Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

pethoxamid (ISO); 2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide

EC Number: -
CAS Number: 106700-29-2

CLH-O-0000007269-65-01/F

Adopted

16 March 2023

16 March 2023

CLH-O-0000007269-65-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

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

EC Number: **-**

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

The proposal was submitted by **Austria** and received by RAC on **11 March 2022.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Austria has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **11 April 2022**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **10 June2022**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Anna Biró**

Co-Rapporteur, appointed by RAC: **Irina Karadjova**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **16 March 2023 by consensus.**

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	616-145-00-3	pethoxamid (ISO); 2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide		106700-29-2	Acute Tox. 4* Skin Sens. 1 Aquatic Acute 1 Aquatic Chronic 1	H302 H317 H400 H410	GHS07 GHS09 Wng	H302 H317 H410		M=100	
Dossier submitters proposal	616-145-00-3	pethoxamid (ISO); 2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide		106700-29-2	**Retain** Aquatic Acute 1 Aquatic Chronic 1 **Modify** Acute Tox. 4 Skin Sens. 1A	**Retain** H302 H317 H400 H410	**Retain** GHS07 GHS09 Wng	**Retain** H302 H317 H410		**Retain** M=100 **Add** oral: ATE = 983 mg/kg bw M=10	
RAC opinion	616-145-00-3	pethoxamid (ISO); 2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide		106700-29-2	**Retain** Aquatic Acute 1 Aquatic Chronic 1 **Modify** Acute Tox. 4 Skin Sens. 1A	**Retain** H302 H317 H400 H410	**Retain** GHS07 GHS09 Wng	**Retain** H302 H317 H410		**Retain** M=100 **Add** oral: ATE = 980 mg/kg bw M=10	
Resulting Annex VI entry if agreed by COM	616-145-00-3	pethoxamid (ISO); 2-chloro-*N*-(2-ethoxyethyl)-*N*-(2-methyl-1-phenylprop-1-enyl)acetamide		106700-29-2	Acute Tox. 4 Skin Sens. 1A Aquatic Acute 1 Aquatic Chronic 1	H302 H317 H400 H410	GHS07 GHS09 Wng	H302 H317 H410		oral: ATE = 980 mg/kg bw M=100 M=10	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

The absorption, distribution, metabolism and excretion of pethoxamid in rats were investigated with the active substance radiolabelled in the phenyl ring. The studies indicated greater than 80% absorption following oral administration. Pharmacokinetic parameters were not dose-dependent, and males and females showed no significant differences. Tissue distribution: the highest radioactivity occurred in whole-blood, plasma, liver and kidneys after single low or high dose administration. The highest concentrations in all tissues were observed 12 hours after dosing. Pethoxamid was extensively metabolised by glutathione-S-transferase to cysteine conjugates, which were then further oxidised to sulphoxides and sulphones.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Explosives

The explosive properties of pethoxamid have been tested according to EC A.14. As EC A.14 is not comparable to the test methods in Part I of the UNRTDG, the dossier submitter (DS) proposed no classification due to lack of data.

Flammable gases (including chemically unstable gases)

Not applicable.

Oxidising gases

Not applicable.

Gases under pressure

Not applicable.

Flammable liquids

Not applicable.

Flammable solids

Due to the low melting point of pethoxamid, it is not possible to perform the test to assess flammable solids. Therefore, the flash point test has been performed for liquids. The flash point was measured at 182°C at 1008 mbar (100800 Pa). A substance is classified as a flammable liquid when the flash point is <60°C. The DS has concluded that pethoxamid is not highly flammable as it does not meet the criteria for classification as flammable liquid.

Self-reactive substances

According to the DS the substance shall not be considered for classification as 'self-reactive', if it is classified as explosive, oxidising liquid or solid or organic peroxide. No valid data for explosive properties and no data for self-reactive substances is available. The DS has proposed no classification due to lack of data.

Pyrophoric liquids

Not applicable.

Pyrophoric solids

A study is not necessary due to practical experience in handling and use: The substance is known to be stable in contact with air at room temperature for prolonged periods of time, therefore the criteria for classification are not met, according to the DS.

Self-heating substances

According to the CLP Regulation, self-heating properties are tested using methods given in Part III, sub-section 33.3.1.6 of the UN RTGD; results from the test method EC A.15 (autoflammability) are not sufficient to conclude on this hazard class. The DS proposed no classification based on lacking data.

Substances which in contact with water emit flammable gases

No test is necessary since pethoxamid does not contain metals or metalloids. Further experience in handling and use indicates that it will not emit flammable gases on contact with water. The substance does not meet the criteria for classification.

Oxidising liquids

Not applicable.

Oxidising solids

An EC A.17 test has been performed and based on the test results pethoxamid is non-oxidising. As according to the CLP Regulation, oxidising properties should be tested using UN test O.1 or O.3 (CLP Annex I, 2.14.2.1.), the results from test method EC A.17 are not sufficient to conclude on this hazard class. The hazard class can also be assessed based on the screening procedure (see criteria in CLP Annex I, section 2.14.4.1).

It is stated there, that for organic substances or mixtures the classification procedure for this class shall not apply if:

(a) the substance or mixture does not contain oxygen, fluorine or chlorine; or
(b) the substance or mixture contains oxygen, fluorine or chlorine and these elements are chemically bonded only to carbon or hydrogen.

In this case (b) applies. Therefore, the criteria for classification are not met.

Organic peroxides

Not applicable, pethoxamid does not contain peroxo moieties.

Corrosive to metals

The screening procedure for this hazard class is based on the melting point and the chemical nature of the substance. According to the CLP Guidance, section 2.16.4.1, solids that have a melting point lower than 55 °C as well as substances or mixtures containing halogens should be tested. The melting point for pethoxamid is 37-38°C. Therefore, for pethoxamid both of the above-mentioned criteria are met, and no test has been provided. The DS has proposed no classification based on lacking data.

Comments received during consultation

An MSCA commented that data on explosive, flammable, self-heating and oxidizing properties are provided in the RAR (May 2017), the available results are presented in table 8 of the CLH report. The DS replied that According to the CLP Regulation the used methods such as e.g. EC A.14 for explosivity, EC A.15 for flammability and self-heating and EC A.17 for oxidizing properties were not sufficient to conclude on these hazard classes and therefore were not acceptable for classification purposes.

Assessment and comparison with the classification criteria

Explosives

The explosive properties of pethoxamid have been tested according to the EC A.14, meaning it was tested for heat, shock and friction sensitivity. The conclusion of the test was that pethoxamid is not explosive. However, test method EC A.14 is not comparable to the test methods in Part I of the UNRTDG. Nevertheless, according to CLP Annex I, section 2.1.4.3, no classification is warranted if any of the following (a-c) is met:

(a) when there are no chemical groups associated with explosive properties present in the molecule; or

(b) when the substance or mixture contains chemical groups associated with explosive properties which include oxygen, and the calculated oxygen balance is less than -200;

The oxygen balance is calculated for the chemical reaction:

$C_xH_yO_z + [x + (y/4)-(z/2)]\ O_2 \leftrightarrows x\ CO_2 + (y/2)\ H_2O$

using the formula:

$$\text{oxygen balance} = -1600 \times \frac{\left(2x + \frac{y}{2} - z\right)}{\text{molecular weight}};$$

(c) when the organic substance or a homogenous mixture of organic substances contains chemical groups associated with explosive properties, but the exothermic decomposition energy is less than 500 J/g and the onset of exothermic decomposition is below 500 ºC.

Of the three options, (b) is true for pethoxamid, as it contains unsaturated C=C bonds, but the calculated oxygen balance is -221.8, which is less than -200. Therefore, RAC proposes **no classification for explosives.**

Flammable solids

Due to the low melting point of pethoxamid (37-38°C), it is not possible to perform a test to assess flammable solids. Therefore, the flash point test has been performed for liquids. A substance is classified as a flammable liquid when the flash point is <60°C. The flash point of pethoxamid is 182°C at 1008 mbar (100800 Pa). RAC concurs with the DS that pethoxamid is not highly flammable as it **does not meet the criteria for classification as a flammable liquid**.

Self-reactive substances

The substance shall not be considered for classification as 'self-reactive', if it is classified as explosive, oxidising liquid or solid or organic peroxide. There is no data for self-reactive

substances available, therefore RAC concurs with the DS proposal of **no classification as a self-reactive substance due to lack of data.**

Pyrophoric solids

According to the CLP Regulation, this hazard class is assessed based on screening procedure in CLP Annex I, 2.9.4.1, i.e., based on experience in manufacturing or handling. In the case of pethoxamid, a study was not needed due to practical experience in handling and use: the substance is known to be stable in contact with air at room temperature for prolonged periods of time, therefore the criteria for classification are not met. RAC concurs with the DS that data is conclusive but not sufficient for classification and **no classification is warranted as a pyrophoric solid**.

Self-heating substances

According to the CLP Regulation, self-heating properties are tested using methods given in Part III, sub-section 33.3.1.6 of the UN RTGD; results from the test method EC A.15 (autoflammability) are not sufficient to conclude on this hazard class. However, according to the CLP guidance 2.11.4.2., substances with a low melting point (< 160 °C) should not be considered for classification. The melting point of pethoxamid is 37-38°C, therefore **no classification is warranted as a self-heating substance.**

Substances which in contact with water emit flammable gases

No test is necessary since pethoxamid does not contain metals or metalloids. Further experience in handling and use indicates that it will not emit flammable gases in contact with water. RAC concurs with the DS that the substance **does not meet the criteria for classification as a substance which in contact with water emits flammable gases**.

Oxidising solids

An EC A.17 test was performed, but according to the CLP Regulation, oxidising properties should be tested using UN test O.1 or O.3 (CLP Annex I, 2.14.2.1.). Results from test method EC A.17 are not sufficient to conclude on this hazard class. The hazard class can be assessed also based on the screening procedure (CLP Annex I, 2.14.4.1.).

Section 2.14.4.1. states that for organic substances or mixtures the classification procedure for this class shall not apply if:

(a) the substance or mixture does not contain oxygen, fluorine, or chlorine; or
(b) the substance or mixture contains oxygen, fluorine or chlorine and these elements are chemically bonded only to carbon or hydrogen.

In this case (b) applies. Therefore, the RAC concurs with the DS that **classification is not warranted as an oxidising solid.**

Organic peroxides

Not applicable, pethoxamid does not contain peroxo moieties.

Corrosive to metals

The screening procedure for this hazard class is based on melting point and the chemical nature of the substance. According to the CLP guidance 2.16.4.1, solids having a melting point lower than 55 °C as well as substances or mixtures containing halogens should be tested. Both are the case with pethoxamid. No test has been provided. RAC concurs with the DS proposal for **no classification as corrosive to metals based on lack of data.**

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

ACUTE ORAL TOXICITY

Summary of the Dossier Submitter's proposal

One OECD test guideline (TG) 401 and GLP study (Anonymous, 1994a) was summarised in the CLH report. According to the CLH report, at 20°C pethoxamid is crystalline solid, while under some circumstances technical grade pethoxamid can also be a viscous liquid. The CLH report states that the "substance was administered as supplied" but did not mention the physical state. The DS later clarified that in the acute oral toxicity study pethoxamid (95% purity) consisted of an oily solid and was stored at 4°C in the dark. The test substance was melted at 50°C before weighing. Melted test substance was added to the vehicle (methyl cellulose 1%) to generate the desired concentrations. The pethoxamid suspension was shaken or stirred to create a thick suspension. Prior to dosing, the substance was placed in a water bath at 50°C to melt, then left in a water bath at 25°C for 20 minutes.

In a preliminary study, a dose of 2500 mg/kg bw was administered by oral gavage to 2 male and 2 female Sprague Dawley (CD) rats. At 2500 mg/kg bw 3/4 rats died (or were sacrificed for humane reasons) on day 2 after dosing. In the main study, groups of fasted rats (5 males and 5 females) received doses of 800, 1260 or 2000 mg/kg bw. The substance (purity 95%) was administered as supplied, in dose volumes of 0.72, 1.13, or 1.8 mL/kg. At 2000 mg/kg bw, deaths occurred in 5/5 males and 4/5 females between days 1 and 3. At 1260 mg/kg bw, deaths occurred in 3/5 males and 2/5 females on days 2 or 3. At 800 mg/kg bw, deaths occurred in 2/5 males on day 2, while no females died. The LD_{50} was 1472 mg/kg bw for females, and 983 mg/kg bw for males.

Clinical signs included piloerection, abnormal body carriage, abnormal gait, lethargy, decreased respiratory rate, partially closed eyelids, pallor of extremities, soft/liquid faeces and increased salivation in all or the majority of rats. Unsteadiness, body tremors, dilation of pupils, cold to touch and anogenital staining was observed in rats at 1260 and 2000 mg/kg bw. In the one surviving rat dosed at 2000 mg/kg bw, clinical signs persisted until day 15. Recovery of surviving rats was complete by day 3 (females at 800 mg/kg bw), day 4 (all rats at 1260 mg/kg bw) or day 6 (males at 800 mg/kg bw). The mean body weights of surviving animals increased within the normal range by day 15. There were no abnormalities at macroscopic examination of surviving animals on day 15. The DS proposed Acute Tox 4, H302 Harmful if swallowed; with an ATE of 983 mg/kg bw.

Comments received during consultation

A Company-Manufacturer agreed with the proposed classification.

Assessment and comparison with the classification criteria

In the available GLP and OECD TG 401 compliant study, the LD_{50} was 1472 mg/kg bw for females and 983 mg/kg bw for males. The substance was administered by oral gavage "as supplied", which was clarified by the DS to mean a thick suspension in 1% methyl cellulose. The LD_{50} values obtained are in the range for Category 4 ($300 < LD_{50} \leq 2000$ mg/kg bw) for acute oral toxicity.

Therefore, RAC agrees with the DS's proposal that a **classification as Acute Tox 4, H302 Harmful if swallowed; with an ATE rounded to 980 mg/kg bw, is warranted**.

ACUTE DERMAL TOXICITY

Summary of the Dossier Submitter's proposal

One guideline (OECD TG 402) and GLP compliant study (Anonymous, 1994b) was summarised in the CLH report. The substance (purity 95%) was administered to Sprague Dawley (CD) rats as supplied but did not mention the physical state of the substance. The DS later clarified that in the acute dermal toxicity study the substance was placed in a water bath at 50°C to melt prior to dosing, and thus was administered in liquid form. The animals (5/sex/group) were exposed to a single limit dose of 2000 mg/kg pethoxamid for 24 hours under a semi-occlusive dressing. At the end of the 24-hour exposure period, the dressing was removed, and the application site was rinsed with warm water. There were no deaths, signs of systemic toxicity or local skin effects. The mean body weights of the animals increased within the normal range throughout the study period and there were no adverse macroscopic findings at necropsy on day 15. The DS concluded that as the LD_{50} value of pethoxamid was > 2000 mg/kg, the substance does not meet the criteria for classification.

Comments received during consultation

A National Authority commented that the latest version of the OECD TG 402 states that solid test items should be moistened sufficiently, preferably with water or, where necessary, a suitable vehicle to ensure good contact with the skin. In contrast, it is stated in the CLH report that the test material was administered as supplied to the rats at 2000 mg/kg bw (application volume 1.8 mL/kg). The test material was pethoxamid, purity 95%. As pethoxamid is solid at room temperature, its direct application as supplied constitutes a deviation from the above-mentioned guideline. It seems unclear whether good contact between the test item and the skin can be ensured under such circumstances and whether robust conclusions with regard to dermal toxicity can be drawn. They noted that the application volume, if it refers to solid pethoxamid without vehicle, would be equivalent to ~2.14 g/kg considering the relative density of pethoxamid.

Assessment and comparison with the classification criteria

In the available GLP and OECD TG 402 study, a single limit dose of 2000 mg/kg pethoxamid was applied for 24 hours under a semi-occlusive dressing. As clarified by the DS, the substance was preheated prior to dosing and applied as a liquid. No deaths, signs of systemic toxicity or local skin effects were detected. As the LD_{50} value of pethoxamid was > 2000 mg/kg, RAC agrees with the DS's proposal that **no classification for acute dermal toxicity is warranted.**

ACUTE INHALATION TOXICITY

Summary of the Dossier Submitter's proposal

One guideline (OECD TG 403) and GLP compliant study (Anonymous, 1994) was discussed in the CLH report. Sprague Dawley (CD) rats (5/sex/group) were exposed whole body for four hours to a maximum attainable concentration of pethoxamid as a liquid droplet aerosol (analysed concentration of 4.16 mg/L air). The purity of the substance was 95%, the MMAD of the particles was 3.3 µm. There were no deaths. Clinical signs included matted fur, partially closed eyes,

wetness/staining around the eyes, snout and mouth, exaggerated respiratory movements (seen in 1 male), and were observed from 1 hour to 8 days after exposure. All treated animals showed recovery from day 6 in males or day 8 in females. Body weights increased as expected from day two onwards and there were no treatment related macroscopic or microscopic necropsy findings. The DS concluded that a substance is classified for acute inhalation toxicity if the 4 h LC_{50} value is less than 5 mg/L, and as the LC_{50} value of pethoxamid was > 4.16 mg/L (the highest attainable analysed concentration), the substance is of low acute toxicity by the inhalation route and does not meet the criteria for classification.

Comments received during consultation

A Company-Manufacturer agreed with the proposed classification.

Assessment and comparison with the classification criteria

A substance is classified for acute inhalation toxicity if the 4 h LC_{50} value is less than 5 mg/L (dusts and mists). In the available inhalation study, there were no mortalities at the highest achievable concentration (4.16 mg/L). The LC_{50} is higher than 4.16 mg/Land at this concentration there was no mortality, therefore it is reasonable to conclude that the LC_{50} is above 5 mg/L. RAC agrees with the DS that **no classification for acute toxicity via the inhalation route is warranted**.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

There are 4 studies which are relevant to this hazard class in the CLH report: the 3 acute toxicity studies which were summarised in the acute toxicity section and an acute neurotoxicity study in rats (Anonymous, 2014c), which were performed according to OECD TG 424 and were GLP compliant and included a range finding study and a main study.

In the range finding study, Crl:CD(SD) rats (5/sex/group) were administered pethoxamid or the vehicle control (1% methylcellulose and 0.5% Tween® 80) by oral gavage at dose levels of 0, 600, and 800 mg/kg bw. No mortality was observed. 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, and these were statistically significant in males in the 800 mg/kg group and in females in both dose groups. There was also a statistically significant reduction in absolute and relative food consumption observed in male and female rats at 600 and 800 mg/kg bw on the day following dose administration. The clinical signs at 600 and 800 mg/kg bw considered to be related to the test substance were hunched posture, vocalization to the touch, chromorhinorrhea, pale ears, red urine, coldness to the touch, ptosis, mild or moderate dehydration and whole-body tremors.

In the main study, rats (10/sex/group) were administered pethoxamid or the vehicle control by oral gavage on Day 1 at dose levels of 0, 100, 300, or 800 mg/kg bw. At 800 mg/kg bw, two female rats were found dead (on Days 2 and 3). Clinical signs (decreased motor activity, ptosis, mild and moderate dehydration, hunched posture, pale ears and extremities, bradypnea, scant faeces and/or ungroomed coat) were observed only in the two female rats that were found dead. At 800 mg/kg bw, a statistically significant body weight loss was observed in male and female

rats on Days 1 to 2 after which body weight rebounded. This was associated with a statistically significant absolute and relative decreased food consumption in both sexes from Day 1 to 2. At 300 mg/kg bw, decreased body weight gain was observed in male and female rats from Day 1 to 2 (statistically significant in males). This was associated with a statistically significant relative decrease in food consumption in males and females from Day 1 to 2. A functional observation battery (FOB), followed by motor activity evaluation was performed on all rats prior to dose administration, on the day of dose administration at 16-hour post-dose, 7 days after dose administration and 14 days after dose administration. 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. 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. There were no treatment-related effects on motor activity. Absolute and relative brain weights were unaffected at all doses. There were no treatment-related gross or neurohistopathology findings.

The DS summarised that in the acute oral study the observed clinical signs were indicative of general toxicity and did not give any indication of specific target-organ toxicity. In the acute dermal study, a dose of 2000 mg/kg bw did not induce any clinical signs of systemic toxicity and there were no macroscopic findings at necropsy. In the acute inhalation toxicity study the observed clinical signs of toxicity were general indicators of toxicity and respiratory effects that are commonly associated with the inhalation route of exposure, and do not provide specific evidence of an irritant effect on the respiratory tract of rats.

The DS concluded that the available acute studies do not provide any indication that pethoxamid meets the classification criteria for STOT SE category 1, 2 or 3 and thus proposed no classification.

Comments received during consultation

A Company-Manufacturer concurred that classification is not required.

Assessment and comparison with the classification criteria

STOT SE is defined as specific, non-lethal target organ toxicity arising from a single exposure to a substance or mixture.

STOT SE categories 1 and 2 are assigned on the basis of clear evidence of significant or severe toxicity to a specific target organ that arises from a single exposure to a substance.

