. The test material was applied to sandy silt loam soil at a rate equivalent to an agricultural use rate of 1.2 kg/ha and aged under aerobic conditions (at a moisture content of 45 % maximum water holding capacity in darkness at 20 °C) for a period approximately equal to the DT_{50} value (approximately 6 days) for pethoxamid in this soil type.

Table 4.1.6-3: Characteristics of the test soil

Huntingdon Life Sciences batch number	30/498A
Textural classification (USDA classification)	
0.053-2 mm (%)	52.79
0.002-0.053 mm (%)	36.11
< 0.002 mm (%)	11.11
Textural classification	Sandy loam
Organic carbon (%)	2.7
Cation exchange capacity (mEq/100 g)	18.6
pH (1 : 5) in water	6.9
Maximum water holding capacity (%)	54.5
Water holding capacity at 0.33 bar (%)	18.9

Biomass (µg C/g)	
Time of test substance application	533.0
End of ageing period	209.1

The aged soil and (^{36}Cl) sodium chloride (to provide data on column void volume and to confirm elution) was applied to a 30 cm column of untreated soil, pre-saturated with 0.01 M calcium chloride, and the column eluted with 0.01 M calcium chloride. A volume of calcium chloride solution equivalent to rainfall to a depth of 500 mm over the cross sectional area of the column was applied over a period of approximately 48 hours and the leachate collected. Leachate and soil extracts were analysed by reverse phase HPLC and normal phase TLC. Soil residues were combusted and radio assayed (LSC) as were all extracts and leachate samples.

Findings:

The recovery of the radioactivity from the two dishes of treated soil which were taken for analysis at the end of the ageing period of 6 days were in the range 95.7 – 98.4 % AR. Extractable radioactivity represented 67.6 - 70.1 % AR, volatile radioactivity represented 7.4 % AR and non-extractable radioactivity represented 20.7 – 20.9 % AR.

^{14}C-pethoxamid total recovery of the applied radioactivity from the eluted column was 95.5 %. After elution of the equivalent of 200 mm rainfall, 3.7 % of the applied radioactivity was detected in the leachate. This comprised of pethoxamid (0.2 % applied radioactivity) and at least 9 other metabolites, all previously seen in the aerobic soil metabolism study. None of these metabolites exceeded 1 % of the applied radioactivity. After elution, equivalent to 500 mm rainfall, 18 % of the applied radioactivity was detected in the leachate, which contained 6.5 % pethoxamid and no metabolite greater than 3.4 %. Radioactivity was detected in all sections of the column, however the top section contained the majority of the radioactivity, 43.7 % applied radioactivity. Of this approximately half (21.8 % of the applied radioactivity) was unextractable and did not appear to be mobile.

The ^{36}Cl-sodium chloride recovery was 107.0 % applied radioactivity. Examination of the elution profile suggested that the majority of the ^{14}C activity was less mobile than ^{36}Cl.

Table 4.1.6-4: Vertical distribution of radioactivity in a sandy silt loam after application of aged soil treated with ^{14}C-pethoxamid at a nominal rate of 1.2 mg/kg and percolation of 500 mm of 0.01 M calcium chloride solution

Approximate depth of soil layer (cm)	Results expressed as % applied radioactivity[a]		
	Extractable	Non-extractable	Total
0-8[b]	21.9	21.8	43.7
8–13	4.3	1.3	5.6
13–18	3.7	1.2	4.9
18–23	3.8	1.3	5.1
23–28	3.7	0.8	4.5
28–33	5.0	1.3	6.3
Subtotal			70.1
Leachate			18.0
Volatile radioactivity[c]			7.4
Total recovery			95.5

[a] Radioactivity applied to the column at the start of ageing
[b] This section contained the aged soil
[c] Produced during the ageing period

Table 4.1.6-5: Proportions of radioactive components in leachate collected from a soil column after application of aged soil treated with ^{14}C-pethoxamid at a nominal rate of 1.2 mg/kg and percolation of 500 mm of 0.01 M calcium chloride solution

Component	Approximate HPLC retention time (minutes)	Results expressed as % applied radioactivity		
		Leachate		
		Portion 1[c]	Portion 2[d]	Total
		Fractions 7-27	Fractions 28-68	
Polars[a]	2 - 6	< 0.1	0.1	0.1
S1	20.0	0.3	0.4	0.7
S2	22.6	0.7	0.9	1.6
S3	23.5	0.3	0.6	0.9
S4	25.5	1.0	2.4	3.4
S5	27.0	0.4	1.3	1.7
S6	29.0	0.1	0.5	0.6
S7	30.2	0.2	0.5	0.7
S8	31.8	0.3	1.1	1.4
TKC-94	34.0	0.2	6.3	6.5
Others[b]	-	< 0.1	0.2	0.2

[a]Unresolved polar radioactivity eluting at the solvent front
[b]Regions of the chromatogram other than those specified
[c]Represents the radioactivity in leachate after elution of approximately 200 mm of 0.01 M calcium chloride over the cross sectional area of the column
[d]Represents the radioactivity in leachate after elution of approximately 500 mm of 0.01 M calcium chloride solution over the cross sectional area of the column

Summary:

Based on results obtained in this study, aged residues of pethoxamid can be classified as having a moderate potential for leaching in soil.

4.1.6.5 140 PXA (2000)

Reference: A field dissipation and leaching study to monitor the fate of TKC-94 and its relevant soil metabolites when applied, pre-emergence, to fodder maize in the United Kingdom 1998
Author(s), year: Anonymous, 2000
Report/Doc. number: JF4204 (TON 022), 140 PXA
Guideline(s): SETAC, 1995; OEPP/EPPO Bulletin 23, 27-49, 1993; BBA-Richtlinie Teil IV, 4 1, 1986
GLP: Yes
Deviations: None
Acceptability: Yes

Executive Summary (leaching):

A field leaching study has been performed to monitor the fate of pethoxamid and MET-42 when applied pre-emergence to fodder maize in the United Kingdom. The experimental site was established at Barrow-upon-Trent (England) on a sandy soil with shallow groundwater. The site consisted of a single non-treated plot and two replicated treated plots, all cropped with fodder maize. Soil water samples were collected using suction samplers installed at 100 cm depth (six replicates per treated plot, three replicates in the untreated plot) to assess the leaching potential of pethoxamid and MET-42. The product (599 g L^{-1} EC) was applied to each treatment plot at a rate of 2.0 L p.p.p. ha^{-1}. Bromide was also applied as an inert tracer. The leaching plots were monitored on a routine basis (twice monthly for the first 2 months and monthly thereafter) and on a trigger

basis. Trigger conditions were defined as 10 mm of rainfall over a 24-hour period or 15 mm over a 48-hour period. Quantification of pethoxamid and MET-42 in water was performed by LC-MS. The limit of detection for pethoxamid and MET-42 was 0.05 µg L^{-1} and 0.02 µg L^{-1}, respectively. The limit of quantification was 0.1 µg L^{-1} for both compounds. Concentrations of bromide were quantified by ion chromatography. Daily rainfall was recorded at the experimental site using an automated meteorological system.

Climatic conditions during the study were considerably wetter than average with total rainfall for the year after application approximately 132 % of the long-term average. The soil hydrological conditions at the time of application were close to field capacity. Precipitation in the month following application exceeded the long-term average by approximately 170 %. The concentration of bromide in water showed breakthrough to 100 cm depth occurred between 28 to 35 DAT.

Soil water samples were collected on 15 separate occasions following test substance application. Pethoxamid was detected in 2 of the 175 samples collected from 100 cm depth. Both concentrations were at the limit of quantification (0.1 µg L^{-1}) and were detected in the water from the same sampler at 28 and 33 DAT. Residues of MET-42 were detected in 151 of the 171 soil water samples collected, at concentrations ranging from 0.1 to 11.2 µg L^{-1}. Average concentrations for individual samplers over the study were between 0.6 and 3.2 µg L^{-1}.

Test system:

Product: ASU 96520H; Batch no. 9802, Composition: TKC-94 599 g/l formulated as an emulsifiable concentrate.

The experimental site was established at Barrow-on-Trent (England) on a sandy soil with shallow groundwater. Details of the soil characteristics and microbial biomass are given in the table below.

Table 4.1.6-6: Test site soil characteristics

Parameter	Soil Horizon (depth in cm)					
	0 - 31	31 - 45	45 - 58	58 - 80	80 - 112	112 - 125
Sand (%) 2000 – 63 µm	61.0	65.3	82.6	91.3	95.5	92.4
Silt* (%) 63 – 2 µm	24.2	22.9	11.0	4.2	1.7	2.2
Clay (%) < 2 µm	14.8	11.9	6.4	4.7	2.9	5.4
pH (1:5) in water	7.2	7.2	7.0	7.3	7.2	7.3
Cation exchange capacity (mEq/100 g)	20.2	13.6	7.5	3.1	1.1	1.8
Organic carbon (%)	3.0	0.7	0.4	0.2	0.1	0.1
Soil microbial biomass (mg C/100 g soil), topsoil only	Prior to application:			18.82 (Plot JF4204-03)		
	End of study:			14.98 (Plot JF4204-02)		
				27.41 (Plot JF4204-02)		

* UK silt split

The site consisted of a single non-treated plot (JF4204-03) and two replicated treated plots (JF4204-01 and JF4204-02), all cropped with fodder maize. Each plot was divided into 12 equally sized sub-plots. Eleven were designated for the collection of soil cores to monitor the dissipation of pethoxamid and its metabolite MET-42. The remaining sub-plot was designated for evaluation of the leaching potential of pethoxamid and MET-42. Soil water samples were collected using suction samplers installed at 100 cm depth (six replicates per treated plot, three replicates in the untreated plot) to assess the leaching potential of pethoxamid and MET-42. Prior to application, the hydrological status of the site was monitored. The soil microbial biomass was determined prior to application and at the end of the field phase.

On 8th of May 1998 (pre-emergence), ASU 96520H was applied to each treatment plot at a nominal rate of 2000 ml/ha using a research backpack boom sprayer. Potassium bromide was applied as an inert tracer to each leaching sub-plot within two hours of the application of pethoxamid. The leaching sub-plots were monitored

on a routine basis (twice monthly for the first 2 month and monthly thereafter) and on a trigger basis. Trigger conditions were defined as 10 mm of rainfall over a 24-hour period or 15 mm over a 48-hour period.

Soil samples for residue analysis were taken from each plot using an automated 'zero contamination' soil coring system. Soil samples were taken at predetermined intervals. At each sampling occasion, a total of 20 soil cores (5 cm diameter by 50 cm length) were collected from one sub-plot, combined and frozen on the day of sampling until analysis. A separate sub-plot was used on each sampling occasion. Plot dimensions and treatment detail are shown in the Table below.

Table 4.1.6-7: Plot dimensions and treatment detail

Plot number	Treatment	Plot width (m)	Plot length (m)	Plot area (m^2)
JF-4204-01	Treated, cropped	17	36	612
JF-4204-02	Treated, cropped	17	36	612
JF-4204-03	Untreated, cropped	17	36	612

For analysis, each soil sample was cut into 0-10, 10-20 and 20-30 cm horizons. Pethoxamid and MET-42 were quantified in the soil from the 0-10 and 10-20 cm horizons. The soil was sieved and extracted with aqueous acetone and concentrated. Clean up of the water samples was performed by solid phase extraction. Quantification of pethoxamid and MET-42 was performed by LC-MS. The limit of detection (LOD) was 2 ng/g for both compounds in soil. The LOD for pethoxamid and MET-42 in water was 0.05 µg/l and 0.02 µg/l respectively. The limit of quantification (LOQ) for pethoxamid and MET-42 was 10 ng/g in soil and 0.1 µg/l in water. Concentrations of bromide in sub-samples of soil water were quantified by ion chromatography. The limit of detection for this technique was 1.10 mg/l.