In the available acute oral toxicity study, rats (5/sex/group) received doses of 800, 1260 or 2000 mg/kg bw. Clinical signs included piloerection, abnormal body carriage, abnormal gait, lethargy, decreased respiratory rate, partially closed eyelids, pallor of extremities, soft/liquid faeces and increased salivation in all or the majority of rats. In rats at 1260 and 2000 mg/kg bw unsteadiness, body tremors, dilation of pupils, cold to touch and anogenital staining was observed. There were no abnormalities at macroscopic examination of surviving animals on day 15.

In the available acute dermal toxicity study, rats (5/sex/group) were exposed to a single limit dose of 2000 mg/kg. There were no deaths, signs of systemic toxicity or local skin effects, and there were no adverse macroscopic findings at necropsy on day 15.

In the available acute inhalation study, rats (5/sex/group) were exposed to a maximum attainable concentration of pethoxamid as a liquid droplet aerosol (4.16 mg/L air). There were no deaths. Clinical signs included matted fur, partially closed eyes, wetness/staining around the eyes, snout and mouth, exaggerated respiratory movements (seen in 1 male), and were

observed from 1 hour to 8 days after exposure. All treated animals showed recovery from day 6 in males or day 8 in females.

In the acute neurotoxicity study dose levels of 0, 600, and 800 mg/kg bw (range finding study) or 0, 100, 300, or 800 mg/kg bw (main study) was administered by gavage. Systemic toxicity was observed (body weight loss and reduction in absolute and relative food consumption at 600 and 800 mg/kg bw on the day following dose administration). Nevertheless, there were no statistically significant or biologically important effects of pethoxamid on the FOB parameters at any time point that were considered to be test substance related. There were no treatment-related effects on motor activity. Absolute and relative brain weights were unaffected at all doses. There were no treatment-related gross or neurohistopathology findings. There were no signs of specific target-organ toxicity in any of the available acute toxicity studies, therefore RAC agrees with the DS that no classification for STOT SE category 1 or 2 is warranted.

STOT SE category 3 covers 'transient effects' occurring after single exposure, specifically respiratory tract irritation and narcotic effects. There was no indication in the acute toxicity studies of either respiratory irritation or narcotic effects; therefore, RAC agrees with the DS's proposal that **no classification for STOT SE is warranted**.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

One guideline (OECD TG 404) and GLP compliant study (Anonymous, 1994a) was summarised in the CLH report. The CLH report did not indicate the physical state of the substance. The DS later clarified that in the skin corrosion/irritation study the substance was placed in a water bath at 50°C prior to dosing, and thus was administered in liquid form. Six male New Zealand White rabbits were exposed to 0.5 mL pethoxamid under a semi-occlusive dressing for 4 hours. The purity of the substance was 95%. Very slight erythema (score 1) was present on day 1 in all 6 animals (approximately 30 mins after removal of the dressings). Very slight erythema and very slight edema was present in 1 rabbit on days 2 and 3. All reactions had resolved by Day 5. No other cutaneous reactions and no signs of toxicity were observed during the study. Mean scores over 24, 48 and 72 hours for each animal were 0.0, 0.0, 0.0, 0.0, 1.0 and 0.0 for erythema and 0.0, 0.0, 0.0, 0.0, 0.66 and 0.0 for edema. Since the average scores for each animal were 0 or 1 for erythema and 0 or 0.66 for oedema, the DS concluded that pethoxamid does not meet the criteria for classification.

Comments received during consultation

There were two comments on this endpoint. A Company-Manufacturer concurred that pethoxamid is not a skin irritant and that classification is not required.

A National Authority pointed out that the latest version of the OECD TG 404 states that when testing solids, the test chemical should be moistened with the smallest amount of water or other suitable vehicle, sufficient to ensure good skin contact. In contrast, in the CLH report the rabbits received 0.5 mL of the test substance, which was administered as supplied to the shaved skin of each animal. The test material was pethoxamid, purity 95%. As pethoxamid is a solid at room temperature, its direct application as supplied constitutes a deviation from the above-mentioned guideline. It seems unclear whether good contact between the test item and the skin can be ensured under such circumstances and whether robust conclusions with regard to potential dermal irritation/corrosion can be drawn. Also, the application volume indicated, if it refers to solid pethoxamid without vehicle –is equivalent to ~0.6 g considering the relative density of

pethoxamid. This would constitute another deviation from OECD TG 404, which states that a dose of 0.5 g of solid or paste is to be applied to the test site.

Assessment and comparison with the classification criteria

In the available GLP and OECD TG 404skin corrosion/irritation study, 6 male New Zealand White rabbits were exposed to 0.5 mL pethoxamid under a semi-occlusive dressing for 4 hours. The DS clarified that the substance was placed in a water bath at 50°C prior to dosing, and thus was administered in liquid form. Mean scores over 24, 48 and 72 hours for each animal were 0.0, 0.0, 0.0, 0.0, 1.0, 0.0 for erythema and 0.0, 0.0, 0.0, 0.0, 0.66, 0.0 for edema. No other cutaneous reactions and no signs of toxicity were observed during the study.

A substance is classified as a skin irritant category 2 if any of the following criteria are met:

1. mean value of ≥ 2.3 - ≤ 4.0 for erythema/eschar or for edema in at least 2 of 3 tested animals from gradings at 24, 48 and 72 hours after patch removal or, if reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions; or
2. inflammation that persists to the end of the observation period, normally 14 days in at least 2 animals, particularly taking into account alopecia (limited area), hyperkeratosis, hyperplasia, and scaling; or
3. in some cases where there is pronounced variability of response among animals, with very definite positive effects related to chemical exposure in a single animal but less than the criteria above.

As none of the criteria for classification in CLP are met, RAC agrees with the DS's proposal that **no classification for skin corrosion/irritation is warranted**.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

One guideline (OECD TG 405) and GLP compliant study (Anonymous, 1994b) performed with 6 New Zealand White rabbits was summarised in the CLH report. The purity of the substance was 95%. Pethoxamid was warmed in a water bath and 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. There were no signs of gross toxicity, adverse clinical signs or abnormal behaviour following administration of the test substance. Dulling of the cornea was seen in 5/6 animals at 1 hour after instillation. Diffuse conjunctival redness (grade 2), obvious conjunctival chemosis (grade 2) and conjunctival discharge with moistening of the eyelids (grade 2/3) were noted in all six animals at 1 hour after instillation. At 24 hours after instillation, slight conjunctival chemosis (grade 1) was noted in 3/6 rabbits and slight conjunctival redness (grade 1) was noted in 5/6 rabbits. There were no signs of ocular irritation in any animals at 48 hours after instillation. The mean scores calculated for each animal over 24, 48 and 72 hours were 0.0, 0.0, 0.0, 0.0, 0.0 and 0.0 for corneal opacity and iris lesions, 0.3, 0.0, 0.3, 0.0, 0.3, 0.0 for conjunctival chemosis and 0.3, 0.3, 0.3, 0, 0.3, 0.3 for conjunctival redness. The ocular reactions were fully reversible within 48 hours after application. The DS concluded that the substance does not meet the criteria for classification.

Comments received during consultation

A Company-Manufacturer concurred that pethoxamid is not an eye irritant and that classification is not required.

Assessment and comparison with the classification criteria

In the available guideline and GLP study in 6 New Zealand White rabbits, each animal received 0.1 mL of the (preheated) test substance, by a singular ocular instillation without irrigation to the lower everted lid of one eye. There were no signs of gross toxicity, adverse clinical signs or abnormal behaviour following administration of the test substance. One hour after instillation the following were noted: dulling of the cornea in 5/6 animals, diffuse conjunctival redness (grade 2), obvious conjunctival chemosis (grade 2) and conjunctival discharge with moistening of the eyelids (grade 2/3) in all six animals. At 24 hours after instillation the following were noted: slight conjunctival chemosis (grade 1) in 3/6 rabbits and slight conjunctival redness (grade 1) in 5/6 rabbits. At 48 hours after instillation there were no signs of ocular irritation in any of the animals. The mean scores calculated for each animal over 24, 48 and 72 hours were 0.0, 0.0, 0.0, 0.0, 0.0, 0.0 for corneal opacity and iris lesions, 0.3, 0.0, 0.3, 0.0, 0.3, 0.0 for conjunctival chemosis and 0.3, 0.3, 0.3, 0, 0.3, 0.3 for conjunctival redness. The ocular reactions were fully reversible within 48 hours after application.

According to the CLP criteria, a substance is classified as an eye irritant category 2 if, when applied to the eye of an animal, the substance produces in at least 2 of 3 tested animals a positive response of:

- corneal opacity ≥ 1 and/or
- iritis ≥ 1, and/or
- conjunctival redness ≥ 2 and/or
- conjunctival edema (chemosis) ≥ 2

calculated as the mean scores following grading at 24, 48 and 72 hours after installation of the test material, and which fully reverses within an observation period of 21 days.
As the criteria for classification in CLP are not met, RAC agrees with the DS's proposal that **no classification for eye corrosion/irritation is warranted**.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

Because of the lack of data, no conclusion on classification for respiratory sensitisation can be drawn.

Comments received during consultation

No comments were received on this endpoint.

Assessment and comparison with the classification criteria

There are no studies relevant to this endpoint, therefore RAC agrees with the DS that **no classification for respiratory sensitisation is warranted due to lack of data.**

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

One Guinea-pig maximisation test (GPMT) (Anonymous, 1998) was summarised in the CLH

report, which was performed according to OECD TG 406 and GLP. Based on the results of a preliminary test, a concentration of 0.5% (v/v) in Alembicol D was selected for intradermal induction, the test substance as supplied (purity 95%) for the topical induction, and concentrations of 25 and 12.5% (v/v) in Alembicol D for topical challenge. Sixty male Dunkin-Hartley guinea pigs were used, 20 test animals, 20 control animals and 10-10 for the positive control (HCA) and control to the HCA group. The test animals all showed slight (11/20), well-defined (8/20) or moderate erythema (1/20) following topical induction. No erythema was seen in the control guinea pigs. Following the challenge exposure, 4/20 control animals showed slight erythema at 24 or 48 hours. In the test group, 19/20 animals showed skin reactions at 24 and/or 48 hours, which were more marked than those seen for controls and were considered positive responses. The remaining animal showed a similar response to the controls and therefore was considered a negative response. As 95% of the test animals responded to a 0.5% intradermal induction dose, the DS concluded that classification in sub-category 1A is warranted.

Comments received during consultation

A Company-Manufacturer agreed with the proposed classification.

Assessment and comparison with the classification criteria

In the available study, which used a concentration of 0.5% (v/v) in Alembicol D for intradermal induction, the test substance as supplied (purity 95%) for topical induction, and concentrations of 25 and 12.5% (v/v) in Alembicol D for topical challenge, 19/20 animals showed skin reactions at 24 and/or 48 hours after topical challenge. The concentrations used were based on the results of a preliminary study, in which 0.5% in Alembicol D was the highest concentration that caused irritation suitable for the intra-dermal induction and 25 and 12.5% was the maximum non–irritant concentration for the challenge application.

Concentration for intradermal induction (% w/v)	Incidence sensitised guinea pigs (%)	Potency	Resulting sub-category
≤ 0.1	≥ 60	Extreme	1A
≤ 0.1	>30 - <60	Strong	1A
>0.1 - ≤ 1.0	≥60	Strong	1A
>0.1 - ≤ 1.0	>30 - <60	Moderate	1B
> 1.0	≥ 30	Moderate	1B

According to the CLP criteria, in a GPMT, when an intradermal induction concentration of >0.1% - ≤1.0% induces responses in ≥60% of the guinea pigs, Skin Sens. 1A is warranted. In the GPMT with pethoxamid, at an intradermal induction concentration of 0.5% (w/v), a response was seen in 95% of the animals. Therefore, RAC agrees with the DS that a **classification as Skin Sens. 1A is warranted**.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The studies summarised in the CLH dossier for this endpoint consisted of four 28-day studies (1 oral and 1 dermal study in rats, 1 oral study in mouse and a maximum tolerated dose and four

week constant dose study in dogs), three 90 day oral studies (rat, mouse, dog), a dog 52-week study, a rat chronic- and carcinogenicity study, a mouse carcinogenicity study, and finally a 90-day rat study to evaluate potential mechanisms underlying the observed thyroid gland changes.

***Table**: Repeated dose toxicity studies (Table 54. in the CLH report)*

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, route of exposure, dose levels, duration of exposure**	**Results**	**Reference**
Rat oral 28-day feeding study (via diet) OECD TG 407 Deviations: Some omissions in range of haematology parameters, organ weights recorded, tissues preserved and tissues examined microscopically. GLP Rat Crl:CD(SD)BR 5/sex/group	Pethoxamid Batch: TB-930727 Purity: 95.2%. Dose levels: 0, 500, 2500, 5000, 7500 ppm in diet for 28 days.	<u>7500 ppm (699 mg/kg bw/d males, 737 mg/kg bw/d females)</u> ↓ Body weight Week 4: 35.2% males, 20.5% females ↓ Body weight gain Week 0-4: 88.9% males, 64.4% females ↓ Food consumption Week 1-4: 28% males, 19% females ↓ Water consumption Week 3: 25% males (single value) Haematology: ↓ Platelets 14.4% males; ↓ White blood cell counts 31.0% males; ↓ lymphocytes 36.3% males; ↓ haemoglobin 7.0% females; ↓ MCHC 4.7% females Clinical chemistry: ↑ Cholesterol 228% males, 173% females; ↓ Phosphorus 18.8% males; ↑ ALT 66.7% females; ↓ Glucose 16.1% males, 21.2% females; ↑ Globulin 11.4% males, 20.6% females ↑ Liver weights: Adjusted 63% males, 44% females Pathology findings: Centrilobular enlargement of hepatocytes 5/5 males and females (0/5 controls); Periportal hepatocytes with eosinophilic inclusions 4/5 males and 3/5 females (0/5 controls). <u>5000 ppm (482 mg/kg bw/d males, 535 mg/kg bw/d females)</u> ↓ Body weight Week 4:17.2% males, 8.8% females ↓ Body weight gain Week 0-4: 42.4% males 30.1% females ↓ Food consumption Week 1-4: 13% males Haematology: ↓ Platelets 13.9% males; ↓ lymphocytes 30.3% males; ↓ haemoglobin 6.3% females Clinical chemistry: ↑ Cholesterol 162% males, 117% females; ↓ Phosphorus 10.4% males; ↓ Glucose 16.1% females; ↑ Globulin 20.0% males, 17.6% females ↑ Liver weights: Adjusted 69% males, 45% females Pathology findings: Centrilobular enlargement of hepatocytes 5/5 males and 3/5 females (0/5 controls); Periportal hepatocytes with eosinophilic inclusions 3/5 males and 1/5 females (0/5 controls). <u>2500 ppm (227 mg/kg bw/d males, 266 mg/kg bw/d females)</u> ↓ Body weight gain Week 0-4: 23.6% males ↓ Food consumption Week 1-4: 11% males Haematology: ↓ Platelets 18.9% males Clinical chemistry: ↑ Cholesterol 121% males, 51.9% females ↑ Liver weights: Adjusted 58% males Pathology findings: Centrilobular enlargement of hepatocytes 1/5 males (0/5 controls); Periportal hepatocytes with eosinophilic inclusions 4/5 males (0/5 controls). <u>500 ppm (45.3 mg/kg bw/d males, 52.9 mg/kg bw/d females)</u> Clinical chemistry: ↑ Cholesterol 55.2% males **No NOAEL established.**	Anonymous (1994); 69 PXA
Mouse oral 28 day feeding study (via diet) OECD 407 Deviations: no haematology or clinical chemistry. Additional liver enzyme investigations. Only macroscopic abnormalities and liver and thyroid	Pethoxamid Batch: TB-930727 Purity: 95%. Dose levels: 0, 100, 500, 3000, 10000 ppm in diet for 28 days.	<u>10000 ppm (1786 mg/kg bw/d males, 2206 mg/kg bw/d females)</u> ↓ Body weight gain: Week 0-4 112% males; Week 0-1 weight loss 2.5 g males (controls gained 2.3 g), 1.2 g females (controls gained 0.6 g) ↓ Food consumption: Week 1 30% males, 24% females ↑ Liver weights: Adjusted 45% males, 27% females Hepatic enzyme activities: ↑ both sexes EROD, PROD, lauric acid 11 hydroxylase (LA11), p-nitrophenol UDP glucuronyltransferase (UDPGT) Pathology findings: Hepatocellular hypertrophy- Centrilobular 7/16 males (1/16 controls); generalised 9/16 males (0/16 controls); periportal 16/16 females (0/16 controls) <u>3000 ppm (539 mg/kg bw/d males, 679 mg/kg bw/d females)</u> ↓ Body weight gain: Week 0-4 52% males; Week 0-1 weight	Anonymous (1996a); 70 PXA

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
examined microscopically. GLP Mouse Crl: CD-1 (ICR) 16/sex/group		loss 0.2 g males (controls gained 2.3 g) ↓ Food consumption: Week 1 20% males, 14% females ↑ Liver weights: Adjusted 27% males, 16% females Hepatic enzyme activities: ↑ EROD, PROD both sexes; LA11 and UDPGT females only Pathology findings: Hepatocellular hypertrophy- Centrilobular 5/16 males (1/16 controls); generalised 5/16 males (0/16 controls); periportal 14/16 females (0/16 controls) 500 ppm (85 mg/kg bw/d males, 114 mg/kg bw/d females) ↑ Liver weights: Adjusted 14% males Hepatic enzyme activities: ↑ PROD both sexes; EROD females only Pathology findings: Centrilobular hepatocellular hypertrophy 4/16 males (1/16 controls) 100 ppm (17 mg/kg bw/d males, 22 mg/kg bw/d females) Hepatic enzyme activities: ↑ PROD both sexes **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 observed, but this was not considered an adverse effect.**	
Dog maximum tolerated dose and four weeks constant dose study via capsule administration No guideline for this study type in non-rodents GLP Dog Beagle MTD phase: Dose given to initial 2 dogs increased every 3 to 4 days. Constant dose phase: Further pair of dogs	Pethoxamid Batch: TB 930727 Purity: 96.0% and Batch: TB 951005 Purity: 95.1%. Orally by capsule MTD phase: 25, 50, 100, 200, 400, 800, 1000 and 1600 mg/kg bw/d given for 3 to 4 days. Constant dose phase: 50, 200 and 800 mg/kg bw/d given to for 28 days. Due to adverse observations at 800 mg/kg bw/d, dosing suspended for 7 days and then restarted at 400 mg/kg bw/d for 28 days.	**MTD phase:** 25-1600 mg/kg bw/d Liquid faeces observed in both sexes at all dose levels. Mucoid/red stained faeces observed from 400 mg/kg bw/d. Vomiting observed from 800 mg/kg bw/d. Salivation observed in males from 1000 mg/kg bw/d. Female was subdued after 2 doses of 1600 mg/kg bw/d and dosing discontinued. ↓ Body weight: Slight in female from 800 mg/kg bw/d ↓ Food consumption: From 800 mg/kg bw/d ↑ Liver weights: Relative (to body weight) female Pathology findings: Centrilobular hepatocellular hypertrophy and prominent thyroid microfollicles (male only) **Constant dose phase:** 800 mg/kg bw/d Liquid/red faeces, vomiting, salivation, subdued behaviour, marked loss of body weight and decreased food consumption at 800 mg/kg bw/d. Dosing suspended after 7 days. Following 7 days off dose, animals given 400 mg/kg bw/d for 28 days. 400 mg/kg bw/d Vomiting, liquid/mucoid faeces, salivation and subdued behaviour. Decreased haemoglobin and related parameters. Haematology: ↓ Slight in red cell parameters; ↑ platelets; ↑ reticulocytes Clinical chemistry: ↓ Slight cholesterol ↑ Liver weights: Relative (to body weight) female Hepatic enzyme activities: ↓ EROD male; PROD female; lauric acid 11 hydroxylase (LA11) male; lauric acid 12 hydroxylase (LA12) male and female; p-nitrophenol UDP glucuronyltransferase (UDPGT) male and female Pathology findings: Minimal centrilobular hepatocyte hypertrophy female only 200 mg/kg bw/d Vomiting, liquid/mucoid faeces both male and female Subdued behaviour female ↑ Liver weights: Relative (to body weight) female Pathology findings: Minimal centrilobular hepatocellular hypertrophy, male and female 50 mg/kg bw/d Liquid/mucoid faeces both dogs	Anonymous (1996b); 72 PXA

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		Conclusion: Dosages of 400 mg/kg bw/d and above are not suitable for further investigations on dogs. No induction of drug metabolizing enzymes in dogs	
Rat oral 90 day feeding study (via diet) OECD 408 Deviations: No FOB or motor activity, thymus weight not recorded, no microscopic examination of accessory sex organs, skin or peripheral nerve. GLP Rat Crl: CD (BR) 10/sex/group	Pethoxamid Batch: TB-930727 Purity: 95.2%. Dose levels: 0, 100, 500, 2500, 5000 ppm in diet for 13 weeks	5000 ppm (388 mg/kg bw/d in males, 426 mg/kg bw/d in females) ↓ Body weight gain Week 0-13: 31% males, 25% females ↓ Food consumption: Week 1 49% males, 24% females; Week 1-13: 15% males ↓ Water consumption Week 12: 16% males Haematology: ↓ Platelets 11% males Clinical chemistry: ↑ Cholesterol 108% males, 75% females; ↓ Glucose 13% males; ↑ Total protein 11% males, 7% females Liver enzyme activity: ↑ cyt P450, EROD, PROD, LA11 and UDPGT in both sexes; LA12 in females only. Increase in PROD in females 791-fold. ↑ Liver weights: Adjusted 42% males, 42% females Liver pathology: Periportal hepatocyte margination of cytoplasm 10/10 males and 10/10 females (0/10 controls); Occasional concentric intracytoplasmic inclusions 7/10 males (0/10 controls); Minimally generalised hepatocyte enlargement 4/10 males and 4/10 females (0/10 controls); Fat deposition in periportal hepatocytes 6/10 males (0/10 controls). ↑ Thyroid weights: 15% absolute males; 34% adjusted females. Thyroid pathology: Follicular cell hypertrophy 9/10 males, 4/10 females (2/10 control males, 0/10 control females); Sparse colloid 7/10 males (1/10 controls). 2500 ppm (196 mg/kg bw/d in males, 207 mg/kg bw/d in females) ↓ Body weight gain Week 0-13: 24% males, 15% females ↓ Food consumption Week 1: 23% males ↓ Water consumption Week 12: 20% males Haematology: ↓ Platelets 12% males Clinical chemistry: ↑ Cholesterol 46% males, 37% females Liver enzyme activity: ↑ cyt P450, EROD, PROD, LA11 and UDPGT in both sexes; LA12 in females only. Increase in PROD in females 276-fold. ↑ Liver weights: Adjusted 27% males, 21% females Liver pathology: Periportal hepatocyte margination of cytoplasm 9/10 males (0/10 controls); Fat deposition in periportal hepatocytes 5/10 males (0/10 controls). Thyroid pathology: Follicular cell hypertrophy 7/10 males (2/10 control). 500 ppm (36.2 mg/kg bw/d in males, 41.6 mg/kg bw/d in females) ↓ Body weight gain Week 0-13: 12% males Liver enzyme activity: ↑ EROD, PROD, in both sexes; cyt P450, LA11, LA12 and UDPGT in females only. Increase in PROD in females 12.4-fold. No statistically significant increase in organ weights or pathology findings. 100 ppm (7.5 mg/kg bw/d in males, 8.0 mg/kg bw/d in females) No effects. **NOAEL 100 ppm (7.5 mg/kg bw/d) based on decreased body weight gain. 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.**	Anonymous (1996); 61 PXA
90-day rat study to evaluate potential mechanisms underlying the observed thyroid gland changes. General design similar to	Pethoxamid Batch: 21082018 Purity: 99.4% Dose levels: 0, 400, 1600, 5000 ppm (0, 24, 96, 308 mg/kg w/day) in diet for 13	5000 ppm (308 mg/kg bw/d) ↓ Body weight in the 5000 ppm group, 7.5% lower than controls at the end of study. ↑ Mean TSH values in the 5000 ppm groups on Days 15, 29, 57, and 89 ↓ Time-dependent decrease in total T4 relative to pre-treatment values during the first 29 days ↑ Thyroid weight in the 5000 ppm group on Days 30 (absolute 31% and relative to body weight 58%) and 93/94 (absolute 27% and relative to body weight 39%)	Anonymous (2020); 2018TOX-PXA4560

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
OECD 408. Thyroid hormone assessed on Days -3, 15, 29, 57 and 89. Total T3 and T4, TSH and rT3 levels analysed. At termination liver, thyroid and pituitary weighed and histopathology performed. Hepatic UDP-Glucuronosyltransferase (UGT) activity (T3-glucuronidation and T4-glucuronidation) and protein concentration assessed GLP Rat Crl:CD(SD) Sprague Dawley 15 males/group termination after 90 days 5 males/group termination after 30 days	weeks Positive control: Phenobarbital 1000 ppm.	↑ Liver weight in the 5000 ppm group on Day 30 (absolute 6% and relative to body weight 30%) and 5000 ppm (absolute 35% and relative to body weight 47%) group on Day 93/94. ↑ Thyroid follicular cell hypertrophy in the 5000 ppm group (15/15 compared with 0/15 in control) on Day 93/94 ↑ Hepatocellular hypertrophy in the 5000 ppm group on Days 30 (3/5 compared with 0/5 in control) and 93/94 (5/15 compared with 0/15 in control) ↑ T4-glucuronidation activity was observed in the 5000 ppm group on Days 30 (4.9 fold increase) and 93/94 (3.2 fold increase) ↑ T3-glucuronidation activity was elevated in the 5000 ppm group on Day 30 (1.7 fold increase) and on Day 93/94 (1.9 fold increase) 1600 ppm (96 mg/kg bw/d) ↑ Mean TSH values in the 1600 ppm group on Days 15, 29, 57, and 89 ↑ Liver weight in the 1600 ppm group (absolute 15% and relative to body weight 16%) on Day 93/94. ↑ Thyroid follicular cell hypertrophy in the 1600 ppm group (3/14 compared with 0/15 in control) on Day 93/94 ↑ T4-glucuronidation activity was observed in the 1600 ppm group on Days 30 (2.7 fold increase) and 93/94 (1.9 fold increase) ↑ T3-glucuronidation activity was elevated in the 1600 ppm group on Day 30 (1.9 fold increase) 400 ppm (24 mg/kg bw/d) No effects. phenobarbital Data shown in data summary table in the Carcinogenicity Section Overall, it can be concluded that liver enzyme induction, leading to an increase in T4 glucuronidation and clearance of T4, elicited a feedback response on the thyroid via an increase in TSH. Further, the increased TSH and associated thyroid follicular cell hypertrophy resulted in functional compensation by the thyroid in the Pethoxamid-treated rats. **The NOAEL (NOEL) was 400 ppm (24 mg/kg bw/d) based on increased liver weight of liver and thyroid follicular cell hypertrophy.**	
Mouse oral 13 week feeding study (via diet) OECD 408 Deviations: Uterus weight, thymus weight not included; several organs not examined microscopically and not all accessory sex organs preserved. Supplementary electron microscopy of liver. GLP Mouse Crl: CD-1 (ICR) BR 10/sex/group	Pethoxamid Batch: TB-960306 Purity: 95.0% Dose levels: 0, 50, 400, 3000, 10000 ppm in diet for 13 weeks	10000 ppm (2354 mg/kg bw/d in males and 2492 mg/kg bw/d in females) ↓ Body weight gain Week 0-1: loss of 2.7 g males, 1.1 g females; controls gained 2.3 and 1.2 g, respectively ↓ Body weight gain Week 0-12: 102% males, 80% females Haematology: ↓ Haemoglobin 11% males, 8% females; ↓ PCV 8% males; ↓ Red cell count 12% males, 8% females; ↑ MCV 5% males; ↓ MCHC 3% males; ↓ lymphocytes 41% males (affected WBC). Clinical chemistry: ↑ Cholesterol 33% males, 108% females; ↓ Total protein 11% males, 6% females; ↓ Albumin 10% males, 13% females; Changes in plasma ion concentrations in males (↓ K and Ca; ↑ P and Cl). ↓ Urinary protein 75% females. ↑ Thyroid weights: Adjusted 23% females ↑ Liver weights: Adjusted 38% males, 48% females Liver pathology: Hepatocyte hypertrophy- centrilobular midzonal 8/10 males (0/10 controls); periportal 10/10 females (0/10 controls) ↓ Spleen weight: Absolute 33% males, 27% females Spleen pathology: Hemosiderosis 10/10 males (3/10 controls), 9/10 females (7/10 controls); Extramedullary haemopoiesis reduced severity; Reduced cellularity of the white pulp – marginal zone 9/10 males (2/10 control) Thymus pathology: Involution/atrophy 9/9 males (5/9 controls), 5/10 females (8/10 controls) GIT pathology: Villous epithelial cells swollen + cytoplasmic rarefaction in duodenum 10/10 males, 9/10 females; in jejunum 4/10	Anonymous; (1998) 71 PXA and Anonymous L. (1997a); 1288 PXA

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		in males and females (no findings in control group). 3000 ppm (610 mg/kg bw/d in males and 724 mg/kg bw/d in females) ↓ Body weight gain Week 0-12: 42% females Haematology: ↑ MCV 5% males Clinical chemistry: ↑ Cholesterol 33% males, 74% females; ↓ Albumin 10% males, 6% females; Changes in plasma ion concentrations in males (↓ Ca; ↑ Cl). ↑ Thyroid weights: Adjusted 20% females ↑ Liver weights: Adjusted 26% males, 22% females Liver pathology: Hepatocyte hypertrophy - periportal 10/10 females (0/10 controls) GIT pathology: Villous epithelial cells swollen + cytoplasmic rarefaction in duodenum 6/10 males (0/10 control). 400 ppm (70.5 mg/kg bw/d in males and 93 mg/kg bw/d in females) No treatment-related effects. 50 ppm (9.1 mg/kg bw/d in males and 12.0 mg/kg bw/d in females) No treatment-related effects. **The NOAEL (NOEL) was 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.**	
Dog 13 week study via capsule administration OECD TG 409 Deviations: Clinical chemistry: no ornithine decarboxylase GLP Dog Beagle 4/sex/group	Pethoxamid Batch: TB-960306 Purity: 95.0%. Dose levels: 0, 8, 50, 300 mg/kg bw/d for 13 weeks by oral capsule Due to poor clinical condition of 300 g/kg bw/d animals, treatment stopped on Day 4 of Week 2. Following a recovery period of 4 days, animals dosed with 200 mg/kg bw/d for the remainder of the study.	300/200 mg/kg bw/d Clinical signs Week 12: Liquid faeces 22 incidences in males, 23 incidences in females; Salivation 14 incidences in males, 20 incidences in females; Vomiting 1 incidence in males, 7 incidences in females Body weight Week 0-1: loss of 0.3 kg males, 0.8 kg females (control weight gain 0.4 kg in both sexes). Lower terminal body weight 25% males 17% females, neither statistically significant ↓ Body weight gain Week 0-13: 76% males, 59% females ↓ Food consumption: Week 0-1: 15% males, 46% females; Week 3-13 6% males, 8% females Haematology Week 12: ↑ Platelets 34% males, 37% females; ↑ Reticulocytes 4.5-fold males, 5.5 fold females; ↓ Hb 15% males, 13% females; ↓ PCV 12% females; ↓ RBC 16% males 14% females Clinical chemistry: ↓ Albumin 16% males, 12% females; ↓ALT activity 37% females [↓ ALT both sexes approx. 50% week 6, but ↑ males week 12]. Organ weight differences considered to be due to the marked body weight difference of control and high dose group dogs. Pathology findings: Vacuolation cortical tubules in kidney 4/4 both sexes (2/4 controls); Thymus involution 3/4 both sexes (0/4 controls); Spleen minimal haemosiderosis 2/4 males, 3/4 females (1/4 controls) Glycogen depletion in the liver (4/4 both sexes); myeloid atrophy in the bone marrow (3/4 males, 4/4 females); reduced lymphoid cellularity in the lymph nodes (2/4 male), 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 male) considered related to poor clinical condition, and not a direct effect. 50 mg/kg bw/d Clinical signs Week 12: Liquid faeces 19 incidences in males, 17 incidences in females Haematology Week 12: ↓ RBC 8% females Clinical chemistry: ↓ Albumin 8% females 8 mg/kg bw/d Clinical signs Week 12: Liquid faeces 6 incidences in males, 6 incidences in females. Haematology Week 12: ↓ RBC 11% females	Anonymous; (1997b); 73 PXA

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		NOAEL 8 mg/kg bw/d based on post dose liquid faeces	
Dog 52-week study via capsule administration OECD TG 452 GLP Dog Beagle 4/sex/group	Pethoxamid Batch: TB-960306 Purity: 95.0%. Dose levels: 0, 2, 20, 150 mg/kg bw/d for 12 months by oral capsule.	150 mg/kg bw/d One male and one female killed moribund weeks 47 and 38 respectively. Clinical observations in these animals were loss of appetite, bloody stool, salivation, dehydration signs, bradypnea, hypothermia, pale oral mucosa, pale conjunctiva, emaciation, decrease in spontaneous activity, prone and lateral position. Surviving animals had increased incidences of soft stool, diarrhoea and vomiting. ↓ Body weight Week 54: 16% females (surviving animals only). Final body weights of the sacrificed animals were 52 (male) and 64% (female) of their maximum values. Clinical chemistry Week 53: ↓ Albumin 4% males, 6% females; ↑ ALP activity 64% males, 151% females. Organ weights: ↑ Relative liver weight 38% males, 67% females; ↑ Relative kidney weights 59% females. GIT pathology: Stomach (pylorus) - micronecrosis mucosa 1/3 males, vacuolation and mononuclear cell infiltration in muscle layer 1/3 males, epithelium hyperplasia mucosa 1/3 males (0/4 in controls). Large intestines vacuolation and mononuclear cell infiltration in muscle layer 2/3 males (0/4 controls). 20 mg/kg bw/d Clinical signs: Soft stool days 162 to 293 and diarrhoea days 20 to 141. Organ weights: ↑ Relative liver weight 42% females. 2 mg/kg bw/d No treatment related effects. **Target organ gastrointestinal tract. The NOAEL in this study was 2 mg/kg bw/d based on increased liver weight in females and slightly increased frequency of diarrhoea**	Anonymous (1999); 74 PXA
Rat 28-day dermal administration toxicity study OECD TG 410 GLP Rat Crl: CD (SD) 10/sex/group	Pethoxamid technical Batch: P1351-JaK-T2-23-6 Purity: 95.80% Dose levels: 0, 100, 300, 1000 mg/kg bw/d for a 6-hour exposure period for 28 days. Test material applied neat and covered with a semi-occlusive wrap. After 6 hours, the wrap was removed and site washed.	1000 mg/kg bw/d Increase in the number of male and female rats with residue (presumed to be test substance) within the treated area. Increase in the number of male rats group observed with signs of irritation (incidence / animals); 21/7 erythema grade 1 and 45/4 flaking grade 1 [8/3 and 6/2 respectively in control group]. Decreased numbers of anagen-phase follicles (0.9 in males and 0.6 in females, 1.6 males and 2.9 females in controls) with some associated minimal to mild hyperkeratosis (1/10 in male and 6/10 in female, 0/10 in controls) were noted within sections of treated skin from the male and female rats. No systemic toxicity. 300 mg/kg bw/d Increase in the number of male and female rats with residue (presumed to be test substance) within the treated area. No systemic toxicity. 100 mg/kg bw/d Increase in the number of male and female rats with residue (presumed to be test substance) within the treated area. No systemic toxicity. **NOAEL for systemic toxicity is 1000 mg/kg bw/d**	Anonymous (2014d); 1216 PXA

The DS considered the 90-day studies to be the key studies. In these there was a consistent pattern of toxicity in rats and mice: the liver is considered to be the main target organ, and changes are characterised by altered clinical chemistry values (increased cholesterol) and increased activity of hepatic enzymes. These changes are accompanied by increased liver weights and histopathology findings of hepatocyte hypertrophy. Changes in organ weight and histopathology occur after 90 days at and above dose levels of 196 mg/kg bw/d in male rats, 207 mg/kg bw/d in female rats; 610 mg/kg bw/d in male mice and 724 mg/kg bw/d in female.

Hepatomegaly as a consequence of hepatocellular hypertrophy without histologic or clinical pathology alterations indicative of liver toxicity was considered an adaptive and a non-adverse reaction. Corresponding to the adaptive changes in the liver, there was evidence of changes in the thyroid (increased TSH, increased weight and follicular cell hypertrophy (Anonymous, 2019; 2018MET-4538 PXA)). It is well established that certain chemicals cause induction of liver enzymes, resulting in increased hepatic clearance of thyroid hormone and thus affecting thyroid hormone homeostasis (Anonymous, 2020; FMC-54841). The DS states that there is sufficient quantitative evidence on the basic physiological processes to conclude that thyroid tumours, induced by a process involving increased hepatic clearance of thyroid hormone and altered thyroid homeostasis in rodents, will not lead to an increase in susceptibility to thyroid tumour development in humans (see discussion in the section "Carcinogenicity").

In the 90-day study in mice, additional histopathological changes were observed in the spleen, thymus and duodenum, however, effects in the spleen (hemosiderosis) and thymus (involution/atrophy of the thymus in males) were only seen at a very high dose level, 2354 mg/kg bw/d in males and 2492 mg/kg bw/d in females. Effects on the duodenum were seen at or above 610 mg/kg bw/d in males, and 724 mg/kg bw/d in females.

The dog oral studies consisted of a 28-day dose range finding study with a limited number of animals, and 90-day and 12-month OECD TG 452 studies. Body weight was reduced at higher dose levels in all studies. In the 90-day study, liver weights were increased (not statistically significantly) in both sexes. After 1-year of administration, liver weights were markedly increased in males at 150 mg/kg bw/d and females starting at 20 mg/kg bw/d. However, there were no histopathological liver findings. Liver enzyme induction was analysed only during the 28-day dose range finding study, when no induction of hepatic enzymes could be detected. Findings in the 90-day study included increased vacuolation of the cortical tubules of the kidneys and hemosiderosis in the spleen (together with reduced red blood cell parameters, indicating anaemia) at the top dose of 200 mg/kg bw/d. In the 1-year study, at the high dose of 150 mg/kg bw/d, the gastrointestinal tract was affected, showing vacuolation, atrophy and mononuclear cell infiltration in the muscle layer of the stomach as well as the small and large intestines. The changes were seen at high dose levels in the dog 90-day- and 1-year studies, and it should be noted that the high dose level in these studies was at or above the MTD.

In the 28-day dermal toxicity study in rats, there was no evidence of systemic toxicity. The systemic no-observed-adverse-effect-level (NOAEL) was, therefore, set at 1000 mg/kg bw/d for male and female rats.

***Table**: Effects seen at doses below guidance cut-off values (adapted from Table 57 in the CLH report)*

Study	**(Adjusted) guidance value category 1/2 (mg/kg bw/d)**	**Effects at doses below guidance cut-off values**
28-day rat oral (dietary) study Anonymous, (1994) 69 PXA	30 / 300	Category 2: At 45.3 mg/kg bw/d increased cholesterol in males (adaptive)
28-day mouse oral (dietary) study Anonymous, (1996a) 70 PXA	30 / 300	Category 1: At 17/22 mg/kg bw/d increased hepatic enzyme activity: ↑ PROD both sexes (adaptive) Category 2: At 85/114 mg/kg bw/d increased adjusted liver weight (14% males), centrilobular hepatocellular hypertrophy 4/16 males (1/16 controls), increased hepatic enzyme activity: ↑ PROD both sexes (adaptive)
MTD and 28-day dog oral (capsule) study Anonymous, (1996b) 72 PXA	30 / 300	Category 2: At 200 mg/kg bw/d increased relative liver weight in females, minimal hepatocellular hypertrophy both sexes (adaptive). Vomiting, liquid/mucoid faeces and subdued behaviour

90-day rat oral (dietary) study Anonymous, (1996) 61 PXA	10 / 100	Category 2: 36.2/41.6 mg/kg bw/d decreased body weight gain (12% males) and increased hepatic enzyme activity both sexes (adaptive)
90-day male rat oral (dietary) thyroid mechanistic study Anonymous, (2020) 2018TOX-PXA4560	10 / 100	Category 2: 96 mg/kg bw/d in males = ↑ mean TSH, increased liver weight (abs and rel), thyroid follicular cell hypertrophy (3/14 compared with 0/15 in control), ↑T4- and T3-glucuronidation activity (adaptive)
90-day mouse oral (dietary) study Anonymous, (1998) 71 PXA	10 / 100	Category 2: 70.5/93 mg/kg bw/d in males/females = No treatment-related effects.
90-day dog oral (capsule) study Anonymous, (1997b) 73 PXA	10 / 100	Category 1: 8.0 mg/kg bw/d Liquid faeces in both sexes, decreased RBC count in females Category 2: 50.0 mg/kg bw/d Liquid faeces in both sexes, decreased RBC count and decreased albumin in females
1 year dog oral (capsule) study Anonymous, (1999) 74 PXA	2.5 / 25	Category 2: 20.0 mg/kg bw/d Soft stools and diarrhoea in both sexes, increased relative liver weight in females (adaptive)
2 year rat chronic and carcinogenicity study Anonymous, (2000a) 80 PXA	2.5 / 25 (one-year) 1.25 / 12.5 (two-years)	Category 1: 1.0/1.4 mg/kg bw/d increased thyroid weights in males week 53 (adaptive) Category 2: 17/23.3 mg/kg bw/d increased thyroid weights in males week 53 and 79 (adaptive)
Mouse carcinogenicity study 95 weeks males, 92 weeks females Anonymous.(2000b) 82 PXA	2.5 / 25 (one-year) 1.4 / 14.0 (92 or 95 weeks)	Category 2: 4.0 mg/kg bw/d in males = low incidence of swelling/rarefaction of villous epithelium in duodenum at termination and 52 week interim kill.