Daily rainfall was recorded at the experimental site using an automated meteorological system. The mean minimum and maximum air temperature and monthyl rainfall totals for the duration of the experimental phase are summarised in the table below. On occasions of malfunction, daily rainfall from Sutton Bonington (approximately 16 km east-south-east of the experimental site) was used instead of site measurements, as indicated.

Table 4.1.6-8: Meteorological data

Month/year	Monthly mean minimum air temperature (°C)	Monthly mean maximum air temperature (°C)	Monthly rainfall Total (mm)
May 1998	7.6	17.2	18.3 (1)
June 1998	10.5	18.4	144.4
July 1998	11.6	19.7	22.2
August 1998	12.1	20.9	64.0
September 1998	11.2	18.4	67.0
October 1998	7.9	13.9	110.6
November 1998	2.7	8.6	30.2
December 1998	2.3	8.7	61.8
January 1999	2.5	8.5	86.2 (2)
February 1999	2.2	8.2	39.2 (3)
March 1999	4.5	10.9	96.2
April 1999	5.2	13.8	58.4 (4)
May 1999	8.7	17.4	57.8
June 1999	9.3	18.4	80.6
July 1999	12.5	22.9	16.2

(1) 1-8 May 1998 rainfall obtained from Sutton Bonington

(2) 5-31 January 1999 rainfall obtained from Sutton Bonington
(3) 1-28 February 1999 rainfall obtained from Sutton Bonington
(4) 26-30 April 199 rainfall obtained from Sutton Bonington

Findings:

Climatic conditions during the study were considerably wetter than average with total rainfall for the period May 1998 to April 1999, approximately 132 % of the long term average. The soil hydrological conditions at the time of application were close to field capacity. Following application, precipitation during June 1998 exceeded the long-term average by approximately 170 % and thus resulted in unseasonable leaching conditions very soon after application. The concentrations of bromide in water showed breakthrough to 100 cm depth occurred between 28 to 53 DAT, providing supportive data that leaching conditions were present at the site soon after application. Results for both plots show two marked periods of leaching with peak concentrations being detected either in early June 1998 or during the period October to December 1998. Between June and September 1998 greater leaching occurred on plot JF4204-01 than JF4204-02. From October to December 1998 concentrations in soil water were broadly similar for both treated plots.

Soil samples
Procedural recoveries for the analysis of pethoxamid and MET-42 in soil and water samples all fell within the range 70 – 110 %.

No quantifiable residues of pethoxamid or MET-42 were detected in any of the samples (soil or water) taken from the control plot. Mean concentrations of pethoxamid and MET-42 found in the 0–10 cm and 10–20 cm soil horizons for the treated plots are summarised in the table below.

Table 4.1.6-9: Concentrations (ng/g) of pethoxamid and MET-42 found in 0–10 cm and 10–20 cm horizons from treated plots

Plot number	DAT	Amount of pethoxamid (ng/g)		Amount of MET-42 (ng/g)	
		0–10 cm	10–20 cm	0–10 cm	10–20 cm
Plot JF4204-01 + JF4204-02	-1	n.d	n.d	n.d	n.d
Plot JF4204-01 + JF4204-02	0	808	< LOQ	n.d	n.d
Plot JF4204-01 + JF4204-02	3	715	n.d	n.d	n.d
Plot JF4204-01 + JF4204-02	7	692	< LOQ	< LOQ	n.d
Plot JF4204-01 + JF4204-02	14	492	< LOQ	< LOQ	n.d
Plot JF4204-01 + JF4204-02	28	445	< LOQ	< LOQ	n.d
Plot JF4204-01 + JF4204-02	60	67	n.d	10	< LOQ
Plot JF4204-01 + JF4204-02	90	35	n.d	< LOQ	n.d
Plot JF4204-01 + JF4204-02	119	28	n.d	< LOQ	< LOQ
Plot JF4204-01 + JF4204-02	182	n.d	< LOQ	n.d	n.d
Plot JF4204-01 + JF4204-02	362	n.d	< LOQ	n.d	n.d

DAT = days after treatment; Plot 1 + 2 = replicated treated plots; n.d = not detected (< 2 ng/g); LOQ = limit of quantification (< 10 ng/g)

Water samples
To investigate the leaching potential of pethoxamid and MET-42, soil water samples were collected on 15 separate occasions following test substance application. Pethoxamid was detected in 2 of the 175 samples of soil water collected from 100 cm depth from the two treated plots. Both concentrations were at the limit of quantification (0.1 µg/l) and detected in the water from the same sampler at 28 and 33 DAT. Residues of MET-42 were detected in 151 of the 171 soil water samples collected post-application from the treated plots, at concentrations ranging from 0.1 to 11.2 µg/l. Maximum concentrations of MET-42 were detected either in early June 1998 or later between December 1998 and January 1999. Average concentrations for individual samplers over the study were between 0.6 and 3.2 µg/l. Concentrations of MET-42 in soil water collected at 100 cm from each plot are summarised in the table below.

Table 4.1.6-10: Concentrations (µg/l) of MET-42 in soil water collected at 100 cm depth from treated and control plots

Sampler ID	Timepoint (DAT)																
	Results are expressed as µg/l																
	Plot	PT	28	33	40	53	109	123	172	207	223	243	256	294	308	340	350
S1	1	n.d	0.8	2.3	7.5	5.6	1.4	0.8	1.7	3.3	6.7	3.9	4.0	1.6	1.3	1.2	1.0
S2	1	n.d	1.3	3.1	11.2	4.3	3.4	1.4	1.5	1.9	2.5	3.4	2.6	2.6	1.6	1.1	1.2
S3	1	n.d	0.3	2.3	8.7	5.5	6.4	2.5	2.9	3.3	4.7	3.4	2.7	2.0	1.3	0.8	0.6
S4	1	n.d	1.8	1.5	1.7	1.1	0.2	0.2	0.3	1.7	3.8	3.3	5.1	3.0	1.9	1.4	1.7
S5	1	n.d	3.4	2.3	3.8	3.9	1.5	i.s	2.5	3.4	3.6	2.6	3.1	2.5	1.5	0.8	0.9
S6	1	n.d	n.d	0.4	1.0	1.9	n.s	i.s	1.0	1.8	1.4	2.6	1.9	2.2	1.8	1.3	0.9
S7	2	n.d	n.d	0.3	1.1	1.2	1.1	0.8	0.5	2.0	2.7	3.1	3.8	3.0	1.8	1.5	1.3
S8	2	n.d	n.d	n.d	n.d	0.5	n.d	n.d	0.1	1.0	2.2	5.5	3.9	1.9	1.2	0.9	0.9
S9	2	n.d	n.d	n.d	n.d	n.d	0.2	0.2	0.1	0.4	0.7	0.7	1.5	1.9	1.3	0.9	0.8
S10	2	n.d	0.2	1.3	7.8	5.8	0.5	n.s	n.s	4.3	5.0	3.0	2.3	1.4	1.1	0.9	0.7
S11	2	n.d	n.d	n.d	n.d	n.d	n.d	i.s	n.d	1.0	2.5	1.1	2.1	1.8	2.1	1.6	1.6
S12	2	n.d	n.d	n.d	0.4	n.s	0.1	i.s	0.3	n.d	n.s	2.8	3.5	2.5	1.8	1.6	1.3
S13	3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S14	3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
S15	3	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

n.d – not detected (LOD – 0.02 µg/l); n.s- no sample; i.s – insufficient sample; PT – pre-treatment; Plot 1 + 2 - replicated treated plots; Plot 3 – control plot

Summary:

The experimental conditions in this study are considered to give a worst-case for leaching. Despite this, pethoxamid showed negligible potential for leaching to 100 cm depth with only 2 of 175 soil water samples from the treated plots containing residues (each at 0.1 µg/l). MET-42 showed a significant potential to leach to 100 cm depth under the worst-case conditions of the study.

4.2 Bioaccumulation

4.2.1 Estimated bioaccumulation

4.2.1.1 41 PXA (1996)

Reference: Determination of the Partition Coefficient of TKC-94 (neat) in n-Octanol/Water.
Author(s), year: 41 PXA, 1996
Report/Doc. number: Study No: AB 90001- PC-054. Cheminova A/S Report No.: 41 PXA.
Guideline(s): B.2.7/01 EEC A8/ OECD 107 (Shake flask method)
GLP: Yes
Deviations: No
Acceptability: Yes

Materials:

Lot/batch number: TP- 940421
Purity: ≥99.9%

Complete phase separation reported. Log P_{ow} = 2.963 ± 0.02 at 20°C (pH 5).The effect of pH was not necessary because compound is not ionized between pH 4 and 10.

4.2.2 Bioaccumulation test on fish

4.2.2.1 154 PXA (2000)

Reference: TKC-94 : Bioconcentration in Rainbow Trout.
Author(s), year: 154 PXA, 2000
Report/Doc. number: Report No. TON 054/992423. CHA Doc. No. 154 PXA.
Guideline(s): OECD 305
GLP: Yes
Deviations: The reported BCF value was normalised for 6% lipid content and not 5%. This deviation is not considered to impact the validity of the BCF value.
Acceptability: Yes

Test substance:	technical pethoxamid
Radiochemical Purity:	> 97%
Batch:	CFQ10530
Guideline:	OECD 305
Test species:	*Oncorhynchus mykiss*
Exposure mode:	flow-through
Conc. levels (nom.):	0.0015, 0.015 mg/L
Conc. levels (meas.):	> 80 %
Duration depuration phase:	56 d
Results related to:	nominal concentrations
Maximum BCF:	33 after 14 d (steady-state achieved)
Depuration:	elimination > 90 % after 56 d
ct50/ct90:	- / - d

The bioaccumulation of radioactivity by Rainbow Trout was studied during 28 days exposure, under dynamic conditions, to TKC-94. Nominal exposure levels of 0.0015 and 0.015 mg/L were used. The elimination of radioactivity was studies during a depuration period of 56 days.

Steady state BCF = 33 after 14 d (kinetic BCF = 47 – 50). Elimination >90% after 56 d.

TKC-94, MET-30, MET-42 and MET-47 were identified using reverse phase HPLC and normal phase TLC.