Very few changes were seen at dose levels corresponding to the maximum guidance values for STOT RE category 1 or 2, and there were no changes which were considered to represent severe target organ toxicity. Increased hepatic enzyme activity, clinical chemistry and organ weights, and pathological changes in the liver and thyroid, indicate adaptive responses to ingestion of the test material in the 28-day mouse and 28-day, 90-day and 2-year rat studies, were considered not to be evidence of significant target organ toxicity. Where the response to a substance is considered to be purely adaptive, with no evidence of dysfunction, no classification is appropriate. Therefore, these findings were considered not to justify classification. Increased thyroid weights are consistent with the mechanism of increased metabolism of thyroid hormones, and thyroid stimulation, and are associated with the adaptive liver changes described. The DS did not propose to classify pethoxamid for STOT RE.

Comments received during consultation

A National Authority commented that OECD TG 410 states that when testing solids, which may be pulverised if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. As pethoxamid is solid at room temperature, its direct application as supplied constitutes a deviation from the above-mentioned guideline. It seems unclear whether good contact between the test item and the skin can be ensured under such circumstances and whether robust conclusions with regard to dermal toxicity can be drawn. The DS responded that in the original study report the test material was described as a "Brown viscous liquid".

Assessment and comparison with the classification criteria

STOT RE means specific, target organ toxicity arising from a repeated exposure to a substance or mixture. All significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed, are included. STOT RE, category 2, is assigned on the basis of a substance demonstrating evidence of significant or severe toxicity, generally at or below the oral guidance value of 100 mg/kg bw/d obtained in a 90-day rat study. Additionally, Haber´s rule is used to adjust the standard guidance values for studies of longer or shorter duration. The guidance values for a classification for STOT RE in category 2 under CLP are: ≤ 300 mg/kg bw/d from subacute studies (28 days), ≤ 100 mg/kg bw/d from subchronic studies (90 days), ≤ 25 mg/kg bw/d from 1-year studies and ≤ 12.5 mg/kg bw/d from long term studies. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Potentially relevant effects were observed in repeated dose toxicity studies in the liver (rats, mice and dogs), thyroid (rats) and gastrointestinal tract (mice) at doses below the guidance values for STOT RE 2.

Liver

In the 28-day mouse dietary study, at 17/22 and 85/114 mg/kg bw/d doses, there was an increased hepatic enzyme activity (PROD) in both sexes. At the higher dose, increased adjusted liver weight (14%) and centrilobular hepatocellular hypertrophy were found in males (4/16 *vs* 1/16 in controls).

In one of the two 90-day rat dietary studies, increased hepatic enzyme activity in both sexes was found at 36.2/41.6 mg/kg bw/d. In the 90-day thyroid mechanistic study, using only males, at 96 mg/kg bw/d, increased absolute and relative liver weight and increased T4- and T3-glucuronidation activity were also found. There were no liver effects in the 2-year rat dietary study below the guidance value for STOT RE 2.

In the 28-day dog oral study, at 200 mg/kg bw/d there was an increased relative liver weight in females, and minimal hepatocellular hypertrophy in both sexes. In the 1-year dog study, increased relative liver weight was found at 20.0 mg/kg bw/d in females. There were no liver effects in the 90-day dietary study below the guidance value for STOT RE 2.
Increased hepatic enzyme activity, liver weights, and hepatocellular hypertrophy indicate adaptive responses and are not regarded as significant target organ toxicity.

Thyroid

In the 90-day thyroid mechanistic (male) rat dietary study, at 96 mg/kg bw/d, increased mean TSH levels and thyroid follicular cell hypertrophy was observed (3/14 *vs* 0/15 in the control). In the other 90-day rat dietary study, at lower doses (36.2/41.6 mg/kg bw/d), no thyroid effects were observed. In the 2-year rat dietary study at 1.0/1.4 and 17/23.3 mg/kg bw/d, there was an increase in thyroid weights in males.
The 90-day thyroid mechanistic rat dietary study established that liver enzyme induction leading to an increase in T4 glucuronidation and clearance of T4 elicited a feedback response on the thyroid via an increase in TSH. Further, the increased TSH and associated thyroid follicular cell hypertrophy resulted in functional compensation by the thyroid in the Pethoxamid-treated rats. Induction of hepatic UDPGT and subsequent disturbance of thyroid hormone homeostasis is a well-known, threshold-mediated, species-specific MoA that is generally not considered to be relevant to human hazard assessment.

Gastrointestinal tract

In the mouse carcinogenicity study, at 4.0 mg/kg bw/d, there was an increased incidence of swelling of villous epithelium cells and subsequent rarefaction of the cytoplasm in the duodenum of male mice at the 52 week interim kill (5/8 vs 0/8), and at termination (6/47 vs 0/48). Similar effects can be seen in the 90-day mouse dietary study at 610 mg/kg bw/d in 6/10 males, well above the guidance value (100 mg/kg bw/d) for STOT RE 2. At the low dose of 70.5 mg/kg bw/d there were no treatment related effects.

In the 1-year dog study, at 150 mg/kg bw/d, which is well above the guidance value of 25 mg/kg bw/d, gastrointestinal effects were observed: micronecrosis of the mucosa in the stomach (pylorus)(1/3 males), vacuolation and mononuclear cell infiltration in the muscle layer (1/3 males), epithelium hyperplasia of the mucosa (1/3 males vs 0/4 in controls). In the large intestines, vacuolation and mononuclear cell infiltration in muscle layer (2/3 males) was seen. But these effects were seen only at the high dose, showing systemic toxicity and near the MTD (one male and one female out of four were killed moribund after weeks 47 and 38 respectively). The gastrointestinal tract effects in mice (swelling of the villous epithelium cells and subsequent rarefaction of the cytoplasm in the duodenum) are of concern, as they are rare findings, but not enough in themselves to warrant classification.

Therefore, RAC agrees with the DS's proposal that **classification for STOT RE is not warranted.**

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

There are five *in vitro* and two *in vivo* studies discussed in the CLH dossier. All studies were conducted according to OECD TG and GLP.

In vitro tests

Two bacterial reverse mutation assays (Anonymous, 1994, Anonymous, 2012) were performed according to OECD TG 471, with and without S9 mix, using 95% or 93.5% substance with DMSO as solvent. The first did not include the E. coli WP2 uvrA tester strain, but the second assay had a full range of the tester strains. Cytotoxicity showed that adequate test concentrations had been used. Both bacterial reverse mutation assays were negative.

An *in vitro* chromosome aberration test in human lymphocytes (Anonymous, 1994) performed according to OECD TG 473 was carried out with and without S9 mix. Without S9 mix, cells were exposed continuously for 18 hours. In the presence of S9 mix, exposure was limited to three hours. Duplicate tests were carried out. In the first test the concentrations for metaphase analysis were 2.0, 7.8 and 15.6 µg/mL without S9 mix, and 3.9, 15.6, and 31.3 µg/mL with S9 mix. No cells survived, either in the presence or absence of S9 mix, at concentrations of 62.5 µg/mL and above. In the absence of S9 mix, a statistically significant increase in chromosomal aberrations occurred at the highest dose level (15.6 µg/mL), indicative of a clastogenic activity. In the second test the concentrations used for metaphase analysis were 3.75, 20, and 37.5 µg/mL without S9-mix and 7.5, 45 and 80 µg/mL with S9-mix. Death of all cells was observed 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 second test, statistically significant increases in chromosomal aberrations occurred at the intermediate and high dose levels in the absence of S9 mix. The percentage of cells with aberrations (excluding gaps) at 0, 3.75, 20, and 37.5 µg/mL was 0.25, 1.5, 5.5*** and 15.5***,

respectively (*** = p<0.001). In the presence of S9 mix, a statistically significant increase in chromosomal aberrations occurred at all dose levels analysed. The percentage of cells with aberrations (excluding gaps) at 0, 7.5, 45 and 80 µg/mL was 2.5, 6.0*, 14.0*** and 41.5***, respectively (*** = p<0.001). The second experiment showed a distinct dose-response relationship, indicating clastogenic activity.

The fourth *in vitro* test was a Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/TK+/-) in Mouse Lymphoma L5178Y Cells (Anonymous, 2015), performed according to OECD TG 476 on 94.5% substance. Two parallel tests were carried out, with metabolic activation and without. The result of this assay was negative.

The last *in vitro* test was a mammalian cell gene mutation assay in V79 cells (HGPRT Test) according to OECD TG 476 (Anonymous, 1992). It was carried out with 98.6% test substance, with and without metabolic activation. Without S9 mix, cells were exposed for 24 hours, while in the presence of S9 mix, the exposure was limited to two hours. The test concentrations were 0, 1, 3, 10, 20, 30 µg/mL (without S9 mix) and 0, 10, 30, 100, 200, 300 µg/mL (with S9 mix). The assay was negative.

In vivo studies

The first *in vivo* study was a mouse micronucleus test (Anonymous, 1994) conducted according to OECD TG 474. Deviations compared to this guideline: only 1000 cells were assessed. CD-1 mice were used, 15/sex/dose, at doses of 0, 320, 640, or 1280 mg/kg bw via gavage. The purity of the test substance was 95%. The vehicle (1% aqueous methyl cellulose and 0.5% Tween 80) served as the negative control and Mitomycin C as the positive control. Within the first hour after dosing clinical signs were hunched posture and piloerection in all groups. The high dose group also showed lethargy and ptosis; furthermore, 8 female and 3 male mice died after treatment with the high dose and were replaced. No significant increase in the number of micronucleated immature erythrocytes was observed at 24, 48 or 72 hours. The positive control showed the expected increase in micronuclei. No effect was observed on the proportion of immature erythrocytes, indicating no bone marrow toxicity. In conclusion, pethoxamid did not show any evidence of chromosomal or other damage leading to micronucleus formation in this *in vivo* test.

The second study was an *in vivo* rat liver DNA repair test (Anonymous, 1994) conducted according to OECD TG 486 and TG 482 in Albino Hsd/Ola Sprague-Dawley male rats. Single doses of 600, 1200 (provisional group, not evaluated for UDS) and 2000 mg/kg bw were used. The purity of the substance was 95%. 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. The test compound did not cause any significant increase in either the gross or net nuclear grain count at any dose level at the 2 hours expression time. At the 14 hours expression time, statistically significant increases in gross nuclear grain counts were obtained at 600 and 2000 mg/kg bw but not in net nuclear grain counts, i.e. were not indicative for unscheduled DNA synthesis. Positive control group animals showed the expected significant increase in the gross and net nuclear grain count.

The DS concluded that pethoxamid does not meet the criteria for classification in Category 2 because four of five *in vitro* tests demonstrated negative results, only the *in vitro* chromosomal aberration test indicated potential clastogenic effects. In addition, both *in vivo* studies were found negative, including a mouse micronucleus study, supporting the conclusion that clastogenic effects are not manifested *in vivo*.

Comments received during consultation

A National Authority questioned whether in the *in vitro* chromosome aberration test the

conclusion that pethoxamid demonstrates clastogenic activity both in the absence and presence of S9 mix is based on the assessment criteria laid out in the latest version of the OECD TG 473, as it is unclear whether a trend test was performed and/or the results were compared to the distribution of the historical negative control data. The National Authority also commented that the ADME studies were conducted in rats, while the micronucleus test was conducted in mice. Thus, possible interspecies differences may occur, and the available data do not appear to enable it to be concluded that bone marrow exposure occurred in mice, and hence, clastogenic effects observed *in vitro* may not have occurred *in vivo* because the bone marrow of mice was not (sufficiently) exposed to the test substance. The DS replied that clinical signs were reported, which showed a dose-dependent increase in severity indicating systemic exposure at all dose levels (piloerection in the low dose group; piloerection and hunched posture in the mid-dose group; piloerection, hunched posture, ptosis, and lethargy in the highest dose group). At the highest dose of 1280 mg/kg also mortalities were reported. The DS concluded that there is sufficient evidence to demonstrate bone-marrow exposure, based on the clinical signs and not on toxicokinetic data derived from rats.

A Company-Manufacturer concurred with the DS that pethoxamid does not warrant classification.

Assessment and comparison with the classification criteria

According to the CLP Regulation classification in Category 1A is based on positive evidence from human epidemiological studies. As no such evidence exists, classification in Category 1A is not supported. No germ cell mutagenicity studies are available with pethoxamid, thus classification in Category 1B is also not warranted.

The classification in Category 2 is based on:

- Positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from:
    - Somatic cell mutagenicity tests *in vivo*, in mammals; or
    - Other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays.

There are 5 *in vitro* assays (2 bacterial reverse mutation assays, an *in vitro* chromosome aberration test in human lymphocytes, a Mammalian Cell Gene Mutation Assay (Thymidine Kinase Locus/TK+/-), and a mammalian cell gene mutation assay in V79 cells (HGPRT Test), as well as 2 *in vivo* (a micronucleus test in mice and a rat liver DNA repair test) genotoxicity tests with pethoxamid, all performed according to the appropriate OECD test guidelines and under GLP. Of the 5 *in vitro* assays, one was positive, namely the chromosome aberration test, but the two *in vivo* assays were found to be negative. RAC agrees with the DS's proposal that **no classification for mutagenicity is warranted**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The CLH dossier includes 2 studies relevant to this endpoint: a rat combined chronic toxicity and carcinogenicity study, and a mouse carcinogenicity study. Both were performed according to the OECD TG 453 and under GLP.

***Table**: Summary table of animal studies on carcinogenicity (adapted from Table 38 in the CLH report)*

Method, guideline, deviations if any, species, strain, no/group	**Test substance, dose levels, duration of exposure**	**Results**	**Reference**
Rat chronic and carcinogenicity study via the oral (dietary) route OECD TG 453 + electron microscopy of liver. Deviations: MCH not presented; uterus not weighed; Coagulating gland and peripheral nerve not preserved. GLP Rat Crl: CD BR (IGS) Main groups: 50 /sex/group Up to 10 rats/sex/group examined after the completion of 26, 52 and 78 weeks of treatment.	Pethoxamid Batch: TB-960306 Purity: 95.0% Dose levels: 0, 25, 400, 1600 ppm in diet for 104 weeks	1600 ppm (70 mg/kg bw/d in males and 99 mg/kg bw/d in females) ↓ Body weight gain: Week 0-4 15% males, 15% females; Week 0-88 13% males, 23% females ↓ Food consumption: Week 1-4 8% males, 6% females; Week 1-104 6% males Clinical chemistry: ↑ Cholesterol All timepoints 17-42% males, 33- 46% females; ↓ Globulin First year of study 9-13% females; ↑ γ GT activity All timepoints 1.5-8 x control in males ↑ Liver weights: Adjusted weight 10 and 12% males Weeks 27 and 53; 17 and 16% females Weeks 27 and 105 ↑ Thyroid weights: Adjusted weight 50% and 31% males Weeks 53 and 79. No similar change Week 27 or 105. *Non-neoplastic findings* Liver pathology: Centrilobular hepatocyte hypertrophy 11/50 males, 8/50 females (0/50 controls); Concentric intracytoplasmic inclusions 10/50 males (0/50 controls); Cystic degeneration 24/50 males (12/50 controls); Clear cell hepatocytes 15/50 males (6/50 controls). Thyroid pathology: Not statistically significant. Follicular cell hyperplasia 4/50 males (0/50 controls); Follicular cell cystic hyperplasia 7/50 males (4/50 controls). 400 ppm (17.0 mg/kg bw/d in males and 23.3 mg/kg bw/d in females) ↓ Body weight gain: Week 0-88 11% females (not statistically significant but considered treatment related) ↓ Food consumption: Week 1-4 4% males ↑ Thyroid weights: Adjusted weight 39% and 49% males Weeks 53 and 79. 25 ppm (1.0 mg/kg bw/d in males and 1.4 mg/kg bw/d in females) ↑ Thyroid weights: Adjusted weight 38% males Week 53. **The NOAEL was considered to be 25 ppm (1.0 mg/kg bw/d), based on the decreased body weight gain in females at 400 ppm.** *Neoplastic findings*: see Table: Neoplastic findings in rat chronic and carcinogenicity study (below) **The only statistically significant evidence of tumourigenicity was a higher incidence of thyroid follicular cell adenoma in males at the high dose level of 1600 ppm. Mechanistic studies demonstrate that the mode of action is not relevant to humans.**	Anonymous (2000a); 80 PXA (Anonymous, 2000-amdt-1; 80 PXA amdt-1) (Anonymous, 2003; 80 PXA suppl- 1) (Anonymous, 2016; 80 PXA suppl- 2) (Anonymous, 2003; 203 PXA)
Mouse carcinogenicity study via the oral (dietary) route. OECD TG 453, however OECD TG 451 acceptable for second rodent species Deviations from OECD TG 453: Slight exceedance	Pethoxamid Batch: TB-960306 Purity: 95.0% and Batch: TB-960306-C Purity: 94.8%.	5000 ppm (982 mg/kg bw/d in males, 1068 mg/kg bw/d in females) ↓ Bodyweight at termination: 16% males, 17% females ↓ Body weight gain: 40% males to Week 95; 34% females to Week 92 ↑ Total food consumed: 21% males ↑ Liver weight: Terminal adjusted 77% males, 36% females ↑ Kidney weight: Terminal adjusted 23% females ↑ Thyroid weight: Terminal adjusted 29% females ↑ Adrenal weight: Terminal adjusted 26% males *Non-neoplastic findings*	Anonymous (2000b); 82 PXA (Anonymous, 2016; 82 PXA suppl- 1) (Anonymous, 2001; 83 PXA) (Anonymous, 2001; 1484 PXA)

of weight variation in females; no organ weights of spleen, uterus; no preservation of the coagulating gland, peripheral nerves; haematology- only blood smears; no clinical biochemistry. GLP Mouse Crl: CD-1 BR 50/sex/group Treated until one group in each sex reached 50% survival i.e. up to 92 week (females) or 95 week (males). Additional 10/sex/group treated for up to 52 weeks.	Dose levels: 0, 30, 400, 5000 ppm in diet.	Liver pathology: Hepatocyte hypertrophy 39/50 males (generalised), 42/50 females (periportal) 0/50 in controls. Kidney pathology <u>both sexes</u>: Cortical tubules basophilic 43/50 males (33/50 controls), 41/50 females (12/50 controls); Medullary tubules dilated with eosinophilic casts: 42/50 males (27/50 controls), 32/50 females (14/50 controls); Cortical mineralisation 46/50 males (32/50 controls), 26/50 females (3/50 controls); Medullary mineralisation 44/50 males (6/50 controls), 31/50 females (0/50 controls); Papillary mineralisation 36/50 males (11/50 controls), 30/50 females (3/50 controls). Kidney pathology <u>males only</u>: Cortical tubular cell hypertrophy (slight) 8/50 (0/50 controls); Cortical fibrosis with tubular collapse and basophilia 37/50 (13/50 controls); Cortical cysts 31/50 (20/50 controls). Duodenum pathology: Swelling/rarefaction of villous epithelium 42/49 males (0/48 controls), 18/49 females (0/45 controls); Villous hypertrophy 27/49 males (0/48 controls). Jejunum pathology: Swelling/rarefaction of villous epithelium 35/49 males (0/48 controls), 14/49 females (0/46 controls); Villous hypertrophy 16/49 males (0/48 controls). <u>400 ppm (56.8 mg/kg bw/d in males, 68 mg/kg bw/d in females)</u> ↑ Liver weight: Terminal adjusted 14% males (not statistically significant), 10% females ↑ Kidney weight: Terminal adjusted 11% females *Non-neoplastic findings* Duodenum pathology: Swelling/rarefaction of villous epithelium 29/47 males (0/48 controls), 12/50 females (0/45 controls); Villous hypertrophy 9/47 males (0/48 controls). Jejunum pathology: Swelling/rarefaction of villous epithelium 25/48 males (0/48 controls), 7/50 females (0/46 controls); Villous hypertrophy 8/48 males (0/48 controls). <u>30 ppm (4.0 mg/kg bw/d in males, 5.0 mg/kg bw/d in females)</u> *Non-neoplastic findings* Duodenum Pathology: Swelling/rarefaction of villous epithelium 6/47 males at termination, 5/8 males at interim kill (0/48, 0/8 controls, respectively). **The LOAEL was considered to be < 30 ppm (<4 mg/kg bw/d).** *Neoplastic findings:* Treatment-related increase in hepatocellular adenomas in male mice at 5000 ppm. Slightly higher (not statistically significant) incidence of hepatocellular carcinomas within historical control incidence. See also Table: Neoplastic findings in mouse carcinogenicity study (below) **The only evidence of tumourigenicity was an increased number of males showing benign hepatocellular liver tumours at the high dose of 5000 ppm. The no effect level for tumourigenicity was set at 400 ppm (56.8 mg/kg bw/d). The DS concluded that mechanistic studies demonstrate a phenobarbitone-like mode of action for pethoxamid.**	(Anonymous, 2001; 1241 PXA)

***Table**: Summary table of other studies relevant for carcinogenicity (adapted from Table 40 in the CLH report)*

Type of study/data	Test substance, species, strain, sex, no/group	Relevant information about the study (as applicable)	Observations	Reference
Additional study on cell proliferation in the livers taken from Carcinogenicity Study by dietary Administration to CD-1 Mice for at least 80 Weeks (Anonymous, 2000b; 82 PXA) GLP	Pethoxamid Batch: TB-960306 Purity: 95.0% and Batch: TB-960306-C Purity: 94.8%. Mouse Crl: CD-1 BR 8-10 male mice/group Dose levels: 0, 30, 400, 5000 ppm in diet for at least 80 weeks.	Original paraffin blocks used in the carcinogenicity study were subjected to immunohistochemistry for proliferating cell nuclear antigen (PCNA). PCNA labelling index (LI) was determined for each animal by counting the number of PCNA-positive (S-phase) cells per approximately 1000 hepatocytes.	Pethoxamid appeared 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.	Anonymous (2001a); 1241 PXA
Evaluation of thyroid function using perchlorate discharge test. Non- guideline study. GLP	Pethoxamid Batch: TB-9603061 Purity: 94.8% Positive controls: Phenobarbital and Propylthiouracil Rat Sprague Dawley 16 males/group Dose levels: 0, 1600, 5000 ppm in diet for 28 days (0, 155, 462 mg/kg bw/d).	Blood samples taken before treatment and on days 12 and 24 for measurement of T3, T4 and TSH. After 28 days of treatment, sodium ^{125}Iodide was given i.p. to each animal. 6 h later potassium perchlorate and saline were given to 6 animals/group i.p. 2½ min later the rat was anaesthetised and blood sampled to measure radioactivity. Animals were killed, the thyroid removed and weighed and the total amount of radioactivity in the thyroid measured.	Elevated TSH, only statistically significant in the 1600 ppm group on day 12. No effects on T3 or T4. Pethoxamid did not cause a significant discharge of thyroid radioactivity by perchlorate; thus, the activity of thyroid peroxidases was not reduced. 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 are similar to that for phenobarbitone (TSH levels, thyroid and whole-blood radioactivity); thus, the DS concluded that the mechanism of action of pethoxamid is similar to that of phenobarbitone.	Anonymous (2000); 94 PXA
Hepatic drug-metabolising enzyme induction and cell proliferation study. Non- guideline study. GLP	Pethoxamid Batch: TB-9603061 Purity: 95% Mouse ICR (crj:CD-1) 18 males/group Dose levels: 0, 30, 400, 5000 ppm in diet for 14 days (0, 3.92, 49.1, 541 mg/kg bw/d).	Hepatic enzyme activity, PCNA labelling index and cell to cell communication in liver by evaluation of gap junction protein connexion 32 (Cx 32) spots per hepatocyte assessed. Measured after 3, 7 and 14 days.	Liver weights increased at 5000 ppm. Microsomal protein and PROD activity increased in 5000 pm group. Cytochrome P-450 isoenzyme contents of CYP1A, CYP2B, CYP3A2 and CYP4A1 increased in 5000 pm group. CYP2B most affected. Increase in PROD in the 400 ppm group. PCNA labeling index increased in 5000 ppm group after 3 and 7 days, but not after 14 days. Decrease in Cx 32 spots (20-30%) in 400 and 5000 ppm groups. Decrease in Cx 32 spots in 30 ppm group after 3 and 7 days, but not 14 days. Based on the results observed, the overall profile of effects are suggestive	Anonymous (2001b); 98 PXA

Type of study/data	Test substance, species, strain, sex, no/group	Relevant information about the study (as applicable)	Observations	Reference
			that pethoxamid 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.	
Evaluation of liver and thyroid effects and their potential reversibility after dietary exposure in mice and rats. Non- guideline study. GLP	Pethoxamid Technical Batch: P1351-Jak-T2-23-6 Purity: 92.6% Mice: CRL:CD 1(ICR) 8 male mice/group Mice dosed at 0, 400 and 5000 ppm for a period of 7 days (0, 82.1, 972 mg/kg bw/d main group). Rats: CRL: CD® Sprague Dawley Rats dosed at 0, 400 and 1600 ppm for a period of 14 days (0, 31.2, 131.6 mg/kg bw/d main group). 15 male rats/group Recovery after 42 days two groups per species (control and high dose).	Mice: Livers weighed and histopathology (general and IHC) performed. BrdU administered to mice by osmotic pump, containing 15 mg/mL BrdU implanted subcutaneously. Incorporation of BrdU into hepatocytes was then measured using a mouse monoclonal anti-BrdU antibody and light microscopy. Rats: Serum thyroid hormones assessed. Thyroids and livers harvested. Thyroids weighed and histopathology (general and IHC) performed. Rats received 10 µg/hr BrdU. Incorporation of BrdU into hepatocytes was then measured using a mouse monoclonal anti-BrdU antibody and light microscopy.	Mice: <u>5000 ppm</u> ↑ Liver weight (absolute and relative) Demonstrated hepatocellular hypertrophy and an increase of the number of BrdU positive cells. Increases were completely reversible in recovery animals evaluated on Day 49. <u>400 ppm</u> minimal effects. Rats: <u>1600 ppm</u> ↑ Thyroid weight (absolute and relative) An increase of the BrdU labelling index in the thyroid gland. Increases were completely reversible in recovery animals evaluated on Day 56. Thyroid follicular epithelium hypertrophy (grade 1) was observed in 2/8. No observed in recovery animals. There were no noteworthy effects of pethoxamid on circulating thyroid hormone <u>400 ppm</u> minimal effects. Conclusion: Pethoxamid administered to mice in their diet at concentrations of 5000 ppm caused reversible changes in liver weight and centrilobular hepatocytes including hypertrophy as well as increased hepatocyte proliferation. Pethoxamid 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.	Anonymous (2016); 1538 PXA
Ex vivo evaluation of liver microsomal Cytochrome P450 induction and UGT expression in rodents Non- guideline study. GLP	Liver samples for this study taken from Anonymous, 2016 (1538 PXA)	Evaluation of effect of Pethoxamid on liver microsomal cytochrome P450 (CYP) enzyme activity and mRNA levels in male mice. Evaluation of effect of Pethoxamid on liver microsomal uridine diphosphate glucuronosyltransferase (UGT) activity and mRNA levels toward the thyroid hormone thyroxine (T4) in male rats.	Mice: <u>5000 ppm</u> ↑ Cytochrome b5 content: 1.39-fold, ↑ Cytochrome P450 content 1.50-fold ↑ 7-ethoxyreorufin-O-dealkylation (Cyp1a1/2) activity: 1.54-fold ↑ testosterone 16β-hydroxylase (Cyp2b10) activity: 1.70-fold ↑ Cyp1a2 mRNA levels: 1.97-fold ↑ Cyp2b10 mRNA levels: 115-fold ↑ Cyp3a11 mRNA levels: 6.90-fold ↑ Cyp4a10 mRNA levels: 9.00-fold Following a 42 day recovery period, measured values were generally comparable between mice previously treated at 5000 ppm pethoxamid and concurrent controls.	Anonymous (2016); 1539 PXA

Type of study/data	Test substance, species, strain, sex, no/group	Relevant information about the study (as applicable)	Observations	Reference
			400 ppm ↑ Cytochrome P450 content: 1.22- fold ↑ 7-ethoxyreorufin-O-dealkylation (Cyp1a1/2) activity: 1.69-fold ↑ testosterone 16β-hydroxylase (Cyp2b10) activity: 1.46-fold ↑ testosterone 6β-hydroxylase (Cyp3a11/13) activity: 4.39-fold ↑ Cyp2b10 mRNA levels: 12.4-fold Rats: 1600 ppm ↑ Cytochrome b5 content: 1.22-fold ↑ Cytochrome P450 content: 1. 63-fold ↑ thyroxine glucuronidase (UGT1A1/6) activity: 1.62-fold ↑ UGT1A1 mRNA levels: 1.23-fold ↑ UGT1A6 mRNA levels 3.78-fold Following a 42-day recovery period, measured values were generally comparable between rats previously treated at 1600 ppm pethoxamid and concurrent controls. 400 ppm ↑ Cytochrome P450 content: 1.17-fold ↑ UGT1A6 mRNA levels: 1.82- fold 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.	
Mode of action and human relevance analysis of rodent-specific tumours	Pethoxamid:	Position paper	Based on results from the repeat-dose toxicity and mechanistic studies, the data strongly support the MoA for pethoxamid induced male mouse liver tumours involving the activation of the nuclear hormone receptor CAR, which mediates the induction of replicative DNA synthesis in the liver. The proposed mouse liver tumour MoA satisfies the conditions of dose and temporal concordance, biological plausibility, coherence, strength, consistency and specificity as described in the IPCS framework. The proposed MoA for the male rat thyroid follicular cell adenomas is based on activation of CAR along with the induction of UGT. This results in disrupted thyroid hormone homeostasis, leading to thyroid follicular cell adenomas in the male rat. The DS concluded that the increased tumour incidence in carcinogenicity studies performed with pethoxamid are not of relevance to humans.	Anonymous (2016); 1540 PXA
Inhibition of thyroperoxidase (TPO) activity *in vitro*	Pethoxamid Technical. Batch: 21082018 Purity: 97.7%	The guaiacol assay of TPO activity was used for this study. Final concentrations: 0, 0.01, 0.1, 0.3, 1, 3, 10,	Pethoxamid did not inhibit TPO activity at any of the tested concentrations.	Anonymous (2019); 2018TOX-PXA4481

Type of study/data	Test substance, species, strain, sex, no/group	Relevant information about the study (as applicable)	Observations	Reference
Non guideline study. GLP	Positive control: TPO inhibitor 6-propyl-2-thiouracil (PTU)	30, 100, 300 and 1000 µM tested in pooled thyroid microsomes prepared from male Sprague Dawley rats.		
90-day rat study to evaluate potential mechanisms underlying the observed thyroid gland changes. General design similar to OECD TG 408. GLP	Pethoxamid Batch: 21082018 Purity: 99.4% Rat Crl:CD(SD) Sprague Dawley Dose levels: 0, 400, 1600, 5000 ppm (0, 24, 96, 308 mg/kg bw/d). Positive control: Phenobarbital 1000 ppm. 15 males/group termination after 90 days 5 males/group termination after 30 days	Thyroid hormone assessed on Days -3, 15, 29, 57, and 89. Total T3 and T4, TSH and rT3 levels analysed. At termination liver, thyroid and pituitary weighed and histopathology performed. Hepatic UDP-glucuronosyltransferase (UGT) activity (T3-glucuronidation and T4-glucuronidation) and protein concentration assessed	<u>Pethoxamid</u> ↓ Body weight in the 5000 ppm group, 7.5% lower than controls at the end of study. ↑ Mean TSH values in the 1600 and 5000 ppm groups on Days 15, 29, 57, and 89 ↓ Time-dependent decrease in total T4 relative to pre-treatment values during the first 29 days → Total T3 or rT3 ↑ Thyroid weight in the 5000 ppm group on Days 30 (absolute 31% and relative to body weight 58%) and 93/94 (absolute 27% and relative to body weight 39%) ↑ Liver weight in the 5000 ppm group on Day 30 (absolute 6% and relative to body weight 30%) and in the 1600 (absolute 15% and relative to body weight 16%) and 5000 ppm (absolute 35% and relative to body weight 47%) groups on Day 93/94. ↑ Thyroid follicular cell hypertrophy in the 1600 (3/14 compared with 0/15 in control) and 5000 ppm (15/15 compared with 0/15 in control) groups on Day 93/94 ↑ Hepatocellular hypertrophy in the 5000 ppm group on Days 30 (3/5 compared with 0/5 in control) and 93/94 (5/15 compared with 0/15 in control) ↑ T4-glucuronidation activity was observed in the 1600 and 5000 ppm groups on Days 30 (2.7 and 4.9 fold increase in the 1600 and 5000 ppm groups respectively) and 93/94 (1.9 and 3.2 fold increase in the 1600 and 5000 ppm groups respectively) ↑ T3-glucuronidation activity was elevated in the 1600 and 5000 ppm groups on Day 30 (1.9 and 1.7 fold increase in the 1600 and 5000 ppm groups respectively) and in the 5000 ppm group on Day 93/94 (1.0 and 1.9 fold increase in the 1600 and 5000 ppm groups respectively) <u>phenobarbital</u> ↑ Mean TSH values on Days 15, 29, 57, and 89 ↓ Total T4 on Day 15 and 29 ↑ Total T3 or rT3 on Day 89 ↑ Thyroid weight on Days 30 (absolute 53% and relative to body weight 69%) and 93/94 (absolute 36% and relative to body weight 36%) ↑ Liver weight on Day 30 (absolute 17% and relative to body weight 32%) and Day 93/94 (absolute 52% and relative to body weight 52%) ↑ Thyroid follicular cell hypertrophy on	Anonymous (2020); 2018TOX-PXA4560

Type of study/data	Test substance, species, strain, sex, no/group	Relevant information about the study (as applicable)	Observations	Reference
			Day 30 (2/5 compared with 0/5 in control) and hypertrophy (15/15 compared with 0/15 in control) and hyperplasia (5/15 compared with 0/15 in control) on Day 93/94 ↑ Hepatocellular hypertrophy Days 30 (5/5 compared with 0/5 in control) and 93/94 (15/15 compared with 0/15 in control) ↑ T4-glucuronidation activity was observed on Days 30 (4.1 fold increase) and 93/94 (2.4 fold increase) ↑ T3-glucuronidation activity on Days 30 (3.7 fold increase) and 93/94 (1.9 fold increase) Overall, it can be concluded that liver enzyme induction, leading to an increase in T4 glucuronidation and clearance of T4, elicited a feedback response on the thyroid via an increase in TSH. Further, the increased TSH and associated thyroid follicular cell hypertrophy resulted in functional compensation by the thyroid in the Pethoxamid-treated rats. The results with pethoxamid were consistent with the results of the positive control phenobarbital, which was included in this study.	
Biliary Excretion of [^{125}I]Thyroxine and Metabolites in Rats. Non guideline study. GLP	Pethoxamid Batch: 21082018 Purity: 99.4% Rat Sprague Dawley Dose levels: 0, 300 mg/kg bw/d. Positive control: Phenobarbital , 100 mg/kg bw/d. 6 bile-duct and jugular vein cannulated male/group termination after 7 days.	Bile-duct cannulated rats were pre-treated with control, phenobarbital (positive control), or pethoxamid once daily for 7 consecutive days. On Day 8, ~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 animal. 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.	<u>Pethoxamid</u> ↑ Liver weight ↓ Serum total radioactivity Cmax and AUC_{0-4} ↑ % administered radioactivity in bile ↑ T4 Glucuronide in bile <u>phenobarbital</u> ↑ Liver weight ↓ Serum total radioactivity Cmax and AUC_{0-4} ↑ % administered radioactivity in bile ↑ T4 Glucuronide in bile Similar response in pethoxamid and phenobarbital-treated animals. Overall, data indicates greater clearance of thyroxine due to liver induced T4 glucuronidation in the pethoxamid-treated rats compared to controls. The results were consistent with those for the phenobarbital positive control.	Anonymous (2019); 2018MET-PXA4538
In vitro mRNA and DNA synthesis induction in cultured mouse and human hepatocytes. Non guideline study. GLP	Pethoxamid Batch: 21082018 Purity: 97.7% Primary hepatocytes isolated from male CD-1 mice and primary cryopreserved hepatocytes	Isolated primary male CD-1 mouse hepatocytes or male primary human hepatocytes (3 donors) were exposed in culture to pethoxamid, phenobarbital or EGF for approximately 96 hours after which cell cytotoxicity was evaluated by quantification of ATP levels, or the cells were harvested and processed	This study was designed to evaluate the hypothesis that pethoxamid induces mouse liver tumours via a phenobarbital-like MoA. Cyp2B and Cyp3A expression is induced by CAR and to a lesser degree pregnane X receptor (PXR). In rodents, activation of CAR by phenobarbital leads to hepatocellular tumours, which is not evident in hamsters, guinea pigs or primates including humans (Elcombe et al., 2014). While CAR/PXR induced gene expression is conserved across	Anonymous (2019); 2018TOX-PXA4482

Type of study/data	Test substance, species, strain, sex, no/group	Relevant information about the study (as applicable)	Observations	Reference
	from three male human donors. Dose levels: Mouse hepatocytes were exposed to pethoxamid (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). Positive control: Phenobarbital , 1000 µM. Epidermal growth factor (EGF; 25 ng/mL) used as positive control for replicative DNA synthesis.	for mRNA analysis of Cyp2b10 and Cyp3a11 (mouse) and CYP2B6 and CYP3A4 (human), or cells were processed for assessment of replicative DNA synthesis. Constitutive androstane receptor (CAR) and pregnane-X-receptor (PXR) activation was assessed by downstream Taqman® mRNA analysis (Cyp2b10 and Cyp3a11 in mouse, respectively; and CYP2B6 and CYP3A4 in human, respectively). Cell proliferation (measured as the change in replicative DNA synthesis (RDS) [S-phase of the cell cycle]) in both mouse and human hepatocytes assessed.	species, difference in replicative DNA synthesis (RDS) occurs in rodent hepatocytes, and not in human hepatocytes. Cultures of primary hepatocytes isolated from male CD-1 mice and cryopreserved human hepatocytes from three individual male donors were used to investigate the potential of pethoxamid to activate CAR and PXR. After a 96-hour treatment period, cells were harvested and processed for mRNA analysis of Cyp2b10 and Cyp3a11 for mice and Cyp2B6 and Cyp3A4 for human cells, corresponding to CAR and PXR receptor activation, respectively. Cell proliferation was measured as the change in both mouse and human hepatocytes following incubation with BrdU and subsequent immunohistochemical staining to determine the number of cells in S phase. phenobarbital was included as a positive control for the activation of CAR and PXR; epidermal growth factor (EGF) was included as a positive control for the induction of cell proliferation. An assessment of cytotoxicity was also performed. phenobarbital, the positive control, induced Cyp2b10 and Cyp3a11 mRNA in mouse hepatocytes, compared with concurrent vehicle controls, respectively (Table 10 of the CLH report). Pethoxamid induced Cyp2b10 or Cyp3a11 mRNA in mouse hepatocytes to a somewhat lesser degree relative to phenobarbital. Therefore, pethoxamid was considered to be a weak activator of CAR and PXR *in vitro*, relative to phenobarbital in mouse hepatocytes. In human hepatocytes, phenobarbital treatment resulted in marked induction of Cyp2B6 mRNA and Cyp3A4 mRNA (Table 11 of the CLH report). In cultures treated with pethoxamid, overt cytotoxicity was observed at 10–20 µM, depending on the specific human donor hepatocytes. Cyp2B6 mRNA was induced at 10 or 20 µM. Cyp3A4 mRNA was similarly induced. In cultures of primary male CD-1 mouse hepatocytes, phenobarbital and pethoxamid both induced RDS at all concentrations tested (Table 12 of the CLH report). Neither phenobarbital nor pethoxamid induced RDS in human hepatocytes. The positive RDS control, EGF, induced RDS in both mouse hepatocytes and human hepatocytes. Overall, pethoxamid is a weak activator of CAR and/or PXR *in vitro*. Pethoxamid has been shown to induce RDS in mouse hepatocytes, but not in	

Type of study/data	Test substance, species, strain, sex, no/group	Relevant information about the study (as applicable)	Observations	Reference
			human hepatocytes. The responses observed with pethoxamid are similar to that of phenobarbital. These data are supportive of the lack of human relevance for mouse liver tumour formation following treatment with pethoxamid.	
Pethoxamid: Human Relevance Framework Assessment of Induced Rodent Liver and Thyroid Tumours	Pethoxamid:	Position paper *NB: an updated position paper to assess the mode of action and human relevance of the rodent liver and thyroid tumours to include the additional data generated to further characterise the mechanistic bases for the increased tumour incidence.*	Review of all available data from toxicological studies and mode of action (MoA) studies strongly demonstrate that the hepatocellular adenomas/carcinomas and thyroid follicular cell adenomas observed in rodents treated with high doses of pethoxamid are not relevant to humans. In an *in vitro* species comparison assay, pethoxamid induced cell proliferation in mouse hepatocytes, but not in human hepatocytes. Based on the difference in biological response in humans and rodents to CAR activation, any hepatocellular adenomas developed in mice through activation of these nuclear receptors by pethoxamid in mice, are not of relevance to humans. Therefore, it can be concluded that pethoxamid does not pose a hepatic carcinogenic hazard in humans. Overall, it is considered that a concordant and highly plausible MoA has been established for pethoxamid-induced rat thyroid follicular cell adenomas and that this MoA is not relevant to humans. In conclusion, the extensive experimental data demonstrate that pethoxamid does not pose a carcinogenic hazard to humans.	Anonymous (2020); FMC-54841

In the rat (combined chronic- and carcinogenicity) study, pethoxamid was administered to 50 Crl:CD/sex/dose rats via the diet for up to 104 weeks at 0, 25, 400 or 1600 ppm, corresponding to 1.0/1.4, 17.0 /23.3 or 70/99 mg/kg bw/d, respectively, in males/females (Anonymous, 2000a; 80 PXA). Satellite groups were sacrificed at 27, 53 and 79 weeks and subjected to histopathological examination (Deviation from guideline: HCM not present; uterus not weighed; coagulating gland and peripheral nerve not preserved). Clear toxicity (reduced body weight gain, correlated with reduced food consumption, and increased liver weight, in both sexes and at various time points) was observed at the highest dose level of 1600 ppm. In males, there was also a statistically significant dose-related increase in thyroid weight at all doses. The NOAEL was considered to be 25 ppm (1.0 mg/kg bw/d), based on the decrease in body weight gain in females at the intermediate dose level of 400 ppm. The incidence of thyroid (follicular cell) adenomas was statistically significantly (trend-test) increased in male rats at the highest dose level. Slightly higher incidence of pancreatic islet cell carcinomas was also observed but not dose-dependent (at high- and low-, but not at mid-dose levels). Based on a review of the data, assessment of the relevance of the rat thyroid follicular cell tumours to humans, and differences in thyroid physiology between rodents and humans, the DS considered the thyroid tumours in rodents to be consequent to the phenobarbitone-like mode of action and thus not relevant to humans.