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

4.3.1.1 151 PXA (1999a)

Reference: TKC-94: Acute Toxicity for Rainbow Trout (*Oncorhynchus mykiss*) **Author(s), year**: 151 PXA, 1999a **Report/Doc. number**: TON 038/984981; Cheminova A/S Report No.: 151 PXA + amdt1 (3 pages) **Guideline(s)**: OECD Guideline 203 (1992); EPA Guideline 72-1 **GLP**: Yes **Deviations**: None relevant **Validity**: Acceptable

Material and methods:

Test substance: Petoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: Rainbow Trout (*Oncorhyncus mykiss*)

Number of organisms: 10 fish per test concentration and control, 2 replicates

Age, length, weight: Age not reported; 3.6 cm (SD 0.2 cm), 0.81 g (SD 0.08g)

Loading: 0.41 g bw/L

Type of test: Semi-static acute toxicity test, daily renewal

Applied concentrations:
Nominal: 0; 1; 1.8; 3.2; 5.8; 10 mg/L
Measured (mean): 0; 1.1; 1.7; 2.5; 4.7; 8.3 mg/L

Solvent: None

Test conditions:
Water quality: Purified tap water
Conductivity: 360 µs/cm
Temperature: 12-15 °C
pH: 7.4 - 7.8
O_2 content: 8.0 - 8.7 mg O_2/L
Light regime: 16 h light / 8 h dark
Feeding Feeding with fry pellets during acclimatisation until 48 hours before exposure, no feeding during exposure.

Methods: The test was carried out in glass aquaria of ca. 20 litre capacity with approx. dimensions of 25 x 46 x 25 cm. At the initiation of the study ten fish were allocated at random to each test vessel.

Test parameters: Observations on mortality and subjective assessments for sublethal effects at 3, 6, 24, 48, 72 and 96 h.

Analytical measurements: Samples of fresh and expired test solutions were taken for quantitative and qualitative analysis.

Statistics: EC_{50} and 95 % confidence limits calculated (Thompson and Weil, 1952)

Results:

Analytical data: Mean measured concentrations ranged from 86 to 111 % of nominal at 0 h and 82 to 92 % at 96 h.

Biological effects: Increased pigmentation, resting on the bottom of the tank, loss of equilibrium, hyperventilation, gasping, swimming at surface, erratic swimming behaviour were observed starting at the 3.2 mg/L concentration group.

Mortality : 70 % at 3.2 mg/L (nom.) and 100 % at higher concentration levels.

Conclusion: The 96h- LC_{50} for rainbow trout was 2.2 mg a.s./L (mean measured). NOAEC 1.7 mg a.s./L

4.3.1.2 152 PXA (1999b)

Reference: TKC-94 : Acute Toxicity for Bluegill Sunfish (*Lepomis macrochirus*)
Author(s), year: 152 PXA., 1999b
Report/Doc. number: TON 037/984980; Cheminova A/S Report No.: 152 PXA + amdt1 (3 pages)
Guideline(s): OECD Guideline 203 (1992); EPA Guideline 72-1
GLP: Yes
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: Bluegill Sunfish (*Lepomis macrochirus*)

Number of organisms: 10 fish per test concentration and control, 2 replicates

Age, length, weight: Age not reported; 3.8 cm (SD 0.2 cm), 1.54 g (SD 0.27g)

Loading: 0.77 g bw/L

Type of test: Semi-static acute toxicity test, daily renewal

Applied concentrations:
Nominal: 0; 1; 1.8; 3.2; 5.8; 10; 18 mg/L
Measured (mean): 0.81, 1.6, 2.7, 5.1, 8.5, 15 mg/L

Solvent: None

Test conditions:
Water quality: Purified tap water
Conductivity: 360 µs/cm
Temperature: 20-22°C
pH: 7.0 – 7.4
O_2 content: 5.1 - 9.0 mg O_2/L
Light regime: 16 h light / 8 h dark
Feeding Feeding with fry pellets during acclimatisation until 48 h before exposure, no feeding during exposure

Methods: The test was carried out in glass aquaria of ca. 20 litre capacity with approx. dimensions of 25 x 46 x 25 cm. At the initiation of the study ten fish were allocated at random to each test vessel.

Test parameters: Observations on mortality and subjective assessments for sublethal effects at 0.25, 3, 6, 24, 48, 72 and 96 h.

Analytical measurements: Samples of fresh and expired test solutions were taken for quantitative and qualitative analysis.

Statistics: EC_{50} and 95 % confidence limits calculated (Thompson and Weil, 1952)

Results:

Analytical data: Mean measured concentrations ranged from 81 to 91% of nominal at 0 h and 85 to 92 % at 96 h.

Biological effects: Increased pigmentation, resting on the bottom of the tank, swimming at surface were observed starting at the 5.8 mg/L concentration group.

Mortality: Complete mortality at 8.5 and 15 mg/L.

Conclusion: The 96h- LC_{50} for *L. macrochirus* was 6.6 mg a.s./L (mean measured). NOAEC 2.7 mg a.s./L

4.3.1.3 1177 PXA (2013)

Reference: Pethoxamid Technical: Acute Toxicity to the Sheepshead Minnow, *Cyprinodon variegatus*, Determined Under Static Conditions in Warm Salt Water
Author(s), year: 1177 PXA, 2013
Report/Doc. number: 69648; Cheminova A/S, Unpublished report No.: 1177 PXA
Guideline(s): OECD Guideline 203 (1992); EPA Guideline OPPTS 850.1075
GLP: Yes
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Sheepshead minnow (*Cyprinodon variegatus*)

Acclimation/holding: 14 days (<5% mortality)

Number of organisms: 10 fish per test concentration and control, 2 replicates

Age, length, weight: Age not reported; 2.9 cm (SD 0.14 cm), 0.439 g (SD 0.09g)

Loading:0.549 g bw/L

Type of test: static

Applied concentrations:
Nominal: 0 (control), 0.65, 1.3, 2.5, 5.0, and 10 mg a.s./L
Measured (mean): < MQL (control), 0.608, 1.20, 2.36, 4.94, and 10.2 mg a.s./L

Solvent: None

Test conditions:

Water quality: commercial saltwater mix with laboratory freshwater; Hardness: 130 to 160 mg/L $CaCO_3$, Salinity: 19.5 – 19.9 $^0/_{00}$
Conductivity: not reported
Temperature: 21.8 - 22.4°C
pH: 7.6 – 8.1
O_2 content: 61 to 97% saturation (4.6 to 7.4 mg/L)
Light regime: 16 h light / 8 h dark, 1064 lux
Feeding: fish food and brine shrimp at least once per day during acclimation, no feeding during exposure

Methods: 10-L glass jars containing approximately 8 L of medium

Test parameters: Mortality and other observations were made after 0, 6, 24, 48, 72 and 96 hours. Test solutions were sampled at time 0, 48-h and 96-h.

Analytical measurements: Stock solutions were analysed after preparation. Test solutions were sampled at the start of the test and at 24-hour intervals and analysed using HPLC-UV.

Statistics: All statistical analyses were performed with SAS software (version 9.3). Estimates of LC_{50} values and their 95% confidence limits were calculated using the probit method and Trimmed or Untrimmed Spearman-Karber method. The NOEC was determined by using Fisher's one-tailed exact test and was based on both mortality and sub-lethal effects (if observed).

Results:

Analytical data: Measured mean concentrations in the fortified samples were 101 to 105% of nominal and overall measured concentrations of the test concentration were 92 to 102% of nominal (between the start and the end of the study). Hence, results may be expressed based on nominal concentrations.

Biological effects: After 96 hours of exposure, mortality was 0, 0, 0, 0, 95, and 100% in the 0 (control), 0.65, 1.3, 2.5, 5.0, and 10.0 mg a.s./L treatments (nom.), respectively. Sublethal effects were observed in the 10.0 and 5.0 mg a.s./L treatments after 6- and 24-hours, respectively, and throughout the remainder of the test. Sub-lethal effects included discoloration, surfacing, fish on the bottom of test chambers, and loss of equilibrium.

Table 4.3.1-1: Mortality

Test concentration [mm, mg a.s./L]	Mortality [%] (no. of dead fish / 20 treated fish)					
	6 h	24h	48 h	72 h	96 h	Mean % mortality
Control	0	0	0	0	0	0
0.608	0	0	0	0	0	0
1.20	0	0	0	0	0	0
2.36	0	0	0	0	0	0
4.94	0	0	1	13	19	95
10.2	0	0	20	20	20	100

Conclusion: The 96-hour LC_{50} of pethoxamid technical to sheepshead minnow was 3.66 mg a.s./L based on nominal concentrations (equivalent to 3.54 mg a.s./L based on mean measured concentrations). The corresponding NOEC was 2.5 mg a.s./L (2.36 mg a.s./L).

4.3.2 Short-term toxicity to aquatic invertebrates

4.3.2.1 155 PXA (1999a)

Reference: TKC-94: Acute Toxicity to *Daphnia Magna*
Author(s), year: 155 PXA, D.C. (1999a)
Report/Doc. number: TON '042/984982 ; Cheminova A/S Report No.: 155 PXA + amdt 1 (3 pages)
Guideline(s): OECD Guideline 202 I, EPA 72-2
GLP: Yes
Deviations: None
Validity: Acceptable

Material and methods:

Test substance:	Pethoxamid techn. (TKC-94), Lot-No.: TB-960306-C; purity: 94.8%
Test species:	Water flea (*Daphnia magna*)
Number of organisms:	4 replicates per treatment and control, each replicate containing 5 daphnids
Age:	First instar, < 24 h old
Type of test, duration:	Static test, 48 hours
Applied concentrations:	
Nominal:	0; 1; 1.8; 3.2; 5.8; 10; 18; 32 mg a.s./L
Mean measured:	0; 0.82; 1.6; 2.9; 5.1; 9.1; 17; 29 mg a.s./L
Solvent:	None
Test conditions:	
Water quality:	Reconstituted Elendt M4 medium
Temperature:	19-20 °C
pH:	7.4 – 7.7
O_2 content:	8.5 – 8.6 mg O_2/L
Light regime:	16 hours light / 8 hours darkness
Food:	none during exposure
Test parameters:	Observations for immobilised daphnids were recorded at 24 and 48 hours of exposure.
Analytical measurement:	Test concentrations were verified by chemical analysis at 0 and 48 h.
Statistics:	EC50 and 95% confidence limits calculated (Thompson and Weil, 1952)

Results:

Analytical measurements:	Measured concentrations ranged from 86 to 92% of nominal at 0h and from 79 to 92% of nominal at 48h.
Effects:	Cumulative immobilisation in the groups 0, 0.82, 1.6, 2.9, 5.1, 9.1, 17 and 29 mg/L was 0, 5, 10, 20, 15, 10, 0 and 90 %. At least 5 % immobilisation occurred in all test groups < 17 mg/L, however, a level of < 10 % immobilisation is considered not

biological significant. At 2.9 and 5.1 mg/L 20 and 15 % immobilisation, respectively, was observed after 48 h. However, as no significant effects were observed at the two higher test groups this is considered anomalous and has not been considered in the determination of the NOEC.

Conclusion: EC_{50} (48 h) 23 mg a.s./L (mean measured)
NOEC 17 mg a.s./L

4.3.2.2 1176 PXA (2014a)

Reference: Pethoxamid Technical: Acute Toxicity Test with the Mysid Shrimp, *Americamysis bahia*, Determined Under Static Conditions
Author(s), year: 1176 PXA, 2014a
Report/Doc. number: 69650; Cheminova A/S, Unpublished report No.: 1176 PXA
Guideline(s): U.S.EPA OPPTS 850.1035
GLP: Yes (certified laboratory)
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Mysid shrimp (*Americamysis bahia*)

Number of organisms: 4 replicates each with 5 mysid shrimp per treatment and control

Age: Juveniles, < 24 hours old

Type of test, duration: Static test, 96 hours

Applied concentrations:
Nominal: 0 (control), 0.65, 1.3, 2.5, 5.0 and 10.0 mg a.s./L
Mean measured: - (control), 0.637, 1.26, 2.47, 4.96 and 10.3 mg a.s./L

Solvent: none

Test conditions:
Water quality: Synthetic sea water , salinity 19.5 - 20.2 $^{0}/_{00}$, hardness 130- to 160 mg/L $CaCO_3$
Temperature: 24.1 - 25.2°C
pH: 7.6 - 8.2
O_2 content: 42 to 110% saturation (3.0 to 7.9 mg/L)
Light regime: 14 hours light / 10 hours darkness, 654 Lux

Test parameters: Assessment of mortality and sub-lethal effects were performed at 24-h intervals. Mysids were provided with brine shrimp nauplii daily as a food source.