***Table**: Neoplastic findings in the rat chronic and carcinogenicity study*

Finding	**Dietary Concentration (ppm)**				**Historical control incidence (range) 16 studies 1996-1999**
MALES	0 (control)	25	400	1600	
Thyroid follicular cell adenoma	2/50 4%	2/50 4%	2/49 4%	9/50[$] 18%	30/954 3.14% (0%-12%)
Thyroid follicular cell carcinoma	2/50 4%	0/50 0%	0/49 0%	0/50 0%	7/954 0.73% (0%-5.1%)

[$] Trend test statistically significant

In the mouse (lifetime carcinogenicity) study pethoxamid was administered to 50 CD-1 mice/sex/dose via the diet for up to 95 weeks (males), or 92 weeks (females), at 0, 30, 400 or 5000 ppm, corresponding to 4.0/5.0, 56.8/68 or 982/1068 mg/kg bw/d in males and females, respectively (Anonymous, 2000b; 82 PXA). An additional 10 animals/sex/group were treated for up to 52 weeks. (Deviation from guideline: 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; no clinical biochemistry). Clear toxicity (reduced body weight gain and increased liver and kidney weights) was observed at both the highest and intermediate doses (5000 and 400 ppm). Histopathological findings included generalized hypertrophy of hepatocytes in males and periportal hypertrophy of hepatocytes in females. Hepatocyte hypertrophy was also observed in interim kill mice at 52 weeks. The LOAEL was considered to be <30 ppm (<4 mg/kg bw/d). At the highest dose level the incidence of hepatocellular adenomas was statistically significantly increased in male mice (19/50, 15/50, 18/49 and 34/50 at 0, 30, 400 and 5000 ppm, respectively). At the same dose, a slightly, however not statistically significant, increased number of hepatocellular carcinomas was observed in male mice, which was within the historical control incidence range. The no effect level for tumourigenicity was set at 400 ppm (56.8 mg/kg bw/d).

***Table**: Neoplastic findings in the mouse carcinogenicity study*

Finding	**Dietary Concentration (ppm)**				**Historical control incidence (range) 16 studies 1996-1999**
MALES	0 (control)	30	400	5000	
Hepatocellular adenoma	19/50 38%	15/50 30%	18/50 36%	34/50** 68%	199/867 22.95% (8.3%-42%)
Hepatocellular carcinoma	3/50 6%	3/50 6%	4/50 8%	6/50 12%	70/867 8.07% (3.6%-22%)

Statistical significance: **p<0.01

In an additional investigation of cell proliferation in the livers taken from this study, pethoxamid appears to have no influence on cell proliferation in the liver at 52 or 95 weeks. Based on a review of the data, assessment of the relevance of the mouse liver tumour to humans (Anonymous, 2020; FMC-54841; Anonymous, 2016; 1540 PXA; Table 40 of the CLH report) and comparing with the mechanistic data [mode of action (MoA)] of phenobarbital , the DS concluded that pethoxamid shows a similar pattern of tumorigenesis to phenobarbital, demonstrating that pethoxamid does not pose a carcinogenic hazard to humans.

Comments received during consultation

There were three comments on this endpoint. One National Authority commented that, in light of the uncertainty regarding the relevance to humans of the carcinogenic effects induced by phenobarbital in animal experiments and with respect to the phenobarbital-like mode of action

of pethoxamid, the relevance to tumours observed in carcinogenicity studies should not be ruled out. Regarding the thyroid effects observed in rats in particular, they were of the opinion that chemicals that produce thyroid tumours in rodents should be presumed to pose a carcinogenic risk to the human thyroid (EPA/630/R-97/002). Thus, possible interspecies differences may occur, and the available data do not appear to permit to conclude that the carcinogenic effects of phenobarbital in rodents are not relevant to humans.

A manufacturing company and an MSCA agreed with the DS to not classify pethoxamid for carcinogenicity, based on data from various mechanistic studies carried out to elucidate its mode of action (annex to the CLH Report), and applying the Bradford Hill criteria. The MSCA acknowledged that the slight increase in thyroid adenomas at the maximum dose in male rats is not sufficient to derive a classification.

Assessment and comparison with the classification criteria

According to the CLP Regulation, classification in Category 1 (known or presumed human carcinogen) must be based on positive evidence from epidemiological studies in humans, or sufficient evidence in animals to demonstrate animal carcinogenicity. To demonstrate carcinogenicity classification, Category 2 (suspected human carcinogen), limited evidence is required from human or animal studies, insufficient for Category 1. For pethoxamid, there are no human data, and in the two available studies in rats and mice (see above), treatment-related increases in tumours were observed only in males.

Thyroid follicular cell adenomas in rats

In a combined chronic toxicity and carcinogenicity study, rats were administered pethoxamid via the diet for up to 104 weeks at 0, 25, 400 or 1600 ppm (Anonymous, 2000a; 80 PXA). An increase in relative liver weight was recorded at 1600 ppm, in both sexes at various time points. The incidence of centrilobular hepatocyte hypertrophy was elevated at 1600 ppm: 11/50 in males, and 8/50 in females (0/50 in controls). In males, there was a dose-related increase in relative thyroid weight. At 1600 ppm, a higher incidence of follicular cell adenomas of the thyroid was seen in male rats (2/50, 2/50, 2/49 and 9/50 at 0, 25, 400 and 1600 ppm, respectively), which was statistically significant with the trend test (the Fisher's exact pairwise comparison between control and 1600 ppm groups was not statistically significant). The incidence (18%) falls outside the historical control range (0-12%).

Pethoxamid is non-genotoxic, and one mode of action for non-genotoxic agents to cause thyroid follicular tumours is the phenobarbital-like mode of action, consisting of the alteration of the thyroid-pituitary axis by enhancement of thyroid hormone metabolism via the induction of UDP-glucuronosyltransferase (UDPGT) in the liver, following the following cascade of events:

- Molecular Initiating Event: CAR activation
- Key Event 1: Upregulation of UDP-glucuronosyltransferases (UDPGT's) in liver
- Key Event 2: Increased T4/T3 catabolism and excretion in bile
- Key Event 3: Decreased (initially) serum T4 / T3
- Key Event 4: Increased serum thyroid stimulating hormone (TSH)
- Key Event 5: Thyroid hypertrophy and hyperplasia of follicular cells
- Adverse Outcome: Increased thyroid follicular cell adenoma

This phenobarbital-like mode of action was confirmed in the studies: the upregulation of UDP-glucuronosyltransferase (UDPGT) activity in liver was demonstrated by the rat oral 90 day feeding study (Anonymous, 1996; 61 PXA) and the rat mechanistic study using only male rats (Anonymous, 2020; PXA4560). In a male rat 14-day dietary study, UGT1A1/6 activity was significantly increased, resulting in an increase in thyroxine glucuronidation at 400 and 1600 ppm (Anonymous; 2016 1539 PXA). In the same study, pethoxamid treatment produced an increase

in mRNA for UGT1A1 at 400 and 1600 ppm and an increase in UGT1A6 mRNA at 1600 ppm. The study evaluating the biliary excretion of [^{125}I]Thyroxine and metabolites in male rats (Anonymous, 2019; 2018MET-PXA4538) indicates greater clearance of thyroxine due to liver induced T4 glucuronidation in the pethoxamid-treated rats compared to controls. The 90 day rat mechanistic study (Anonymous 2020 PXA4560) showed a time-dependent decrease in total T4 relative to pre-treatment values during the first 29 days, and also measured increased mean TSH values in the 1600 and 5000 ppm groups on Days 15, 29, 57, and 89. The mechanistic study found increased incidences of thyroid follicular cell hypertrophy in the 1600 ppm group (3/14) and 5000 ppm group (15/15), compared with 0/15 in the control group) and the rat carcinogenicity study found a nonsignificant increase in the incidence of follicular cell hyperplasia (4/50 compared with 0/50 in controls). Male rats dosed for 14 days with 1600 ppm (Anonymous, 2016; 1538 PXA) with a recovery period of 42 days showed a reversible increase in follicular thyroid cell proliferation, and some thyroid follicular epithelium hypertrophy (grade 1) in 2/8 animals. In the rat carcinogenicity study, as mentioned above, there was an increased incidence in thyroid follicular cell adenomas in the high dose (1600 ppm) group of 9/50 vs 2/50 in the control group.

Induction of hepatic UDPGT and subsequent disturbance of thyroid hormone homeostasis is a well-known, threshold-mediated, species-specific MoA that is generally not considered to be relevant to human hazard assessment.

Some alternative modes of action can be ruled out, as Pethoxamid is not genotoxic, and does not inhibit thyroperoxidase (TPO) activity (Anonymous, 2019; PXA 4481).

Conclusion: Although an increased incidence of thyroid follicular cell adenomas was observed in rats in the carcinogenicity studies at the highest dose (1600 ppm) and the incidence (18%) was outside the historical control range (0-12%), factors that reduce the concern are that only benign tumours were observed, and these adenomas were found only in one species (rat) and one sex (male). These findings could be explained by a phenobarbital-like MoA with induction of hepatic UDPGT and subsequent disturbance of thyroid hormone homeostasis, which is considered not to be relevant to human hazard assessment. Therefore, RAC concurs with the DS that a classification is not warranted based on the thyroid adenomas observed in rats.

Liver tumours in mice

In a lifetime carcinogenicity study, mice were administered pethoxamid via the diet at 0, 30, 400 or 5000 ppm (Anonymous, 2000b; 82 PXA). RAC notes that there was clear toxicity at the highest dose: at termination bodyweight was reduced by 16% in males, and by 17% in females, body weight gain was reduced by 40% in males and by 34% in females and an increase in relative liver weights was observed (77% males, 36% females). At the high dose, generalized hepatocyte hypertrophy in males (39/50) and periportal hepatocyte hypertrophy in females (42/50) was observed. Hepatocyte hypertrophy was also observed in the interim kill mice at 52 weeks at this dose. A statistically significantly increased incidence of hepatocellular adenomas was observed in male mice at 5000 ppm. The combined incidence of adenomas and carcinomas was also significantly increased. Although the number of hepatocellular carcinomas was higher in male mice at 5000 ppm than in the concurrent control (6/50 (12%) vs 3/50), it was well within the historical control incidence for the same strain in the same laboratory (3.6%–22%). RAC notes that the dosing of the study was not ideal, since there was more than an order of magnitude between the mid and high dose, with the high dose near the limit dose level. RAC also notes that unfortunately, all the mechanistic studies were performed exclusively in male animals.

Pethoxamid is non-genotoxic, and a mode of action for non-genotoxic agents to cause hepatocellular adenomas in rodents is activation of CAR nuclear receptors resulting in the increase in hepatic cell proliferation leading to hepatocellular tumours (phenobarbital-like

induction of rodent liver tumours). The proposed mode of action is the following:

- Molecular Initiating Event: Constitutive androstane receptor (CAR) activation
- Key Event 1: Altered gene expression specific to CAR activation in hepatocytes (in particular increasing hepatic phase I and II metabolism)
- Key Event 2: Increased cell mitogenic proliferation in hepatocytes
- Key Event 3: Increased preneoplastic foci in hepatocytes
- Adverse Outcome: Increased hepatocellular adenoma

Molecular Initiating Event and Key Event 1: CAR activation and altered gene expression specific to CAR activation in hepatocytes, with increased expression of CYP2B/CYP3A as associative event

In a 7-day male mouse dietary study (Anonymous, 2016; 1539 PXA), there was an increase of cytochrome P450 content, compared with controls, when exposed to pethoxamid at 400 and 5000 ppm, and a particularly high induction of CYP2B10 mRNA at 5000 ppm was observed (115 fold change relative to control). All effects were reversed after a 42-day recovery period. In the same study, activity of the enzyme testosterone 6β-hydroxylation (marker for CYP3A) was increased at 400 and 5000 ppm. In a 14-day male mouse dietary study (Anonymous; 2001b 98 PXA), at 5000 ppm there was an increase of cytochrome P450 content compared with the controls, induction of CYP1A, CYP2B, CYP3A2 and CYP4A1 mRNA was observed, as well as increased activity of PROD (marker for CYP2B and 3A). In a 28-day mouse study, there was a large induction of PROD activity (marker for CYP2B and 3A) at 100 ppm and above, and also an induction of p-nitrophenol-UDPGT activity at 10 000 ppm in males and from 3000 ppm in females (Anonymous, 1996a; 70 PXA). In isolated primary CD-1 (male) mouse hepatocytes treated with pethoxamid, CYP2B10 or CYP3A11 mRNA induction was observed relative to concurrent vehicle controls (Anonymous, 2019; PXA4482). In the same study, phenobarbital induced mouse CYP2B10 and CYP3A11 over concurrent vehicle control levels. Compared with the pattern of effect by phenobarbital in mouse hepatocytes, the weak induction of CYP2B10 or CYP3A11 in the pethoxamid treated groups is considered to be consistent with results expected for a weak inducer of nuclear CAR and PXR gene expression.

Key Event 2: Transiently increased hepatocellular proliferation, with hepatocellular hypertrophy and increased liver weight as associative events

In a 7-day male mouse dietary study, there was a statistically significant increase in hepatic DNA replication (increased BrdU labelling index) at 5000 ppm, but not at 400 ppm (Anonymous, 2016; 1539 PXA). This effect was reversed after a 42-day recovery period. In a 14-day male mouse dietary study, there was increased hepatic DNA replication (increased PCNA labelling index) at 5000 ppm, but not at 400 ppm (Anonymous, 2001; 98 PXA). In a 28-day (Anonymous, 1996a; 70 PXA) and a 90-day mouse study, increased hepatocyte hypertrophy was observed. In the lifetime carcinogenicity study (Anonymous, 2000b; 82 PXA), at 5000 ppm generalized hepatocyte hypertrophy in males (39/50) and periportal hepatocyte hypertrophy in females (42/50) was observed, with none found in the controls or at lower doses. Hepatocyte hypertrophy was also observed in the interim kill mice at 52 weeks at the high dose. Increased liver weight was noted in multiple studies following exposure of mice to pethoxamid: the 7-day (Anonymous, 2016; 1538 PXA) and 14-day (Anonymous, 2001b; 98 PXA) mechanistic studies, the 28-day (Anonymous, 1996a; 70 PXA) and 90-day feeding studies (Anonymous, 1998; 71 PXA) and the mouse carcinogenicity study (Anonymous, 2000b; 82 PXA).

Key event 3: Increased preneoplastic foci in hepatocytes

Hepatic preneoplastic foci were not observed in the long-term dosing studies with pethoxamid, however, these are transient, as they develop into neoplasia (adenomas) and the chances of seeing foci in the routine toxicology studies are low.

Adverse outcome: Increased hepatocellular adenoma

In the mouse carcinogenicity study, an increased incidence of hepatocellular adenoma was observed in males at 5000 ppm (982 mg/kg bw/d) (Anonymous, 2000b; 82 PXA).

Human non-relevance of the MoA for liver tumours

Cultures of primary hepatocytes isolated from 4 male CD-1 mice and cryopreserved human hepatocytes from 3 individual male donors were used to investigate the potential of pethoxamid to activate CAR and PXR (Anonymous, 2019; PXA4482). In these *in vitro* assays hepatocytes were tested at four different concentrations (3 to 10 µM in two human donors and 3 to 20 µM in the remaining human donor, and from 1 to 20 µM in mouse cells). After a 96-hour treatment period, cells were processed for mRNA analysis of CYP2B10 and CYP3A11 for mice and CYP2B6 and CYP3A4 for human cells, corresponding to CAR and PXR receptor activation, respectively. Cell proliferation was measured by BrdU incorporation and subsequent immunohistochemical staining. Phenobarbital was included as a positive control for the activation of CAR and PXR; epidermal growth factor (EGF) was included as a positive control for the induction of cell proliferation. An assessment of cytotoxicity was also performed.

In mouse hepatocytes, phenobarbital induced CYP2B10 and CYP3A11 mRNA, compared with concurrent vehicle controls. Pethoxamid induced CYP2B10 or CYP3A11 mRNA to a somewhat lesser degree relative to phenobarbital. Therefore, pethoxamid was considered to be a weak activator of CAR and PXR *in vitro*, relative to phenobarbital in mouse hepatocytes.

In human hepatocytes, phenobarbital treatment resulted in marked induction of CYP2B6 mRNA and CYP3A4 mRNA. In cultures treated with pethoxamid, overt cytotoxicity was observed at 10–20 µM, depending on the specific human donor hepatocytes. Both CYP2B6 mRNA and CYP3A4 mRNA was induced at 10 or 20 µM.

In cultures of primary male CD-1 mouse hepatocytes, phenobarbital and pethoxamid both induced replicative DNA synthesis (RDS) at all concentrations tested, while neither phenobarbital nor pethoxamid induced RDS in human hepatocytes. The positive RDS control, EGF induced RDS in both mouse and human hepatocytes.

The conclusion of the DS and the position paper titled "Pethoxamid: Human Relevance Framework Assessment of Induced Rodent Liver and Thyroid Tumors" was that overall, pethoxamid is a weak activator of CAR and/or PXR *in vitro*, has been shown to induce RDS in mouse hepatocytes, but not in human hepatocytes, thus the responses observed with pethoxamid are similar to that of phenobarbital. In their opinion these data are supportive of the lack of human relevance for mouse liver tumour formation following treatment with pethoxamid. The main focus of the argument is that since a proliferative effect is a necessary key event for liver tumour formation, and pethoxamid did not cause proliferation in human hepatocytes, the phenobarbital-like CAR mode of action is not relevant to humans.

RAC does not fully agree with this conclusion. It is unclear whether the use of primary human hepatocytes is suited for the evaluation of hepatocellular proliferation. The process of isolation, preservation and culturing of primary human hepatocytes is complex, their quality is highly donor-dependent, and their functionality can be compromised. The use of EGF as positive control is also debatable, as it is not known how similar this growth factor and the liver carcinogen in question are in their MoA, with respect to, for example, receptor activation and triggering subsequent cell proliferation. Therefore, taking into account all the presented data concerning pethoxamid, RAC does not fully rule out the human relevance of this mode of action. RAC also notes that there are no *in vivo* studies with CAR/PXR-knock out animals or humanised-CAR animals for confirmation of CAR mediated effects.

Alternative modes of action

- Genotoxicity: Pethoxamid is not genotoxic (see section on germ cell mutagenicity).
- Peroxisome proliferation: Liver tumours in rodents conclusively linked to peroxisome proliferation are not considered to be relevant to humans (ECHA Guidance on the Application of the CLP Criteria).
- AhR receptor activation: Treatment with pethoxamid resulted in slightly increased EROD activity and CYP1A isoform expression at 7 days of exposure (Anonymous, 2016; 1539 PXA) but at 14 days CYP1A isoform expression increased to a 4.29-fold difference at 5000 ppm compared to the control (Anonymous, 2001b; 98 PXA). Furthermore, EROD activity was not investigated in human hepatocytes.
- Estrogenic stimulation: There is no structural similarity between pethoxamid and estrogen, therefore a similar MoA of hepato-carcinogenesis is unlikely. There is no evidence for estrogenic activity in any of the studies included in the CLH dossier, including in the guideline compliant rat two-generation reproductive toxicity study (Anonymous, 2000; 85 PXA).
- Statins: liver HMG-CoA-reductase activity was not measured, there was a slight increase in CYP4A protein expression (Anonymous, 2016; 1539 PXA and Anonymous, 2001b; 98 PXA), but there was no evidence of periportal atypia or bile duct hyperplasia in male rats.
- Cytotoxicity: cytotoxicity is unlikely to be involved given the absence of consistent and significant necrosis.
- Iron/copper overload: there was no evidence that pethoxamid produces cell damage with regeneration in liver tissue.
- Increased apoptosis: There are no data on hepatocellular apoptosis in *in vivo* or *in vitro* studies.