Analytical measurements: Samples of fresh stock treatment solutions and of composite 96-h aged test solutions were taken for analysis by a validated HPLC-UV method to determine pethoxmid concentration. There were no visible signs of undissolved precipitate or surface film in any treatment group.

Statistics: LC_{50} estimates were made using probit analysis with Trimmed Spearman-Karber method where Goodness of fit during probit resulted in P<0.05. The NOEC estimate was performed using Fisher's Exact Test (one-tailed). All statistical analysis was performed using SAS version 9.3.

Results:

Analytical measurements: Recoveries were 102 to 106% of nominal at test start and 90 to 101% after 96 hours.

Biological effects: After 96 hours mortality was 5.0% in the control compared to 0.0, 0.0, 0.0, 40 and 100% at nominal test concentrations of 0.65, 1.3, 2.5, 5.0 and 10.0 mg a.s./L, respectively. There were no sub-lethal observations in any treatment group at any time-point during the study.

Table 4.3.2-1: Summary of *Mysid* mortality (%) over 96-hours exposure to pethoxamid

Nominal concentration (mean measured) mg a.s./L	Mortality (%) (n initial =5)			
	24 hours	48 hours	72 hours	96 Hours
0 (control)	0	0	5	5
0.65 (0.637)	0	0	0	0
1.3 (1.26)	0	0	0	0
2.5 (2.47)	0	0	0	0
5.0 (4.96)	0	10	25	40*
10.0 (10.3)	85	95	95	100*
*Statistically significant				

Table 4.3.2-2: LC_{50} estimates for *Mysid* shrimps exposed to pethoaxmid over 96 hours (nominal concentrations)

Time period	Nominal Concentration (mg a.s./L)		
	LC_{50}	95% confidence limits	NOEC
24 hours	7.5	7.0 – 8.1	-
48 hours	6.8	5.9 – 7.9	-
72 hours	6.2	5.2 – 7.3	-
96 hours	5.4	4.6 – 6.3	2.5

Table 4.3.2-3: LC_{50} estimates for *Mysid* shrimp exposed to pethoaxmid over 96 hours (mean measured concentrations)

Time period	Mean measured Concentration (mg a.s./L)		
	LC_{50}	95% confidence limits	NOEC
24 hours	7.62	7.03 – 8.27	-
48 hours	6.83	5.86 – 8.07	-
72 hours	6.19	5.21 – 7.41	-
96 hours	5.40	4.62 – 6.31	2.47

Conclusion: In a 96-h acute toxicity test with the Mysid shrimp, *Americamysis bahia*, the LC_{50} estimate for pethoxamid was 5.4 mg a.s./L (nominal and mean measured) and the 96-h NOEC was 2.5 mg a.s./L (nominal) and 2.47 mg a.s./L (mean measured).

4.3.2.3 1207 PXA (2014b)

Reference: Pethoxamid Technical: Effect on new shell growth of the Eastern Oyster (*Crassostrea virginica*)
Author(s), year: 1207 PXA, 2014b
Report/Doc. number: 69649; Cheminova A/S, Unpublished report No.: 1207 PXA
Guideline(s): U.S.EPA OPPTS 850.1025
GLP: Yes (certified laboratory)
Deviations: None relevant
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Eastern oyster (*Crassostrea virginica*), 31.1 to 41.8 mm in valve height and acclimated before test start

Number of organisms: 2 replicates with 10 organisms per treatment and control groups

Type of test, duration: Flow-through, 96 hours

Feeding: A marine microalgal concentrate was added periodically during each day as a food source and was also provided via the diluter water.

Applied concentrations:
Nominal: 0 (control), 1.3, 2.2, 3.6, 6.0 and 10.0 mg a.s./L
Mean measured: <LOQ (control), 1.18, 2.01, 3.33, 5.53 and 9.53 mg a.s./L

Solvent: None

Test conditions:
Water quality: Laboratory saltwater, hardness: 130 - 160 mg/L CaCO3
Temperature: 19.8 – 20.9 °C
pH: 7.5 – 8.2
O_2 content: 59% to 97% saturation (4.7 to 7.7 mg/L) (one replicate at 59% at test termination)
Salinity: 19.6 – 20.0 $^{0}/_{00}$
Light regime: 16 hours light / 8 hours darkness, 756 lux
Test system: Total daily volume through-flow was approximately 52 L, or 6 chamber additions. Chambers were glass tanks 21.7 x 37.0 x 17.8 cm, media volume and depth were maintained at approximately 8.4 L and 10.5 cm, respectively, each contained a pump to continuously recirculate the media. Prior to introduction each Oyster was prepared by grinding 3-5 mm of the ventral shell edge.

Test parameter: Observations on mortality and signs of test substance effects were performed daily and new shell growth was measured at test termination using Vernier calipers to the nearest 0.1 mm. Test solution temperature, dissolved oxygen, salinity and pH were measured daily. Test solution samples were collected at prior to test initiation, at initiation and at termination using a validated HPLC-UV method.

During the test, monitoring of water quality parameters included: daily measurement of temperature, pH and dissolved oxygen concentrations in the control and each test solution until test termination or until 100% mortality had occurred.

Analytical measurements: Test solutions were collected at test start and test end, and diluter stock at each sampling point, for analysis of pethoxamid using a validated HPLC-UV method.

Statistics: EC_{50} estimates for new shell growth were performed using four-parameter logistic (sigmoid-shaped) model, two parameters fixed (100 and 0% inhibition), fit to the data with percent inhibition as the dependant variable and log concentration as the independent variable. The NOEC for new shell growth was determined using oneway ANOVA and a one-tailed Dunnett's and William's test ($\alpha = 0.05$). All statistical analysis was performed using SAS version 9.3.

Results:

Analytical measurements: Stock solutions were 101% to 102% of nominal. Recoveries were 87% to 94% of nominal at test start and 85% to 96% after 96 hours, treatment mean measured recoveries for the 96h duration were 91% to 95%.

Validity Criteria: Control mean survival was 100%, new growth was 3.6 mm achieving the criteria of 90% and ≥2 mm, respectively, therefore the control validity criteria was achieved. No evidence of spawning.

Biological effects: After 96 hours there was no mortality in the control or in any treatment group.

Fecal matter was observed in all control and test substance chambers. The most fecal matter was observed in the 0, 1.18, and 2.01 mg a.s./L treatment concentrations, while the 3.33, 5.53, and 9.53 mg a.s./L treatments had reduced fecal matter as compared to the next lower treatment.

Mean new shell growth was 3.6 mm in the control compared to 3.0, 2.6, 1.9, 0.9 and 0.6 mm in the 1.18, 2.01, 3.33, 5.53 and 9.53 mg a.s./L treatments, respectively.

No evidence of spawning was observed in any treatment group.

The NOEC could not be determined using William's test (P<0.05) due to a 17% reduction in shell growth at the lowest concentration (Dunnett's Test gave a NOEC of 1.18 mg a.s./L).

Table 4.3.2-4: Summary of new shell deposition in the Easter Oyster during 96-hours exposure to pethoxamid

Nominal concentration (mean measured) mg a.s./L	**Mean length (mm)**	**Percent inhibition compared to the control**
0 (control)	3.6	-
1.3 (1.18)	3.0	17[a]
2.2 (2.01)	2.6	28*[a]
3.6 (3.33)	1.9	47*[a]
6.9 (5.53)	0.9	75*[a]
10.0 (9.53)	0.6	83* [a]
*Statistically significant (Dunnett's test p<0.05) [a]Statistically significant (William's test p<0.05)		

Table 4.3.2-5: EC_{50} and NOEC estimates for new shell deposition in the Easter Oyster, *Crassostrea virginica* during 96h exposure to pethoxamid

Results based on:	Time	EC_{50} (mg a.s./L)	95% confidence limits (mg a.s./L)	NOEC (mg a.s./L)
Mean measured concentrations	96h	3.28	2.73 to 3.82	*1.18*[a]
Nominal concentrations	96h	3.6	3.0 to 4.1	*1.3*[a]
[a] Not significant based on Dunnett's test, significant based on William's test (p<0.05).				

Conclusion: In a 96-h shell deposition test with the Easter Oyster, *Crassostrea virginica* the EC_{50} estimate for pethoxamid was 3.6 mg a.s./L (nominal) and 3.28 mg a.s./L (mean measured). No NOEC could be determined.

4.3.2.4 1348 PXA (2014)

Reference: Pethoxamid Technical: Pethoxamid Technical: Effect on new shell growth of the Eastern Oyster (*Crassostrea virginica*) **Author(s), year**: 1348 PXA, 2014 **Report/Doc. number**: 81338; Cheminova A/S, Unpublished report No.: 1348 PXA **Guideline(s)**: U.S.EPA OPPTS 850.1025 **GLP**: Yes (certified laboratory) **Deviations**: None **Validity**: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 96.2 % w/w. Batch No. : P1351-JaK-T2-23-6

Test species: Eastern oyster (*Crassostrea virginica*), 31.7 to 49.7 mm in valve height and acclimated before test start

Number of organisms: 2 replicates with 10 organisms per treatment and control groups

Type of test, duration: Flow-through, 96 hours

Feeding: A marine microalgal concentrate was added periodically during each day as a food source and was also provided via the diluter water.

Applied concentrations:
Nominal: 0 (control), 0.42, 0.76, 1.4, 2.5, 4.4 and 8.0 mg a.s./L
Mean measured: <LOQ (control), 0.366, 0.668, 1.20, 2.12, 3.96 and 7.29 mg a.s./L

Solvent: None

Test conditions:
Water quality: Laboratory saltwater, hardness: 130 - 160 mg/L $CaCO_3$
Temperature: 19.6 – 20.8°C
pH: 7.5 – 8.3
O_2 content: 62% to 97% saturation (4.9 to 7.5 mg/L)
Salinity: 19.5 – 20.0 $^0/_{00}$
Light regime: 16 hours light / 8 hours darkness, 462 - 693 lux

Test system: Total daily volume through-flow was approximately 72 L, or 8.7 chamber additions. Chambers were glass tanks 21.7 x 37.0 x 17.8 cm, media volume and depth were maintained at approximately 8.4 L and 10.5 cm, respectively, each contained a pump to continuously recirculate the media. Prior to introduction each Oyster was prepared by grinding 3-5 mm of the ventral shell edge.

Test parameter: Observations on mortality and signs of test substance effects were performed daily and new shell growth was measured at test termination using Vernier calipers to the nearest 0.1 mm. Test solution temperature, dissolved oxygen, salinity and pH were measured daily. Test solution samples were collected at prior to test initiation, at initiation and at termination using a validated HPLC-UV method. During the test, monitoring of water quality parameters included: daily measurement of temperature, pH and dissolved oxygen concentrations in the control and each test solution until test termination or until 100% mortality had occurred.