Conclusion: In a lifetime carcinogenicity study, statistically significantly increased incidence of hepatocellular adenomas was observed in male mice at the high dose of 5000 ppm. The combined incidence of adenomas and carcinomas was also significantly increased at this dose level. Although the number of hepatocellular carcinomas was higher in male mice at 5000 ppm than in the concurrent control (6/50 (12%) vs 3/50), the increase was not statistically significant, and it was well within the historical control incidence for the same strain in the same laboratory (3.6%–22%). The CAR mode of action is demonstrated as plausible in mice, but the MoA is not sufficiently demonstrated in humans, as there are no *in vivo* studies with CAR/PXR-knock out animals or humanised-CAR animals for confirmation of CAR mediated effects, and the proliferation of human hepatocytes can not be ruled out by the *in vitro* test performed. Furthermore, other modes of action cannot be ruled out with certainty. The data show a statistically significant increase only in benign tumours (adenomas), at a dose that showed clear toxicity, in one sex of one species, which reduces the concern. Therefore, RAC agrees with the DS's proposal that **no classification for carcinogenicity is warranted.**

RAC evaluation of reproductive toxicity

SEXUAL FUNCTION AND FERTILITY

Summary of the Dossier Submitter's proposal

The CLH dossier includes 2 studies relevant to this endpoint: a two-generation study in rats, done under GLP and according to Guideline OPPTS 870 3800. The study design is compatible with OECD TG 416, and includes oestrus cyclicity, sperm evaluation and sexual development landmarks. The second study is a preliminary study of effects on reproductive performance in rats, which is a non-guideline study, but done under GLP.

***Table**: Summary table of animal studies on adverse effects on sexual function and fertility (Table 42 in the CLH report)*

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Rat preliminary study of effects on reproductive performance. Non-guideline preliminary study. GLP Rat Sprague Dawley 6/sex/group	Pethoxamid Batch: TB-960306 Purity: 95.0% Dose levels: 0, 25, 100, 400, 1600 ppm in diet F0: 15 days prior and through pairing, gestation and lactation Selected F1: Continuously until approximately 6 weeks of age	**F0 Parental Toxicity** 1600 ppm (127-172 mg/kg bw/d) No effects. **Selected F1 Toxicity** 1600 ppm (240-296 mg/kg bw/d) ↓ Body weight: Week 4 16.5% males, 10.6% females ↑ Liver weight (relative to body weight): 24.2% males; 12.0% females 400 ppm No effects. **Offspring Toxicity** 1600 ppm ↓ Body weight: Day 1 14.5% males, 15.6% females; Day 21 17.1% males, 15.6% females. ↓ Body weight gain from day 4 [values not reported; calculated male 3 g gain in control Day 1 to 4 compared with 1.3 g gain at 1600 ppm, female 2.7 g gain in control Day 1 to 4 compared with 1.7 g gain at 1600 ppm] ↑ Liver weight (absolute): 4.7% males, 8.7% females, unselected F1 pups. 400 ppm ↓ Body weight gain from day 4 [values not reported; calculated male 3 g gain in control Day 1 to 4 compared with 1.9 g gain at 400 ppm, female 2.7 g gain in control Day 1 to 4 compared with 1.8 g gain at 400 ppm] 100 ppm No effects. Note: no statistical analysis, body weight gain values not reported.	Anonymous (1998a); 84 PXA
Rat two- generation reproductive study. Guideline OPPTS 870 3800. Study design compatible with OECD TG 416, includes oestrus cyclicity, sperm evaluation, sexual development landmarks. GLP Rat Sprague Dawley 28/sex/group	Pethoxamid Batch: TB-960306 Purity: 95.0% Dose levels: 0, 25, 200, 1600 ppm in diet F0 and F1: 10 weeks prior and through pairing, gestation and Lactation	**F0 Parental toxicity:** 1600 ppm (pre-mating: 85-170 mg/kg bw/d males; 117-181 mg/kg bw/d females) ↓ Body weight gain during gestation: 10.7% Day 0-20 of gestation ↑ Liver weight (absolute; g): 12.9% males, 12.1% females ↑ Liver weight (relative to body weight): 18.3% males; 11.9% females ↓ Spleen weight (relative to body weight): 10% females 200 ppm (pre-mating: 11-22 mg/kg bw/d males; 14-24 mg/kg bw/d females) No effects. **F1 Parental toxicity** 1600 ppm (pre-mating: 97-291 mg/kg bw/d males; 123-303 mg/kg bw/d females) ↓ Body weight Week 10: 9.8% males; 10.1% females ↓ Body weight gain Week 0-10: 10.7% males; 12.3% females ↑ Liver weight (relative to body weight): 15.3% males; 8.5% females 200 ppm (pre-mating: 12-34 mg/kg bw/d males; 16-36 mg/kg bw/d females) ↓ Body weight Week 10: 6.71% females	Anonymous (2000); 85 PXA

		↓ Body weight gain Week 0-10: 9.84% females ↓ Thymus weight (absolute): 15% females ↑ Seminal vesicles (relative to body weight): 12% males These effects were not considered to be treatment related. **Fertility and Reproductive Performance** No adverse effects.	

In the two-generation reproduction study in rats, clear parental toxicity (reduced body weight and body weight gain during gestation; increased liver weight and decreased spleen weight) was observed at the highest dose level of 1600 ppm. No effects were observed at the intermediate dose level of 200 ppm and this was the NOAEL for parental toxicity. Despite the parental toxicity, there was no evidence for any effect of pethoxamid on sexual function and fertility at the highest dose level tested of 1600 ppm (approximately 112 mg/kg bw/d). Systemic toxicity was also observed in the pups at 1600 ppm, with lower body weight and body weight gains evident at the end of the lactation period. The relevant offspring NOAEL was 200 ppm (14 mg/kg bw/d). The DS concluded that there were no adverse effects on sexual function and fertility in the rat to warrant classification of pethoxamid as a potential human reproductive toxicant.

Comments received during consultation

A Company-Manufacturer agreed with the DS that pethoxamid did not cause adverse effects on sexual function and fertility.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility means any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems.

There were no adverse effects on sexual function or fertility in the rat study to justify a classification of pethoxamid as a potential human reproductive toxicant. Therefore, RAC agrees with the DS's proposal that **no classification for adverse effects on sexual function and fertility is warranted**.

DEVELOPMENTAL TOXICITY

Summary of the Dossier Submitter's proposal

There are several developmental toxicity studies discussed in the CLH dossier: 3 rat studies and 4 rabbit studies.

***Table**: Summary table of animal studies on adverse effects on development (from Table 45 in CLH report)*

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Rat preliminary embryo-foetal development study. Non-guideline preliminary study. GLP Rat CD (Sprague Dawley origin) sexually mature, pregnant 6/group Limited parameters retrieved (no skeletal or soft tissue examination of foetuses done)	Pethoxamid Batch: TB- 951005 Purity: 95% Vehicle: 1% w/v methyl cellulose + 0.5% w/v Tween 80 Dose levels: 0, 8, 80, 400, 800 mg/kg bw/d Exposure: Days 6-15 of gestation	<u>800 mg/kg bw/d</u> Maternal mortality: 2/6 females died on Day 9, and 1/6 female killed in extremis on Day 10 ↓ Bodyweight Day 20: 11.0% (surviving 3 females only) ↑ Salivation: 6/6 females <u>400 mg/kg bw/d</u> ↑ Salivation: 6/6 females No other effects <u>80 mg/kg bw/d</u> ↑ Salivation: 6/6 females, however at lower incidence than higher doses No other effects. <u>8 mg/kg bw/d</u> No effects NOEL: 400 mg/kg bw/d No adverse effects on the litter responses and foetal development were observed.	Anonymous (1996); 86 PXA
Rat embryo- fetal development study. No guideline mentioned but consistent with OECD TG 414 except: Shorter administration period (day 6 to 15 of gestation only) and 40% (10/25) of the dams of the high dose group died. However considered acceptable. GLP Rat CD (Sprague Dawley origin) sexually mature, pregnant 25/group	Pethoxamid Batch: TB- 951005 Purity 95% Vehicle: 1% w/v methyl cellulose + 0.5% w/v Tween 80 Dose levels: 0, 8, 80, 600 mg/kg bw/d Exposure: Days 6-15 of gestation	<u>600 mg/kg bw/d</u> Maternal mortality: 10/25 killed in extremis or found dead Day 8-17 Signs prior to termination included piloerection, hunched posture, unresponsive to stimuli ↓ Bodyweight Day 9: 1%. No weight gain for first 3 days after dosing, but overall weight gain to Day 20 similar to control ↑ Salivation: 25/25 females <u>80 mg/kg bw/d</u> ↑ Salivation: 24/25 females No other effects <u>8 mg/kg bw/d</u> No effects Maternal NOEL: 80 mg/kg bw/d Foetal NOEL: 600 mg/kg bw/d	Anonymous (1997a); 87 PXA
Rat developmental toxicity study. OECD TG 414 (1981) OPPTS 870.3700 (1998). GLP Rat Crl:CD(SD) sexually mature, pregnant 25/group, 30 high dose group	Pethoxamid technical Batch: P1351- JaK-T2-23-6 Purity: 95.80% Vehicle: 1% w/v methyl cellulose + 0.5% v/v Tween 80 Doses levels: 0, 10, 75, 500/350/250 mg/kg bw/d Exposure: Days 6-20 of gestation	<u>500/350/250 mg/kg bw/d</u> **Maternal** Maternal mortality: Due to mortality and/or adverse clinical signs of toxicity in 7 rats at the 500 mg/kg bw/d within 3-7 days of dosing, dose level reduced to 350 mg/kg bw/d. Mortality occurred in 5 additional rats at 350 mg/kg bw/d, which resulted in a subsequent reduction of the dose level to 250 mg/kg bw/d. Clinical signs: 21/30 to 26/30 hunched posture; light brown faeces and dehydration.4/30 to 9/30 moderate dehydration; slightly pale and/or pale ears; ungroomed coat; ptosis; thin body condition; urine staining; slight excess salivation; decreased motor activity; pale extremities; coldness to the touch; scant faeces; and ataxia. ↓ Body weight Day 21: 10.5% ↓ Body weight gain: 24.0% Day 0-21; 28.4% Day 6-21 ↓ Food consumption: 17.0% Day 6-21 ↓ Uterine weight: 8% **Foetal:** ↓ Foetal weight: 10.1% (combined sexes) No foetal gross external, soft tissue or skeletal alterations (malformations or variations) considered to be test substance-related. <u>75 mg/kg bw/d</u> No effects	Anonymous (2014a); 1138 PXA

		10 mg/kg bw/d No effects Maternal NOEL: 75 mg/kg bw/d Foetal NOEL: 75 mg/kg bw/d	
Rabbit tolerability study. Non guideline preliminary study. GLP Rabbit: NZW, females	Pethoxamid Batch: TB- 960306 Purity: 95.0% Vehicle: 1% w/v methyl cellulose + 0.5% w/v Tween 80 2 animals dosed 50 mg/kg for 2 days, dose doubled every 2 days to 800 mg/kg bw on days 9 and 10 2 further mated females dosed for 7 days at 300 mg/kg bw/d	Escalating dose to 800 mg/kg bw/d Maternal mortality: One animal found dead, the other killed *in extremis* after two doses of 800 mg/kg bw/d. Following treatment with 400 mg/kg bw/d both females showed ↓ body weight, ↓ food consumption, ↓food cons water intake and ↓ faecal output. No effects at 200 mg/kg bw/d. 300 mg/kg bw/d for 7 days in mated females ↓ Bodyweight: Average 0.48 kg body weight loss Day 0-7. Note only one female pregnant Conclusion: Dosing for preliminary embryo-foetal development study should be ≤300 mg/kg bw/d.	Anonymous (1997b); 89 PXA
Rabbit preliminary embryo-foetal developmental toxicity study. Non guideline preliminary study. GLP Rabbit: NZW pregnant 4/group Limited parameters retrieved (no skeletal or soft tissue examination of foetuses assessed, no weight of gravid uteri recorded)	Pethoxamid Batch: TB-960306 Purity: 95.1% Vehicle: 1%w/v methyl cellulose + 0.5% w/vTween 80 Dose levels: 0,30, 100, 300 mg/kg bw/d Exposure: Days 6-19 of gestation	300 mg/kg bw/d ↓ Bodyweight: Marked decrease in the two days following the start of dosing. The body weight change during the treatment period was negative. Overall weight gain during gestation 45% lower than control ↓ Food consumption: In 3/4 females. Approximately 46% of control during first half of treatment period. Approximately 12% of control during second half of treatment ↓ Faecal output: In 3/4 females No effects on litter parameters 100 mg/kg bw/d No treatment related effects 30 mg/kg bw/d No treatment related effects Conclusion: Marked effects on dams at 300 mg/kg bw/d.	Anonymous (1998b); 90 PXA
Rabbit embryo- foetal development toxicity study No guideline mentioned but consistent with OECD TG 414 except: Shorter administration period (day 6 to 19 of gestation only). GLP Rabbit: NZW pregnant 20/group	Pethoxamid Batch: TB-960306 Purity: 95.1% Vehicle: 1%w/v methyl cellulose +0.5% w/vTween 80 Dose levels: 0, 12.5, 50, or 200 mg/kg bw/d Exposure: Days 6-19 of gestation	200 mg/kg bw/d ↓ Body weight: No weight gain mid treatment then weight loss. Group mean body weight gain approximately 47% of control at end of treatment period. [note overall weight gain for gestation period similar to control.] ↓ Food consumption: Approximately 76% of control during second half of treatment period No effects on litter parameters or upon growth or development of foetuses in utero 50 mg/kg bw/d ↓ Food consumption: Slightly lower than control during second half of treatment period (approximately 90% of control) 12.5 mg/kg bw/d No treatment related effects Maternal NOAEL: 50 mg/kg bw/d Foetal NOEL: 200 mg/kg bw/d	Anonymous (1998c); 88 PXA
Rabbit developmental toxicity study OECD TG 414 (1981) OPPTS 870.3700 (1998). GLP Rabbit: Hra:(NZW)SPF pregnant 25/group	Pethoxamid technical Batch: P1351- JaK-T2- 3-6 Purity: 95.80% Vehicle: 1% w/v methyl cellulose + 0.5% v/v Tween 80 Dose levels: 0, 12.5, 50, 200 mg/kg bw/d Exposure: Days 6 to 28 of gestation	200 mg/kg bw/d **Maternal:** ↑ Abortions: 4/25 (0/25 controls) Clinical observations in rabbits observed to abort: scant faeces, ungroomed coat, thin body condition, mild dehydration and red substance in the cage pan ↓ Body weight: 8.1% Day 29 ↓ Body weight gain: 58.3% Day 0-29; 74.3% Day 6-29 ↓ Food consumption: 28.7% Day 6-29 ↓ Gravid uterine weight: 12.2% **Foetal:**	Anonymous (2014b); 1139 PXA

		↓ Foetal weight: 18.7% (combined sexes) ↑ Incidence of supernumerary thoracic ribs: mean value 12.64 (control 12.38) ↑ Number of thoracic vertebrae mean value 12.70 (control 12.43) ↓ Number of lumbar vertebrae mean value 6.30 (control 6.56) <u>50 mg/kg bw/d</u> No treatment related effects ↑ Number of thoracic vertebrae mean value 12.63 (control 12.43) within HCD ↓ Number of lumbar vertebrae mean value 6.35 (control 6.56) within HCD <u>12.5 mg/kg bw/d</u> No treatment related effects Maternal NOAEL: 50 mg/kg bw/d Foetal NOAEL: 12.5 mg/kg bw/d	

Rat studies

A developmental toxicity study in rats was available, which was consistent with OECD TG 414 except for the shorter administration period (only from day 6 to 15 of gestation), with a preceding preliminary non-guideline range-finding embryo-fetal development study, with limited parameters examined (no skeletal or soft tissue examination of foetuses done). The second, more recent study, is a rat developmental toxicity study, conducted according to OECD TG 414.

In the first rat developmental toxicity study, animals received the test compound at doses of 0, 8, 80 or 600 mg/kg bw/d by oral gavage from day 6 to 15 of gestation. Significant maternal toxicity was seen at 600 mg/kg bw/d. The dose of 8 mg/kg bw/d was considered to be the maternal NOEL, based on the salivation which occurred from 80 mg/kg bw/d. The NOEL for developmental toxicity was 600 mg/kg bw/d. Although, more than 10% of the dams died at the high dose (10/25), the study was considered to be acceptable because a high number of dams (15/25) 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 no developmental toxicity was seen up to the highest dose (800 mg/kg bw/d).

In the more recent rat developmental toxicity study, female Crl:CD(SD) rats (25 or 30/group) were orally administered pethoxamid or the vehicle control once daily by oral gavage on gestation days 6 through 20, at dose levels of 0, 10, 75 or 500 mg/kg bw/d. Due to mortality and/or adverse clinical signs of toxicity in rats at the 500 mg/kg bw/d dose level within the first 3 to 10 days of dosing, the high-dose level was reduced to 350 mg/kg bw/d. Mortality occurred in three additional rats at this dose level, which resulted in a subsequent reduction of the dose level to 250 mg/kg bw/d. There were two additional deaths in the 250 mg/kg bw/d dose group that occurred on gestation day 21 after the last dose on gestation day 20. Based on effects on mortality, clinical signs, body weight and food consumption in maternal females at the high dose, the NOAEL for maternal toxicity was established at 75 mg/kg bw/d. Based on reductions in gravid uterine weight and fetal body weight, the NOAEL for developmental end-points was also established at 75 mg/kg bw/d. Based on results of the study, pethoxamid was considered not to be a developmental toxicant.

Body weights and bodyweight gains in pethoxamid-treated F1 as well as F2 pups were found to be significantly lower at day 21 in the two-generation reproduction toxicity study (around -7% in F1 pups and -12% in F2 pups) in rats as compared to control. However, these changes are considered to be a consequence of direct food consumption.

Rabbit studies

An embryo-fetal development toxicity study in rabbits was available, which was consistent with OECD TG 414, except for the shorter administration period (only from day 6 to 19 of gestation), with two preceding range finding studies (a non-guideline tolerability study, and non- guideline preliminary developmental toxicity study with limited parameters retrieved (no skeletal or soft tissue examination of foetuses done, no weight of gravid uteri recorded). The second, more recent study is a rabbit developmental toxicity study performed according to OECD TG 414.

In the initial rabbit developmental toxicity study, four groups of 20 pregnant New Zealand White 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. At 200 mg/kg bw/d, a reduced body weight gain was observed during the treatment period and at 50 mg/kg bw/d and above, a lower food intake was observed during the treatment period. Therefore, the dose of 50 mg/kg bw/d was considered to be the maternal NOAEL. No dose was associated with adverse effects on in utero survival or embryo-fetal development; thus the NOAEL (NOEL) for developmental toxicity was 200 mg/kg bw/d.

In the more recent rabbit developmental toxicity study, 25 pregnant New Zealand White rabbits were administered pethoxamid or the vehicle control substance once daily by oral gavage on days 6 through 28 of gestation, at dose levels of 0, 12.5, 50, or 200 mg/kg bw/d. An increased incidence in the number of rabbits that aborted and were subsequently euthanized during the study, and reductions in body weight, body weight gain and food consumption values occurred in the 200 mg/kg bw/d dose group. On the basis of these data, the maternal NOAEL for pethoxamid was 50 mg/kg bw/d. In the 200 mg/kg bw/d dose group, foetuses showed decreased 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, respectively, a common variation observed at maternally toxic doses. The developmental NOAEL was 12,5 mg/kg bw/d. Based on results of that study pethoxamid was considered not to be a selective developmental toxicant.

In summary, pethoxamid has been investigated extensively in the pregnant rat and pregnant rabbit. Maternal toxicity was demonstrated at high doses but none of the four main studies produced any indication of adverse effects on foetal development, therefore the DS proposed no classification for development.

Comments received during consultation

A Company-Manufacturer agreed with the DS that pethoxamid was not a developmental toxicant.

An MSCA commented that there is a discrepancy in the foetal NOAEL mentioned in the CLH dossier and the Annex regarding the Anonymous (2014b) rabbit study. The DS responded that the maternal NOAEL for pethoxamid was 50 mg/kg bw/d, while the developmental NOAEL was set at 12.5 mg/kg bw/d.

Assessment and comparison with the classification criteria

Adverse effects on development of the offspring include any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. Such effects shall have been observed in the absence of other toxic effects, or shall not be secondary non-specific consequences of the other toxic effects.

Rat data

In the two developmental toxicity studies in rats, based on effects on mortality, clinical signs, decreased body weight gain and food consumption in maternal females at the high doses, the NOAEL for maternal toxicity was 75 mg/kg bw/d or 80 mg/kg bw/d. At these doses, there was no evidence of intra-uterine toxicity to the embryos / foetuses or variations/malformations. The studies did not demonstrate any developmental toxicity potential of pethoxamid in rats.

In a rat two-generation reproductive toxicity study, F1 and F2 pups showed a significantly decreased body weight at day 21 and a significantly decreased body weight gain during the last week before weaning (days 14 to 21) at the highest dose. Both pup body weight on day 14 and pup body weight gain from day 1 to 14, were similar to the control values. Therefore, the body weight decrements evident at day 21 are considered a consequence of direct consumption of the diet and a consequence of systemic toxicity.