Analytical measurements: Test solutions were collected at test start and test end, and diluter stock at each sampling point, for analysis of pethoxamid using a validated HPLC-UV method.

Statistics: EC_{50} estimates for new shell growth were performed using four-parameter logistic (sigmoid-shaped) model, two parameters fixed (100 and 0% inhibition), fit to the data with percent inhibition as the dependant variable and log concentration as the independent variable. The NOEC for new shell growth was determined using oneway ANOVA and a one-tailed Dunnett's and William's test ($\alpha = 0.05$). All statistical analysis was performed using SAS version 9.3.

Results:

Analytical measurements: Recoveries were 81% to 89% of nominal at test start and 89% to 93% after 96 hours, treatment mean measured recoveries for the 96-h duration were 85% to 91%. Stock solutions were 94% to 96% of nominal.

Validity Criteria: Control mean survival was 100%, new growth was 3.0 mm achieving the criteria of 90% and ≤ 2 mm, respectively, therefore the control validity criteria was achieved. No evidence of spawning.

Biological effects: After 96 hours there was no mortality in the control or in any treatment group.

Slightly reduced faecal matter was observed at 2.5 and 4.4 mg a.s./L and was reduced at 8.0 mg a.s./L.

Mean new shell growth was 3.0 mm in the control compared to 3.1, 2.7, 3.3, 2.3, 1.2 and 0.3 mm at 0.42, 0.76, 1.4, 2.5, 4.4 and 8.0 mg a.s./L, corresponding to mean measured concentrations of <LOQ, 0.366, 0.668, 1.20, 2.12, 3.96 and 7.29 mg a.s./L, respectively.

No evidence of spawning was observed in any treatment group.

Table 4.3.2-6: Summary of new shell deposition in the Easter Oyster during 96-hours exposure to pethoxamid

Nominal concentration (mean measured) mg a.s./L	Mean length (mm)	Percent inhibition compared to the control
0 (control)	3.0	-
0.42 (0.366)	3.1	-5
0.76 (0.668)	2.7	11
1.4 (1.20)	3.3	-9
2.5 (2.12)	2.3	23* [a]
4.4 (3.96)	1.2	61* [a]
8.0 (7.29)	0.3	90* [a]
*Statistically significant (Dunnett's test p<0.05), [a]Statistically significant (William's test p<0.05) - values show an increase compared to the control		

Table 4.3.2-7: EC_{50} and NOEC estimates for new shell deposition in the Easter Oyster, *Crassostrea virginica* during 96h exposure to pethoxamid

Results based on:	Time	EC_{50} (mg a.s./L)	95% confidence limits (mg a.s./L)	NOEC (mg a.s./L)
Mean measured concentrations	96h	3.38	3.03 to 3.74	1.2*
Nominal concentrations	96h	3.8	3.4 to 4.2	1.4*
*significant based on William's test ($p<0.05$).				

Conclusion: In a 96-h shell deposition test with the Easter Oyster, *Crassostrea virginica* the EC_{50} estimate for pethoxamid was 3.8 mg a.s./L (nominal) and 3.38 mg a.s./L (mean measured). The 96-h NOEC was determined to be 1.4 mg a.s./L (nominal) or 1.2 mg a.s./L (mean measured) (William's test $p<0.05$).

4.3.3 Algal growth inhibition tests

4.3.3.1 158 PXA (1999b), 158 PXA suppl.-1 (2016a)

Reference: TKC-94 : Algal Inhibition Test
Author(s), year: 158 PXA, 1999b
Report/Doc. number: TON 039/992200, Cheminova A/S Report No.: 158 PXA
Guideline(s): OECD test guideline 201 (1984), EPA Subdiv. J, 122-2 and 123-2
GLP: Yes
Deviations: None relevant
Validity: Acceptable

Reference: Re-evaluation of the data of the report TKC-94: Algal Inhibition Test
Author(s), year: 158 PXA suppl.-1, 2016a
Report/Doc. number: Fraunhofer report Fh-IME 2016-05-CHE-02; Cheminova A/S Report No.: 158 PXA suppl.-1
Guideline(s): OECD guideline 201
GLP: No
Deviations: Not applicable
Validity: Acceptable

Reference: Re-evaluation regarding validity of the test: TKC-94: Algal Inhibition Test
Author(s), year: 158 PXA suppl.-2, 2016c
Report/Doc. number: Cheminova A/S Report No.: 158 PXA suppl.-2
Guideline(s): Not applicable
GLP: No
Deviations: Not applicable
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: Green algae *Pseudokirchneriella subcapitata (*formerly known as *Selenastrum capricornutum)*

Number of organisms: Approx. 1 x 10^4 cells/mL; 3 replicates per treatment and control and solvent control group

Type of test, duration: Static test, 120 hours

Applied concentrations:
Nominal: 0; 0.000625; 0.00125; 0.0025; 0.005; 0.01 mg/L, plus solvent
Mean measured: 0; 0.00058; 0.0012; 0.0024; 0.0046; 0.0094 mg/L

Solvent: 10 µL/L auxiliary solvent: acetone

Reference: Potassium dichromate

Test conditions:
Water quality: Sterile nutrient medium
Temperature: 24 ± 1 °C
pH: 7.4 – 8.7
Incubation: Continuous illumination at ~ 4000 lux

Analytical measurements: For chemical analysis of the test substance and solvent control, samples of test solution were taken at test initiation and at test termination.

Test parameters: Samples were taken at 0, 24, 48, 72, 96 and 120 hours. The cell densities of the control cultures were determined by direct counting with the aid of a particle counter.

Algistatic extension: Post-exposure regrowth was observed in the cultures within 7 days. This indicates an algistatic effect of the test substance.

Measurements of pH and temperature were made at initiation and at termination.

Statistics: Logistic regression. NOEC determined by William`s test.

Results:

Analytical data: Mean measured concentrations were in the range of 92 - 103 % of nominal at 0 h and from 85 - 95% of nominal concentrations at 120 h.

Morphological effects: No abnormalities were observed in any of the control or treatment groups.

Table 4.3.3-1: Effects of technical pethoxamid on the green algae *Pseudokirchneriella subcapitata*

pethoxamid [µg/L] (nominal)	**Biomass**		**Growth rate**	
	Area under the curve (72h/ 120h)	**% inhibition relative to the control**	**0 – 72h/ 120h**	**% inhibition relative to the control**
Control (solvent only)	4.9 / 49	0	0.043 / 0.041	0
0.625	6.6 / 70	-33 / -42	0.047 / 0.043	-8.5 / -5.3
1.25	3.9 / 42	21 / 15	0.040 / 0.041	6.7 / 1.8
2.5	1.5 / 17	69 / 65*	0.028 / 0.034	35 / 17*
5.0	0.73 / 6.0	85 / 88*	0.019 / 0.023	57 / 44*
10	0.29 / 1.2	94 / 98*	0.0094 / 0.0078	78 / 81*
* Statistically significant compared to the control				

Re-evaluation (Wenzel, 2016a):

Data from the report on effects of TKC-94 (pethoxamid) on the growth of the unicellular green algal species *Selenastrum capricornutum* (Bell, G. and Lodge, D.C. 1999b; 158 PXA) was re-evaluated to obtain EC_{10} and EC_{20} values for growth rate, yield and biomass. These values were not included in the original report.

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was performed with the original cell number values from GLP report 158 PXA (Table 1: Algal cell densities for control and test cultures) as outlined in the OECD guideline 201, as far as possible. The cell numbers of the treated cultures were compared with that of the pooled controls, since there was no statistically significant difference between control and solvent control.

At first, non-linear regression procedures, e.g. a 3-parametric cumulative normal distribution function according to Bruce and Versteeg (1992), were fitted to the data. Subsequently, other non-linear regression models provided by the computer programme ToxRat Professional® were used. If the requirements for a non-linear regression were not fulfilled, the EC values were calculated by linear regression (Probit analysis, based on normal distribution of the data (sigmoid normal), modified for continuous data).

For growth rate 72 h, all yield and biomass results, convergence criteria for non-linear regression models were not fulfilled and/or there were significant lacks of fit. Therefore, linear regression using Probit was performed. Individual replicate responses were used for the regression analysis.

For growth rate after 96 and 120 hours, convergence criteria for the 3-parametric logistic cumulative distribution function were fulfilled and there were no significant lacks of fit.

The cell number in the control cultures increased by a factor of ≥ 16 within the 72 h, 96 and 120 hour test period.

Table 4.3.3-2: Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time.

		Effect concentrations [µg/L]*, mean measured		
		0 - 72 h	**0 - 96 h**	**0 - 120 h**
Yield				
	EC_{10}	0.98	0.95	1.19
95%-CL	lower	0.68	0.70	0.95
	upper	1.20	1.15	1.38
	EC_{20}	1.25	1.22	1.49
95%-CL	lower	0.97	0.97	1.26
	upper	1.47	1.40	1.66
	EC_{50}	2.03	1.93	2.27
95%-CL	lower	1.78	1.72	2.08
	upper	2.30	2.16	2.46
Growth rate			**	**
	EC_{10}	1.19	1.36	1.84
95%-CL	lower	0.70	0.87	1.30
	upper	1.62	1.80	2.30
	EC_{20}	1.82	2.08	2.65
95%-CL	lower	1.25	1.52	2.08
	upper	2.29	2.55	3.11
	EC_{50}	4.08	4.28	4.93
95%-CL	lower	3.41	3.63	4.33
	upper	4.95	4.95	5.54
Biomass integral				
	EC_{10}	0.96	0.96	1.05
95%-CL	lower	0.69	0.72	0.81
	upper	1.17	1.15	1.23
	EC_{20}	1.25	1.23	1.33
95%-CL	lower	0.98	1.00	1.10
	upper	1.45	1.42	1.51
	EC_{50}	2.06	1.98	2.09
95%-CL	lower	1.82	1.77	1.89
	upper	2.32	2.20	2.30

* calculated using Probit normal sigmoid regression, except for 96 h and 120 h growth rate
** 96 h and 120 h growth rate calculated using non-linear regression (3-parametric logistic)

Conclusion: 72 h E_bC_{50} = 2.06 µg a.s./L
72 h E_rC_{50} = 4.08 µg a.s./L
72 h E_yC_{50} = 2.03 µg a.s./L
96 h E_bC_{50} = 1.98 µg a.s./L

96 h E_rC_{50} = 4.28 µg a.s./L
96 h E_yC_{50} = 1.93 µg a.s./L
120 h E_bC_{50} = 2.09 µg a.s./L
120 h E_rC_{50} = 4.93 µg a.s./L
120 h E_yC_{50} = 2.27 µg a.s./L
120 h NOEC = 1.2 µg a.s./L (biomass and growth rate)
based on mean measured concentrations
EC_{10} and EC_{20}-values see table above

4.3.3.2 157 PXA (1999c)

Reference: TKC-94: Algal Inhibition Test (*Anabaena Flos-Aquae*)
Author(s), year: 157 PXA, 1999c
Report/Doc. number: TON 040/992817, Cheminova A/S Report No.: 157 PXA
Guideline(s): OECD test guideline 201 (1984), EPA Subdiv. J, 122-2 and 123-2
GLP: Yes
Deviations: Not all validity criteria acc. to OECD 201 version 2006 are met - According to the validity criteria given in the OECD test guideline (1984) the study is considered not fully acceptable, as the cell concentration in the control groups increased by a factor of less than 16 within 3 days. After 96 and 120 h the increase was >16, however. Therefore the endpoints for 96 and 120 h might be considered reliable.
Validity: Study not valid. Acceptable as supporting information only.

Reference: Re-evaluation of the data of the report TKC-94: Algal Inhibition Test (*Anabaena Flos-Aquae*)
Author(s), year: 157 PXA suppl.-1, 2016b
Report/Doc. number: Fraunhofer report Fh-IME 2016-05-CHE-01, Cheminova A/S Report No.: 157 PXA supplementary report 1
Guideline(s): OECD guideline 201
GLP: No
Deviations: Not applicable
Validity: Acceptable

Reference: Re-evaluation regarding validity of the test: TKC-94: Algal Inhibition Test (*Anabaena Flos-Aquae*)
Author(s), year: 157 PXA suppl.-2, 2016d
Report/Doc. number: Cheminova A/S Report No.: 157 PXA supplementary report 2
Guideline(s): Not applicable
GLP: No
Deviations: Not applicable
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: *Anabaena flos-aquae*

Number of organisms: Approx. 1.3 x 10^5 cells/mL; 3 replicates per treatment and control group

Type of test, duration: Static test, 120 hours

Applied concentrations:
Nominal: 0; 2.2; 4.6; 10; 22; 46 mg/L
Mean measured: 0; 1.6; 3.8; 8.6; 20; 41 mg/L

Solvent: -

Reference: Potassium dichromate

Test conditions:
Water quality: Jaworski`s nutrient medium
Temperature: 24 ± 1°C
pH: 7.8 - 8.7
Incubation: Continuous illumination at ~ 2000 lux

Analytical measurements: For chemical analysis of the test substance and solvent control, samples of test solution were taken at test initiation and at test termination.

Test parameters: Samples were taken at 0, 24, 48, 96 and 120 hours. The cell densities of the control cultures were determined with a haemocytometer.

Algistatic extension: Post-exposure regrowth was observed in the cultures within 7 days. This indicates an algistatic effect of the test substance.

Measurements of pH and temperature were made at initiation and at termination.

Statistics: Logistic regression. NOEC determined by William`s test.

Results:

Analytical data: Mean measured concentrations were in the range of 81 – 89 % of nominal at 0 h and from 67 - 92% of nominal concentrations at 120 h.

Morphological effects: No abnormalities were observed in any of the control or treatment groups.

Table 4.3.3-3: Effects of technical pethoxamid on *Anabaena flos-aquae*

pethoxamid [mg/L] (nominal)	Biomass		Growth rate	
	Area under the curve (72h/ 120h)	% inhibition relative to the control	0 – 72h/ 120h	% inhibition relative to the control
Control	20 / 130	-	0.027 / 0.029	-
2.2	18 / 113	6.8 / 13	0.03 / 0.029	-9.7 / -2.3
4.6	19 / 111	0.8 / 15	0.028 / 0.029	-3.4 / -0.6
10	14 / 77	28 / 41	0.023 / 0.023	16 / 20
22	-2.8 / -8.1	114 / 106	-0.0087 / -0.022	100 / 178
46	- 4.8 / -10	124 / 108	-0.022 / -0.022	100 / 177
* Statistically significant compared to the control				

Re-evaluation (Wenzel, 2016b):

The data from the report on effects of TKC-94 (pethoxamid) on the growth of the cyanobacteria *Anabaena – flosaquae* (Bell, G. and Lodge, D.C., 1999c; 157 PXA) was re-evaluated to obtain EC_{10} and EC_{20} values for growth rate, yield and biomass. These values were not included in the original report.

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was performed with the original cell number values of the GLP report 157 PXA as outlined in the OECD guideline 201, as far as possible. At first, non-linear regression procedures, e.g. a 3-parametric cumulative normal distribution function according to Bruce and Versteeg (1992), were fitted to the data. Subsequently, other non-

linear regression models provided by the computer programme ToxRat Professional® were used. If the requirements for a non-linear regression were not fulfilled, the EC values were calculated by linear regression (Probit analysis, based on normal distribution of the data (sigmoid normal), modified for continuous data).

For growth rate, yield and 72 h and 120 h biomass, convergence criteria for non-linear regression models were not fulfilled and/or there were significant lacks of fit. Therefore, linear regression using Probit was performed.

Individual replicate responses were used for the regression analysis.

For biomass after 96 hours, convergence criteria for the 3-parametric normal cumulative distribution function were fulfilled and there were no significant lacks of fit. The cell number in the control cultures increased by a factor of ≥ 16 within the 96 and 120 hour test period.

Table 4.3.3-4: Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time

			Effect concentrations [mg/L]*, mean measured		
			0 - 72 h	**0 - 96 h**	**0 - 120 h**
1.	**Yield**				
		EC_{10}	5.32	6.07	2.03
	95%-CL	lower	2.47	n.d.	1.08
		upper	6.78	n.d.	2.86
		EC_{20}	6.58	7.15	3.04
	95%-CL	lower	3.94	n.d.	1.93
		upper	7.94	n.d.	3.97
		EC_{50}	9.88	9.79	6.56
	95%-CL	lower	8.26	n.d.	5.25
		upper	12.5	n.d.	8.18
2.	**Growth rate**				
		EC_{10}	8.20	8.39	7.85
	95%-CL	lower	n.d.	n.d.	n.d.
		upper	n.d.	n.d.	n.d.
		EC_{20}	8.84	9.04	8.59
	95%-CL	lower	n.d.	n.d.	n.d.
		upper	n.d.	n.d.	n.d.
		EC_{50}	10.2	10.4	10.2
	95%-CL	lower	n.d.	n.d.	n.d.
		upper	n.d.	n.d.	n.d.
3.	**Biomass integral**				
		EC_{10}	7.48	8.15**	4.50
	95%-CL	lower	n.d.	n.d.	2.21
		upper	n.d.	n.d.	5.88
		EC_{20}	8.18	8.43**	5.72
	95%-CL	lower	n.d.	n.d.	3.49
		upper	n.d.	n.d.	7.04
		EC_{50}	9.71	8.99**	9.08
	95%-CL	lower	n.d.	n.d.	7.50
		upper	n.d.	n.d.	11.1

n.d.: not determined due to mathematical reasons or inappropriate data

* calculated using Probit normal sigmoid regression, except for 96 h biomass integral

** calculated using non-linear regression (3-parametric normal)

Conclusion: 96 h E_bC_{50} = 8.99 mg a.s./L
96 h E_rC_{50} = 10.4 mg a.s./L
96 h E_yC_{50} = 9.79 mg a.s./L

120 h E_bC_{50} = 9.08 mg a.s./L
120 h E_rC_{50} = 10.2 mg a.s./L
120 h E_yC_{50} = 6.56 mg a.s./L
120 h NOEC = 3.8 mg a.s./L (biomass and growth rate)
based on mean measured concentrations
EC_{10} and EC_{20}-values see table above

4.4 Chronic toxicity

4.4.1 Fish early-life stage (FELS) toxicity test

4.4.1.1 1451 PXA (2015)

Reference: Pethoxamid Technical : Early Life-Stage Toxicity Test with the Rainbow Trout, *Oncorhynchus mykiss*, Under Flow-Through Conditions
Author(s), year: 1451 PXA, 2015
Report/Doc. number: 69651; Cheminova A/S, Unpublished report No.: 1451 PXA
Guideline(s): US EPA OPPTS 850.1400; OECD 210
GLP: Yes (certified laboratory)
Deviations: None
Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn., Purity: 95.8 % w/w, re-analysed 96.2 %. Batch No. : P1351-JaK-T2-23-6

Test species: *Oncorhynchus mykiss*

Number of organisms: 4 replicates per test concentration and control, 45 embryos per replicate, reduced to 15 fry post hatch

Age: Embryos were approx. 19 h post-fertilisation at test initiation.

Type of test: Flow-through test, 94 days (60 d post-hatch)

Applied concentrations:
Nominal: 0 (control), 0.095, 0.19, 0.38, 0.75 and 1.5 mg a.s./L
Measured (mean): - (control), 0.0924, 0.177, 0.365, 0.722 and 1.44 mg a.s./L, equivalent to 97, 93, 96, 96 and 96% of the nominal concentration, respectively

Solvent: None

Test conditions:
Water quality: dilution water was a naturally hard well water with well water that was demineralized by reverse osmosis (RO); hardness: 138 to 144 mg/L $CaCO_3$
Temperature: 9.4 – 11.9°C; maintained at 10 ± 2°C for embryos and 12 ± 2°C for larvae and juvenile fish
pH: 8.0 – 8.6
O_2 content: 6.66 to 10.0 mg/L (63 to 93% saturation)
Conductivity: Not specified
Light regime: Semi-darkness until day 42 then light/dark cycle of 16/8, light intensity approximately 276 - 528 lux

Methods: Developing embryos were incubated in approximately 15 cm tall glass cups constructed from 9 cm diameter glass jars with mesh at the base. An incubation cup was suspended within each replicate aquarium and 45 eggs were placed into each cup. To facilitate test solution circulation, the cups were oscillated vertically (approximately 10 cm vertical travel) in each aquarium by means of a rocker arm apparatus driven by an electric motor. Following placement of eggs into the incubation cups, the lights over the test chambers remained off and a curtain shielded the developing embryos from the surrounding light in the laboratory. Developing embryos were kept in semi-darkness until approximately one-week post-hatch, at which point a 16:8 light:dark photoperiod was used.

The eggs were fertilized by the supplier and allowed to water harden two hours before shipment. The eggs were approximately 19 hours post-fertilization upon arrival at the test facility. Upon receipt, the eggs were allowed to adjust from approximately 1.5ºC to 9.2ºC over a period of approximately 3 hours. The acclimated eggs were then sorted to select live eggs i.e., eggs that were not partially or completely white in appearance. The live eggs were used for addition to the test chambers within 24 hours of fertilization. Eggs were impartially selected and distributed four at a time into each replicate incubation cup until the total number of embryos (i.e., 45) was achieved within each replicate.

The embryos were counted on a daily basis during incubation and any dead embryos, identified by a distinct change in coloration, were removed and discarded. The embryos were observed under low light from the surrounding laboratory by partially opening sections of the curtain surrounding the exposure system at observation times. When all living embryos had hatched, length of time to reach 95% hatch in the control group and overall percent hatchability were recorded. Day 0 post-hatch was based on ≥95% hatch in the control group which occurred on day 34. On study day 42 (8 days post-hatch) the number of fry in each replicate was impartially reduced to 15 fry per replicate, which were released into their respective replicate growth chambers. All remaining non-viable eggs were discarded at this time (i.e., study day 42).

Starting on study day 46, fry were fed live brine shrimp (*Artemia* sp.) nauplii, then brine shrimp nauplii and salmon starter starting on study day 55. The fish were fed *ad libitum* three times daily. Food size and/or quantity were increased during testing on the basis of average fish size. The test chambers were scraped and/or siphoned periodically after the first feeding to remove waste material and uneaten food and to minimize biological growth on the sides and bottom of the test chamber for the remainder of the exposure. The cleaning frequency was generally at least twice weekly.

Test parameters: Survival was monitored daily by visually inspecting each test chamber and any behavioural or physical changes were recorded, including abnormalities. Dead fry were removed on the day they were found dead. After 60 days of post-hatch growth (study day 94), surviving fish were removed from each replicate chamber and euthanized with MS-222. All individuals were measured for standard length using a millimeter scale and blotted wet weight using an electronic balance.

Analytical measurements: Test solutions were analyzed for the concentration of pethoxamid technical using a high performance liquid chromatography with ultraviolet detection (HPLC-UV) method. During the test, the concentrations of pethoxamid technical were measured in test solutions samples collected on day 0 and weekly during the test including test termination. At all sampling points during the test, two replicate samples were collected from the control and test substance treatments. The concentrations of pethoxamid technical in the diluter stock solutions were also determined in samples collected on the same days as the test solutions.

Statistics: All statistical analyses were performed using SAS software (version 9.3 for Windows). Inferences of statistical significance were based upon $p < 0.05$. The NOEC and LOEC for egg hatchability and fry survival data were determined by using a Fisher's exact test. In addition, the NOEC for these parameters was estimated using a one-way analysis of variance (ANOVA) procedure and a onetailed Dunnett's test. The time to start of hatch and fry swim-up, and time to complete hatch and fry swim-up were evaluated using an ANOVA procedure and a one-tailed Dunnett's test. The NOEC values for standard length and blotted wet weight were determined using a nested ANOVA procedure.

Results:

Analytical data: The measured pethoxamid concentrations in the test solutions ranged from 82 to 106% of the nominal concentration over the course of the test. The mean measured concentrations of pethoxamid in the test vessels were determined to be 0.0924, 0.177, 0.365, 0.722 and 1.44 mg a.s./L, equivalent to 97, 93, 96, 96 and 96% of the nominal concentration, respectively. No residues of pethoxamid were measured in the control above the LOQ (0.02 mg a.s./L). The results of the test are expressed in terms of the mean measured concentrations.

Biological Results*:* Egg hatch in the control and test substance treatments began on study day 29 and was completed on study day 35. The mean percent hatch in the control treatment was 76% and ranged from 72 to 81% in the test substance treatments. There was no statistically significant delay in time to hatch completion (i.e., the day the last surviving eyed-embryo had hatched), nor was there a statistically significant reduction in hatch success in any treatment group compared to the control. While there was a statistically significant delay in time to hatch start in the 0.722 mg a.s./L treatment this delay was not considered biologically significant because all other treatment concentrations, including the 1.44 mg a.s./L treatment, were found statistically equivalent to the control. The NOEC and LOEC for time to hatch start, time to hatch completion, and hatching success were 1.44 and >1.44 mg a.s./L, respectively. The day fry swim-up started in the control was study day 46 (12 days post-hatch) and all control fry had completed swim-up by study day 54 (20 days post-hatch). The NOEC and LOEC for time to start of swim-up and time to completion of swim-up were 1.44 and >1.44 mg a.s./L, respectively.

Post-hatch survival before reduction was 84% in the control and was 78, 71, 63, 75, and 66% in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. There was a statistically significant reduction in post-hatch survival, before reduction, in the 0.177, 0.365, and 1.44 mg a.s./L test substance treatments. However, the statistically significant reduction at 0.177 and 0.365 mg a.s./L was not considered biologically significant because the 75% survival at the 0.722 mg a.s./L treatment was not statistically significant, indicating a lack of a concentration-dependent dose response. The NOEC and LOEC for post-hatch survival before reduction were 0.722 and 1.44 mg a.s./L, respectively.

Post-hatch survival (after reduction) in the control was 93% and was 87, 95, 88, 82, and 85% in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. There was no statistically significant reduction in post-hatch survival for any of the test substance treatments compared to the control. The NOEC and LOEC for post-hatch survival after reduction were 1.44 and >1.44 mg a.s./L, respectively.

Mean standard length in the control was 48.4 mm and was 48.2, 47.8, 48.3, 47.4, and 48.5 mm in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. Mean blotted wet weight in the control was 1.820 g and was 1.809, 1.752, 1.857, 1.755 and 1.860 g in the 0.0924, 0.177, 0.365, 0.722, and 1.44 mg a.s./L treatments, respectively. No statistically significant reductions in either mean standard length or blotted wet weight were found for any of the test substance treatment groups when compared to the control group. The NOEC and LOEC for standard length and blotted wet weight were 1.44 and >1.44 mg a.s./L, respectively.

Validity criteria: The validity criteria for this study were considered to have been met. The measured concentrations remained within 20% of the nominal concentration, the dissolved oxygen concentrations were between 60 and 100% saturation throughout the test and the water temperature did not differ by more than ±1.5 °C between test chambers (but did differ by more than ±1.5°C between successive days on two occasions). The mean hatching success in the control was 76% which was greater than the criterion of >66% as well as the criterion of at least 75% in the 2013 version of the OECD 210 guideline. The mean control post-hatch survival rate was 84% on the reduction day and 93% at test termination and was therefore greater than the criterion of ≥70% as well as the criterion of at least 75% in the 2013 version of the OECD 210 guideline.

Table 4.4.1-1: Hatching success of rainbow trout embryos exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L)	Rep	Initial Number of Embryos	Days to Hatch Start	Days to Hatch Completion	Number of Hatched Fry	Hatching success (%)
Control	A	45	29	33	34	76
	B	45	29	34	36	80
	C	45	29	35	33	73
	D	45	29	34	33	73
Mean:			**29**	**34**		**76**
0.0924	A	45	29	35	31	69
	B	45	30	34	33	73
	C	45	29	33	32	71
	D	45	29	33	35	78
Mean:			**29**	**34**		**73**
0.177	A	45	30	35	36	80
	B	45	29	34	40	89
	C	45	29	35	33	73
	D	45	29	34	36	80
Mean:			**29**	**35**		**81**
0.365	A	45	29	33	31	69
	B	45	29	34	36	80
	C	45	29	34	32	71
	D	45	29	35	36	80
Mean:			**29**	**34**		**75**
0.722	A	45	30	34	32	71
	B	45	29	34	30	67
	C	45	30	35	36	80
	D	45	30	35	33	73
Mean:			**30 ***	**35**		**73**
1.44	A	45	29	34	35	78
	B	45	29	33	29	64
	C	45	29	33	37	82
	D	45	30	35	28	62
Mean:			**29**	**34**		**72**

* Indicates a statistically significant delay in mean days to start of hatch as compared to the control mean for this parameter (Dunnett's test; p = 0.035). The statistically significant delay in days to hatch start at 0.722 mg a.s./L was not considered to biologically relevant because the highest test substance treatment was equal to the control

Table 4.4.1-2: Post-hatch survival, before reduction, of rainbow trout exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L)	Rep	Total Number of Hatched Fry	Number of Surviving Fry Before Reduction at Day 8 Post-Hatch	Post-hatch survival (%)
Control	A	34	29	85
	B	36	30	83
	C	33	26	79
	D	33	29	88
			Mean:	**84**
0.0924	A	31	24	77
	B	33	26	79
	C	32	29	91
	D	35	23	66
			Mean:	**78**
0.177	A	36	25	69
	B	40	27	68
	C	33	26	79
	D	36	25	69
			Mean:	**71 ***
0.365	A	31	19	61
	B	36	25	69
	C	32	17	53
	D	36	24	67
			Mean:	**63 ***
0.722	A	32	24	75
	B	30	19	63
	C	36	28	78
	D	33	27	82
			Mean:	**75**
1.44	A	35	22	63
	B	29	20	69
	C	37	23	62
	D	28	20	71
			Mean:	**66 ***

* Indicates a statistically significant reduction in percent survival as compared to the control (Dunnett's test; $p < 0.05$). The statistically significant reduction in survival at 0.177 and 0.365 mg a.s./L was not considered to be biologically relevant by study authors because the 75% survival at the 0.722 mg a.s./L treatment was not statistically significant, indicating a lack of a concentration-dependent dose response. Additionally, it is stated that the average post-hatch survival in all nominal test substance treatments during the range-finding test, including the highest 1.0 mg a.s./L nominal treatment, was greater than or equal to the control at the end of the 24-day post-hatch exposure.

Table 4.4.1-3: Post-hatch survival, post reduction, of rainbow trout exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L)	Rep	Total Number of Fry	Number of Surviving Fry at Day 60 Post-Hatch	Post-hatch Survival (%)
Control	A	15	14	93
	B	15	14	93
	C	15	13	87
	D	15	15	100
			Mean:	**93**
0.0924	A	15	12	80
	B	15	15	100
	C	15	13	87
	D	15	12	80
			Mean:	**87**
0.177	A	15	13	87
	B	15	15	100
	C	15	15	100
	D	15	14	93
			Mean:	**95**
0.365	A	15	15	100
	B	15	13	87
	C	15	11	73
	D	15	14	93
			Mean:	**88**
0.722	A	15	11	73
	B	15	12	80
	C	15	14	93
	D	15	12	80
			Mean:	**82**
1.44	A	15	14	93
	B	15	14	93
	C	15	13	87
	D	15	10	67
			Mean:	**85**
Note: There was no statistically significant reduction in percent survival as compared to the control (Dunnett's test; $p \geq 0.05$)				

Table 4.4.1-4: 60-Day post-hatch mean standard length and wet weight of rainbow trout exposed to pethoxamid technical

Mean Measured Concentration (mg a.s./L) Rep	60-Day Post-Hatch	
	Mean Standard Length in mm (Standard Deviation)	Mean Blotted Wet Weight in grams (Standard Deviation)
Control (n=56):	48.4 (4.7)	1.820 (0.465)
0.0924 (n=52):	48.2 (4.9)	1.809 (0.545)
0.177 (n=57):	47.8 (4.8)	1.752 (0.500)
0.365 (n=53):	48.3 (5.3)	1.857 (0.565)
0.722 (n=49):	47.4 (5.1)	1.755 (0.545)
1.44 (n=51):	48.5 (4.8)	1.860 (0.513)
Note: There was no statistically significant difference in mean growth (i.e., standard length or blotted wet weight) as compared to the control mean for this parameter (Dunnett's test; $p \geq 0.05$)		

Table 4.4.1-5: Effect concentrations for hatchability, post-hatch survival, length and wet weight of rainbow trout exposed to pethoxamid technical

Biological Parameter	No-Observed-Effect Concentration (NOEC) [a]	Lowest-Observed-Effect Concentration (LOEC) [a]
Time to Hatch Start	1.44	>1.44
Time to Hatch Completed	1.44	>1.44
Egg Hatchability	1.44	>1.44
Time to Fry Swim-up Start	1.44	>1.44
Time Fry Swim-up Completed	1.44	>1.44
Fry Survival Before Reduction [b]	*0.0924*	*0.177*
Fry Survival Post-Reduction [c]	1.44	>1.44
Standard Length	1.44	>1.44
Blotted Wet Weight	1.44	>1.44
[a] Expressed as mean measured concentration (mg a.s./L)		

Conclusion: Based on mean measured concentrations of pethoxamid technical, the NOEC and LOEC for rainbow trout time to hatch start, time to hatch completion, hatching success, standard length, wet weight and post-hatch survival (post-reduction) were 1.44 and >1.44 mg a.s./L, respectively. The NOEC and LOEC for post-hatch survival (before reduction) were set by the study authors at 0.722 and 1.44 mg a.s./L, respectively.

Deviating from the study author`s assessment, the RMS considers the overall **NOEC to be 0.0924 mg a.s./L based on reduced post-hatch survival starting at 0.177 mg a.s./L.**

Based on the nature of the data-set generated it was not possible to determine reliable EC_{10}/EC_{20} values.

4.4.2 Chronic toxicity to aquatic invertebrates

4.4.2.1 156 PXA (2000)

Reference: TKC-94 : Prolonged Toxicity to *Daphnia magna*
Author(s), year: 156 PXA, 2000
Report/Doc. number: TON 043/992819; Cheminova A/S Report No.: 156 PXA

Guideline(s): OECD 211, EPA 72-4
GLP: Yes
Deviations: None
Validity: Acceptable

Material and methods:	
Test substance:	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C
Test species:	Waterflea (*Daphnia magna*)
Number of organisms:	25 daphnids per treatment and control group, housed individually or in groups of 5
Age:	First instar, < 24 hours old
Type of test, duration:	Semi-static test, Medium renewal 3 times per week, 21 days
Applied concentrations:	
Nominal:	0 (control), 1.4, 3.1, 6.8, 15, 32 mg/L
Mean measured:	- (control), 1.3, 2.8, 6.3, 13, 29 mg/L
Solvent:	None
Test conditions:	
Water quality:	Elendt M4 medium
Temperature:	19-21 °C
pH:	7.7 – 7.9
O_2 content:	7.0 – 8.8 mg/L (> 72% air saturation)
Light regime:	16 hours light / 8 hours darkness
Feeding	Concentrated suspension of *Chlorella vulgaris*
Test parameters:	The live and dead Daphnia of the parental generation and numbers of live or dead neonates were counted daily and recorded together with observations on the general condition and size of the Daphnia as compared with the controls. The number of Daphnia with eggs or young in the brood pouch plus the number of discarded unhatched eggs was also determined. At the end the length of all surviving Daphnia was measured. Temperature, dissolved oxygen, pH and temperature were measured before and after- each test media renewal.
Analytical measurements:	Samples of fresh media were sampled on days 0, 7 and 18. Samples of expired test solutions were sent for analysis on days 2, 9 and 21.
Statistics:	Mortality: probit analysis (Finney), one-sided Fisher`s exact test. Number of young: William`s test. EC_x determined using logistic regression using SAS 6.11.
Results:	
Analytical measurements:	Results of the chemical analysis ranged from 88 to 99 % of nominal in freshly prepared solutions and from 84 % to 95 % of nominal in expired media.
Biological effects:	Adults in all test groups less than 13 mg/L were gravid by day 6. The surviving adult in the 13 mg/L group did not become gravid before it died on day 7. Production of the first brood neonates occurred on day 7 for all test

grpups less to or equal to 2.8 mg/L, whilst the adiults exposed to the 6.3 mg/L concnetration produced their first brood on day 8. At least 5 broods were produced in all test levels less than 13 mg/L by day 21.

Mean body length of daphnids was 4.18 mm, 4.28 mm, 4.31 mmm and 3.98 mm in the control, 1.4, 3.1 and 6.8 mg/L groups.

Table 4.4.2-1: Effects on daphnids (*Daphnia magna*) exposed to pethoxamid

pethoxamid [mg a.s./L] (nominal)	**% survival of P_1**	**no. live young per adult**	**no. dead young**	**no. unhatched eggs**
Control	100	106	1.7	1
1.4	87	130	0	1
3.1	93	131	0	0
6.8	20*	83	1.6	0
15	0	0	0	0
32	0	0	0	0

* Statistically significant compared to the control

Conclusion: LC_{50} (21 d) 4.2 mg a.s./L (based on arithmetic mean measured concentrations)

NOEC (survival, growth, repro) 2.8 mg a.s./L

EC_{50} (repro, 21 d) > 6.3 mg a.s./L

EC_{20} (repro, 21 d) 5.3 mg a.s./L

EC_{10} (repro, 21 d) 4.3 mg a.s./L

4.4.3 Chronic toxicity to algae or aquatic plants

See Section 4.3.3.

4.4.3.1 160 PXA (1999d)

Reference: TKC-94 : Higher Plant (LEMNA) growth Inhibition Test
Author(s), year: 160 PXA., 1999d
Report/Doc. number: TON 041/992818; Cheminova A/S Report No.: 160 PXA
Guideline(s): Draft OECD Guideline “Duckweed, Static Growth Inhibition Test”(1981), March 2006, EPA Guideline 122-2 and 123-3
GLP: Yes
Deviations: Duration 14 days
Validity: Acceptable with minor deficiencies

Reference: Re-evaluation of the data of the report TKC-94: Algal Inhibition Test
Author(s), year: 160 PXA suppl.-1, 2016e
Report/Doc. number: 2016-05-CHE-03; Cheminova A/S Report No.: 160 PXA supplementary report 1
Guideline(s): OECD guideline 221
GLP: No
Deviations: Not applicable

Validity: Acceptable

Material and methods:

Test substance: Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No. : TB-960306-C

Test species: *Lemna minor*

Number of organisms: each test vessel contained 15 fronds with 3 replicates per treatment

Type of test, duration: 14 day semi-static toxicity test (on days 2, 5, 7 and 9 all plants were transferred to vessels containing freshly prepared test media)

Applied concentrations:
Nominal: 0 (control + solvent control); 0.001; 0.0032; 0.01; 0.032; 0.1 mg/L
Mean measured: 0; 0.001; 0.0029; 0.0091; 0.028; 0.085 mg/L

Solvent: acetone

Toxic reference : Potassium dichromate

Test conditions: Glass vessels with approx. 200 mL test medium
Water quality: Reconstituted nutrient medium
Temperature: 25 ± 2 °C (2 deviations of 28 and 29°C)
pH: 4.8 – 6.0
Light regime: Continuous illumination approx. 5000 Lux

Test parameters: At each media renewal, all plants were observed for differences in growth of roots or fronds or general health. Frond deformation, chlorosis and necrosis were also reported. Frond numbers were determined on days 0, 2, 5, 7, 9, 12 and 14. At the end of exposure and re-culturing phase total plant biomass was determined. pH-values were measured at each media renewal, at start and end of the test. The room temperature in the test chamber was measured and recorded continuously. Light intensity was determined at each media replacement.

Analytical measurements: Sampling and analysis of test concentration were carried out on days 0, 7 and 12 (freshly prepared media) and on days 2, 9 and 14 (expired media). All test concentrations and control replicates were analysed.

Statistics: Mean numbers of fronds in each test group was compared to control using Logistic regression model. EC_{50}-value after 14 days was calculated by probit analysis. NOEC-values were determined by using William`s test.

Results:

Analytical data: Measured concentrations ranged from 90 to 124 % of nominal in fresh media and from 64 to 101% of nominal in the expired samples. Overall mean measured concentrations were 85 to 104%.

Morphological observations: By day 7 root lengths were shorter at concentrations of ≥ 2.9 µg/L, and decreased frond size and chlorosis were observed as well as reduced total fresh weight at ≥9.1 µg/L. New fronds appeared very small with dark green coloration at concentrations of ≥ 9.1 µg/L. *Lemna* exposed to 85 µg/L showed brown coloration of fronds (indicating necrosis).

Recovery: After 14 d selected plants were re-cultured for a further 7 days in fresh untreated medium. Fronds were selected from 10, 32 and 100 µg/L groups where > 50% inhibition of growth occurred. Phytotoxicity was indicated at concentrations of ≥ 9.1 µg/L.

Table 4.4.3-1: Inhibition of growth rate and biomass (frond number)

pethoxamid [µg/L] (mean measured)	Frond number at day 14	mean growth rate % inhibition	Mean biomass	
			Total plant weight/vessel at day 14 (mg)	% inhibition
Control	505	-	936	-
Solvent control	447	-	786	-
1.0	429	1.16	683	-1.96
2.9	424	1.49*	538	12.41*
9.1	136	35.08*	121	58.22*
28	49	65.32*	32	88.67*
85	27	82.44*	15	94.83*
* Statistically significant difference from control, William`s test, $p \leq 0.05$				

Re-evaluation (Wenzel, 2016c):

Data from the report on effects of TKC-94 (pethoxamid) on the growth of the macrophyte *Lemna* (Bell, G. and Lodge, D.C., 1999d; 160 PXA) was re-evaluated to obtain EC_{10} and EC_{20} values for growth rate, yield and biomass (area under the growth curve AUC). These values were not included in the original report.

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations was performed with the original frond number values from the GLP report 160 PXA (Table 1 Frond counts per flask (exposure period)) as outlined in the OECD guideline 221, as far as possible. The frond numbers of the treated cultures were compared with that of the pooled controls, since there was no statistically significant difference between control and solvent control.

At first, non-linear regression procedures, e.g. a 3-parametric cumulative normal distribution function according to Bruce and Versteeg (1992), were fitted to the data. Subsequently, other non-linear regression models provided by the computer programme ToxRat Professional® were used. If the requirements for a non-linear regression were not fulfilled, the EC values were calculated by linear regression (Probit analysis, based on normal distribution of the data (sigmoid normal), modified for continuous data. More information on the statistical approaches can be found in OECD 54 and ToxRat manual and validation document.

For growth rate and biomass (AUC), convergence criteria for non-linear regression models were not fulfilled and/or there were significant lacks of fit. Therefore, linear regression using Probit was performed. Individual replicate responses were used for the regression analysis.

For yield, convergence criteria for the 3-parametric normal cumulative distribution function were fulfilled and there were no significant lacks of fit.

Table 4.4.3-2: Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time

			Effect concentrations [µg/L]*, mean measured	
Effect concentrations [µg/L]			**0 - 7 d**	**0 - 14 d**
Yield (frond number)			**	**
		EC_{10}	4.80	2.94
	95%-CL	lower	2.76	1.82
		upper	8.34	4.74
		EC_{20}	7.05	3.83
	95%-CL	lower	4.19	2.44
		upper	11.9	5.99
		EC_{50}	14.7	6.39
	95%-CL	lower	7.75	3.76
		upper	27.5	10.9
Growth rate (frond number)				
		EC_{10}	5.43	2.90
	95%-CL	lower	3.64	2.03
		upper	7.22	3.80
		EC_{20}	9.23	5.34
	95%-CL	lower	6.90	4.11
		upper	11.5	6.56
		EC_{50}	25.5	17.2
	95%-CL	lower	21.8	14.8
		upper	29.9	19.9
Biomass (area under the growth curve AUC) (frond number)				
		EC_{10}	3.79	1.87
	95%-CL	lower	1.99	1.41
		upper	5.60	2.32
		EC_{20}	6.39	2.97
	95%-CL	lower	4.00	2.41
		upper	8.63	3.51
		EC_{50}	17.3	7.24
	95%-CL	lower	13.6	6.42

* Growth rate and Biomass calculated using Probit normal sigmoid regression, except for yield frond number
** yield frond number calculated using non-linear regression (3-parametric normal)

Conclusion: 14 d E_bC_{50} 7.24 µg a.s./L (frond number)
14 d E_rC_{50} 17.2 µg a.s./L (95% C.I. = 15 - 20 µg/L)
14 d E_yC_{50} 6.39 µg a.s./L (95% C.I. = 3.76 - 10.9 µg/L)
14 d NOEC = 1 µg a.s./L (growth rate, biomass and fresh weight)
Based on mean measured concentrations
EC_{10} and EC_{20} see table above