Rabbit data

Doses up to 200 mg/kg bw/d have been investigated in two oral developmental toxicity studies in rabbits. Based on decreased body weight gain and food consumption in maternal females at the high doses, the NOAEL for maternal toxicity was 50 mg/kg bw/d. At this dose, there was no evidence of intra-uterine toxicity to the embryos / foetuses or malformations. In the 200 mg/kg bw/d dose group, foetuses showed decreased weight and a statistically significantly increased incidence of supernumerary thoracic ribs with an associated statistically significant increase and decrease in the numbers of thoracic and lumbar vertebrae, respectively, a common variation observed at maternally toxic doses. In the 50 mg/kg bw/d dose group, there were a statistically significant increase and decrease ($p \leq 0.05$) in the number 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 was considered to be the starting point of effects, being more severe at the high dose. The developmental NOAEL was 12.5 mg/kg bw/d. Supernumerary thoracic ribs and an associated increase and decrease in the number of thoracic and lumbar vertebrae are considered variations, and do not lead to classification as a developmental toxicant.

Based on results of the studies performed in rats and rabbits, RAC agrees with the DS's proposal that **no classification for developmental toxicity is warranted**.

ADVERSE EFFECTS ON OR VIA LACTATION

Summary of the Dossier Submitter's proposal

There is a two-generation reproduction toxicity study in rats (Anonymous, 2000) of pethoxamid with data on effects on or via lactation. The key data are summarised in the table below.

Parameter	F1 pups		F2 pups	
	Control	1600 ppm	Control	1600 ppm
Live born index (%)	89	93	91	95
Pup viability to day 4 (%)	82	94	80	92
Lactation index Day 4-21 (%)	99	97	96	99
	MALES			
Pup weight at birth (g)	6.0	6.3	6.1	5.9
Pup weight Day 14 (g)	30.9	30.2	31.3	29.2

Pup weight Day 21 (g)	51.9	48.6* (-6,4%)	53.2	47.0** (-12%)
Pup weight gain Day 1-14 (g)	24.7	23.7	25.1	23.3
Pup weight gain Day 1-21 (g)	45.8	42.2* (-7,9%)	47.0	41.2** (-12%)
	FEMALES			
Pup weight at birth (g)	5.7	6.0	5.8	5.5
Pup weight Day 14 (g)	29.9	29.0	30.0	27.9
Pup weight Day 21 (g)	50.1	46.7* (-6,8%)	50.9	45.0** (-12%)
Pup weight gain Day 1-14 (g)	24.0	23.0	24.1	22.4
Pup weight gain Day 1-21 (g)	44.2	40.8* (-7,7%)	45.1	39.5** (-12%)

Statistical significance: * P≤0.05; **P≤0.01

There is no evidence in either generation of an effect on pup viability considering either live born index or survival to weaning (Day 21). There is a statistically significant decrease in body weight and body weight gain of pups on day 21, in both generations. Evaluation of the data shows that both pup body weight on day 14 and pup body weight gain from day 1 to 14, were similar to the control. As stated in the report of this study, in the calculation of achieved dosage 'food intake between days 14 and 20 of lactation includes diet eaten directly by the offspring', as well as the dam. Therefore, the body weight decrements evident at day 21 are a consequence of direct consumption of the diet (no body weight decrement was evident at birth or up to day 14) and considered to be a consequence of systemic toxicity, and do not indicate any adverse effect on the offspring due to transfer of the chemical via the milk or to the quality of the milk. The DS did not propose to classify pethoxamid for adverse effects on or via lactation.

Comments received during consultation

A Company-Manufacturer agreed with the DS that pethoxamid did not cause adverse effects on or via lactation.

Assessment and comparison with the classification criteria

The classification is intended to indicate when a substance may cause harm due to its effects on or via lactation and is independent of consideration of the reproductive or developmental toxicity of the substance.

In a rat two-generation reproductive study F1 and F2 pups showed a significantly decreased body weight at day 21, and a significantly decreased body weight gain during the last week before weaning (days 14 to 21) at the highest dose. Both pup body weight on Day 14, and pup body weight gain from Day 1 to 14, were similar to the control values. Therefore, the body weight decrements evident at day 21 are considered a consequence of direct consumption of the diet and a consequence of systemic toxicity. RAC agrees with the DS's proposal that **no classification for effects on or via lactation is warranted.**

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Pethoxamid (ISO); 2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenylprop-1-enyl)acetamide is currently listed in Annex VI of Regulation (EC) 1272/2008 with classification Aquatic Acute 1 with M-factor 100 and Aquatic Chronic 1 (H410).

The DS proposal is to retain this classification and assigned M-factors:

Acute Aquatic Hazard: Aquatic Acute 1 with M-factor 100 based on 72h mean measured EC_{50} of 0.00408 mg/L for growth rate for *Selenastrum capricornutum (Pseudokirchneriella subcapitata)*.

Chronic Aquatic Hazard: Aquatic Chronic 1 (H410), with M-factor 10 based the fact that the substance is not rapidly degradable and due to a 72-h E_rC_{10} for growth rate and biomass for *Selenastrum capricornutum* (*Pseudokirchneriella subcapitata*) of 0.00119 mg/L.

Degradation

Abiotic

Hydrolysis

The available data (Legacy study broadly in line with OECD TG 111, GLP) for pethoxamid has shown that the substance is stable to hydrolysis at all pH values studied (pH 4, 7 and 9; buffer solutions; 50 ºC; sterile conditions).

Biotic

Ready biodegradability

DS concluded that pethoxamid is not readily biodegradable based on the results of a readily biodegradability study, conducted according to the OECD TG 301B, without identified major deficiencies.

Water, water-sediment and soil degradation data (including simulation studies)

Water, water/sediment degradation data

In natural water under aerobic conditions, the degradation rate of radiolabelled pethoxamid is determined with the SFO method (OECD TG 309). The DT_{50} defined is 144 days. DS concluded that substance is not rapidly degradable in aquatic environment. Substance initially metabolised via glutathione conjugation with subsequent loss of glycine and glutamic acid to form an intermediate cysteine conjugate, pethoxamid metabolite MET-30.
The degradation rate of radiolabelled pethoxamid is studied in water/sediment systems, using SFO kinetics (OECD TG 308). The DT_{50} is 7.0 days for Golden Lake test system and 13 days for Goose River test system. Substance metabolise to thiol via beta lyase cleavage, with subsequent methylation to a methyl sulphide, pethoxamid metabolite MET-6, or transformation to pethoxamid metabolite MET-104.

Soil degradation

Aerobic transformation of pethoxamid in soil is studied according to OECD TG 307 with radiolabelled test substance. Results has shown rapid degradation in soil with DT_{50}< 16 days. 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 pethoxamid metabolite MET-101 or a sulfonic acid, pethoxamid metabolite MET-42. MET-42 is then degraded to pethoxamid metabolite MET-100.

Another study for aerobic transformation of pethoxamid in soil has been performed according to OECD TG 307 with radiolabelled test substance. Defined DT 50< 16 days. Pethoxamid is metabolised via reductive de-chlorination to give pethoxamid metabolite MET-22, with subsequent degradation to give pethoxamid metabolite MET-46. Several minor metabolites (< 5 % of AR) were detected, none of them was identified.

The **rate of degradation** of pethoxamid **in anaerobic soil** is slightly slower than in aerobic soil (DT_{50} of 13.7 days *vs* 6.3 days). Formation fraction of pethoxamid metabolite MET-22 was estimated to be 0.076 with MET-22 considered to be stable under conditions of anaerobic soil degradation.

Terrestrial field dissipation studies conducted in locations in Spain and France showed DT_{50} between 4.2-22.2 days. Pethoxamid metabolite MET-42, which was included in the analysis as well, could not be kinetically assessed owing to low occurrence.

Photochemical degradation

Direct photochemical degradation of pethoxamid was studied according to OECD TG 316 with radiolabelled substance. The degradation is fast in aqueous solution buffered at pH 7 when exposed to artificial sun light (average 20.3% AR after 16 days of continuous light exposure). The pethoxamid was stable in dark controls. Pethoxamid is degraded via reductive dechlorination, with subsequent oxidation to give pethoxamid metabolite MET-102. Further photolytic transformations produce the labile compound benzoic acid. The quantum yield of pethoxamid was determined to be 2.85×10^{-1}.

The rate of **photolysis** of pethoxamid radiolabbeled **on the soil surface** (OECD draft 2002) is relatively slow (*DegT50* of 53.0 days) and is not considered a significant dissipation pathway. Only minor metabolites (< 5 % of AR) are formed from the degradation of pethoxamid on soil under irradiation. None of them was identified.

Environmental fate and other relevant information

Long-range transport of pethoxamid is not expectable since the **half-life in air** (for a 12 hour day, 1.6×10^5 OH radicals / cm^3) is less than 2 days, estimated as 1.167 hours.

Pethoxamid is therefore unlikely to partition from the water phase to air **since Henry's Law Constant** of pethoxamid at 20 °C was calculated as 1.18×10^{-3} Pa m^3/mole.

Results from aged residue soil column leaching study has shown that pethoxamid can be classified as having a moderate potential for leaching in soil.

Pethoxamid has shown 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) in a field dissipation study. Pethoxamid metabolite MET-42 has demonstrated a significant potential to leach to 100 cm depth under the worst-case conditions of the study.

Estimated bioaccumulation

The partition coefficient between n-octanol and water, Log P_{ow} = 2.963 ± 0.02 at 20°C (pH 5) is determined using a shake flask method. For pethoxamid as surface active substances it may be difficult to obtain reliable partitioning (log Pow) or bioconcentration factor (BCF) used in performing environmental risk assessments.

Measured partition coefficient and bioaccumulation test data

Bioconcentration study was conducted in a flow-through system. Rainbow trout (*Oncorhyncus mykiss*) were exposed to a nominal concentrations of 1.5 and 15 µg/L ^{14}C-pethoxamid for a period of 28 days (uptake phase), followed by a period of 56 days in fresh water without test substance (depuration phase). Mean measured concentrations of ^{14}C-pethoxamid in the treatment tank was 1.64 and 16.1 µg/L for the nominal 1.5 and 15 µg/L treatments, respectively, indicating that the test substance remained stable in solution throughout the duration of the test. Based on total radioactivity, calculated kinetic BCFs (whole fish) were 50 and 47 in the low and high concentrations, respectively. Mean steady state BCFs (whole fish), normalised for lipid content, were reported as 28 and 32 in the low and high concentrations, respectively (calculated as the mean of days 7-28).

In the dRAR (2017), steady state is considered to have been reached after 14 days. Therefore, a mean steady state whole-fish BCFs of 33 is used, based on the average BCF after 14 days in the 15 µg/L treatment. It is noted in the dRAR (2017) that the reported BCF values were normalised for 6% lipid content and not 5%, as per current OECD Guideline requirements, however this deviation is not considered to impact the validity of the BCF value.

During the depuration period, mean concentrations of radioactivity in fish declined gradually. At the end of the 56 day depuration period, levels of radioactivity measured in fish were 5 – 26% of those measured on the last day of exposure. The reported DT_{90} (time for 90% depuration) was 64.4 and 50.9 days, in the 1.5 and 15 µg/L treatments, respectively. The dRAR (2017) states >90% depuration after 56 days, representing an average of the two values.

DS concluded that, pethoxamid does not meet the classification criteria as a bioaccumulative substance.

Acute aquatic hazard

The suitable acute aquatic toxicity studies for pethoxamid, reviewed under EU Regulation 1107/2009, conformed to GLP certification or used as supporting information is presented in the Table below.

***Table**: Summary of relevant information on acute aquatic toxicity*

Method	Species	Test material	Results[1]	Remarks	Reference
OECD TG 203 (1992)	*Oncorhynchus mykiss*	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No.: TB-960306-C	96 h LC_{50}: **2.2 mg/L** (mm)	Static renewal, mortality	151 PXA plus 151 PXA amdt-1 (1999a)
OECD TG 203 (1992)	*Lepomis macrochirus*	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No.: TB-960306-C	96 h LC_{50}: 6.6 mg/L (mm)	Static renewal, mortality	152 PXA plus 152 PXA amdt-1 (1999b)
OECD TG 203 (1992)	*Cyprinodon variegatus*	Pethoxamid techn., Purity: 95.8 % w/w. Batch No.:	96 h LC_{50}: 3.54 mg/L (mm)	Static, mortality	1177 PXA (2013)

Method	Species	Test material	Results[1]	Remarks	Reference
		P1351-JaK-T2-23-6			
OECD TG 202	*Daphnia magna*	Pethoxamid techn. (TKC-94), Lot-No.: TB-960306-C; purity: 94.8%	48 h EC_{50}: 23 mg/L (mm)	Static, immobility	155 PXA plus 155 PXA amdt-1 (1999a)
OPPTS 850.1035	*Americamysis bahia*	Pethoxamid techn., Purity: 95.8 % w/w. Batch No.: P1351-JaK-T2-23-6	96 h LC_{50}: 5.4 mg/L (mm)	Static, mortality	1176 PXA (2014a)
OPPTS 850.1025	*Crassostrea virginica*	Pethoxamid techn., Purity: 95.8 % w/w. Batch No.: P1351-JaK-T2-23-6	96 h EC_{50}: **3.28 mg/L** (mm)	Flow through, shell growth inhibition	1207 PXA (2014b)
OPPTS 850.1025	*Crassostrea virginica*	Pethoxamid techn., Purity: 96.2 % w/w. Batch No.: P1351-JaK-T2-23-6	96 h EC_{50}: 3.38 mg/L (mm)	Flow through, shell growth inhibition	1348 PXA (2014)
OECD TG 201	*Selenastrum capricornutum (Pseudokirch-neriella subcapitata)*	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No.: TB-960306-C	72 h E_rC_{50}[2] : **0.00408 mg/L** (mm)	Static, growth rate	158 PXA (1999b) and 158 PXA suppl.-1 (2016a) and 158 PXA suppl.-2 (2016c)
OECD TG 201	*Anabaena flos-aquae*	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No.: TB-960306-C	96 h E_rC_{50}[2] : 10.4 mg/L (mean measured)	Static, growth rate	157 PXA (1999c) and 157 PXA suppl.-1 (2016b) and 157 PXA suppl.-2 (2016d)

[1] Endpoints in bold are the critical endpoints for that organism group. The letters to the right of the numbers refer to what concentration of the active substance the endpoint is based on: n – nominal; mm – mean measured; im – initial measured

[2] Endpoints included in the RAR (2017) (based on re-calculation by 157 PXA suppl.-1 (2016b) and 158 PXA suppl.-2 (2016c), as indicated).

Acute toxicity to fish

The toxicity of pethoxamid to Rainbow trout (*Oncorhyncus mykiss*) was tested in a 96h acute toxicity study under static-renewal conditions. The fish were exposed to nominal pethoxamid concentrations of 1, 1.8, 3.2, 5.8 and 10 mg/L alongside a dilution water control, with daily water renewal. Corresponding mean measured concentrations were 1.1, 1.7, 2.5, 4.7 and 8.3 mg/L. The 96h LC_{50} for pethoxamid to *Oncorhyncus mykiss* was 2.2 mg/L (mean measured) with 95% confidence limits of 1.9 to 2.6 mg/L (151 PXA (1999a)).

The toxicity study of pethoxamid to Bluegill sunfish (*Lepomis macrochirus*) was conducted. The fish species were exposed to nominal pethoxamid concentrations of 1, 1.8, 3.2, 5.8, 10 and 18 mg/L in a 96h acute toxicity test, under static-renewal conditions. Corresponding mean measured

concentrations were 0.81, 1.6, 2.7, 5.1, 8.5 and 15 mg/L. Mortality in the control was less than 10% and dissolved oxygen, temperature and pH were maintained at acceptable levels throughout the test. The 96h LC_{50} for pethoxamid to *Lepomis macrochirus* was 6.6 mg/L (mean measured) with 95% confidence limits of 5.1 to 8.5 mg/L (152 PXA (1999b)).

In a 96h acute toxicity test Sheepshead minnow (*Cyprinodon variegatus*) were exposed to nominal pethoxamid concentrations of 0.65, 1.3, 2.5, 5.0 and 10 mg/L alongside a dilution water control under static conditions. Corresponding mean measured concentrations were 0.608, 1.2, 2.36, 4.94 and 10.2 mg/L, there were no mortalities in the control and dissolved oxygen, temperature and pH were maintained at acceptable levels throughout the test. The 96h LC_{50} for pethoxamid to *Cyprinodon variegatus* was 3.54 mg/L (mean measured) with 95% confidence limits of 3.3 to 3.8 mg/L (1177 PXA (2013)).

Acute toxicity to aquatic invertebrates

In a 48h acute toxicity test *Daphnia magna species were* exposed to nominal pethoxamid concentrations of 1, 1.8, 3.2, 5.8, 10, 18 and 32 mg/L alongside a dilution water control. Corresponding mean measured concentrations were 0.82, 1.6, 2.9, 5.1, 9.1, 17 and 29 mg/L, mortality in the control was less than 10% and dissolved oxygen, temperature and pH were maintained at acceptable levels throughout the test. The 48h EC_{50} for pethoxamid to *Daphnia Magna* was 23 mg/L (mean measured) with 95% confidence limits of 20 to 25 mg/L (155 PXA (1999a)).

The toxicity of pethoxamid to Mysid shrimp (*Americamysis bahia*) was studied in a 96h acute toxicity test performed under static conditions. Animals were exposed to nominal pethoxamid concentrations of 0.65, 1.3, 2.5, 5.0 and 10.0 mg/L alongside a dilution media control. Corresponding mean measured concentrations were 0.637, 1.26, 2.47, 4.96 and 10.3 mg/L. Mysids were observed at 24-h intervals for mortality and sub-lethal effects. Test conditions were acceptable. Based on mean measured concentrations, the 96h LC_{50} for pethoxamid to *Americamysis bahia* was 5.4 mg/L with 95% confidence limits of 4.62 to 6.31 mg/L (1176 PXA (2014a)).

Eastern oyster (*Crassostrea virginica*) species were exposed to nominal pethoxamid concentrations of 1.3, 2.2, 3.6, 6.0 and 10.0 mg/L alongside a dilution control in a 96h flow-through new shell growth test. Corresponding mean measured concentrations were 1.18, 2.01, 3.33, 5.53 and 9.53 mg/L. Test conditions were acceptable. Based on mean measured concentrations, the 96h EC_{50} for new shell growth was 3.28 mg/L with 95% confidence limits of 2.73 to 3.82 mg/L (1207 PXA (2014b)).

The toxicity of pethoxamid to Eastern oyster (*Crassostrea virginica*) was determined in a 96h flow-through new shell growth test. Animals were exposed to nominal pethoxamid concentrations of 0.42, 0.76, 1.4, 2.5, 4.4 and 8.0 mg/L alongside a dilution media control. Corresponding mean measured concentrations were 0.366, 0.668, 1.20, 2.12, 3.96 and 7.29 mg/L. The test conditions were acceptable. Based on mean measured concentrations, the 96h EC_{50} for new shell growth was 3.38 mg/L with 95% confidence limits of 3.03 to 3.74 mg/L (1348 PXA (2014)).

Acute (short-term) toxicity to algae or other aquatic plants

Two acute studies on algae species are available. The most sensitive aquatic species in acute tests was the green algae (*Selenastrum capricornutum*, also known as *Pseudokirchneriella subcapitata*) with a reported growth rate (E_rC_{50}), the preferred endpoint for classification purposes, of 0.00408 mg/L (158 PXA (1999b) and 158 PXA suppl.-1 (2016a)).

The toxicity of pethoxamid to green alga (*Selenastrum capricornutum, also known as Pseudokirchneriella subcapitata)* was studied in a 120h acute toxicity test performed under static

conditions with nominal pethoxamid exposure concentrations of 0.000625, 0.00125, 0.0025, 0.0050 and 0.010 mg/L alongside a culture medium control and a solvent control. Corresponding mean measured concentrations were 0.00058, 0.0012, 0.0024, 0.0046 and 0.0094 mg/L. Test conditions were controlled and acceptable. The validity criteria were met, according to the DS. For classification purposes, the 72h results are preferred, so the 72h EC_{50} and EC_{10} endpoints for biomass and growth rate have been subsequently recalculated from the original data. The linear regression using Probit was performed. Individual replicate responses were used for the regression analysis. The re-calculated 72h E_bC_{50} and E_rC_{50} were very similar to the original report, at 0.00206 and 0.00408 mg/L, respectively (with 95% confidence limits of 0.00182 to 0.00232 and 0.00341 to 0.00495 mg/L, respectively). These re-calculated values are considered acceptable in the dRAR. The acute E_rC_{50} of 0.00408 mg/L is considered by the DS reliable for acute classification purposes. (158 PXA (1999b) and 158 PXA suppl.-1 (2016a)).

The blue-green alga (*Anabaena flos-aquae*) species were exposed to nominal pethoxamid concentrations 2.2, 4.6, 10, 22 and 46 mg/L alongside a culture medium control in a 120h acute toxicity test performed under static conditions. Corresponding mean measured concentrations were 1.6, 3.8, 8.6, 20 and 41 mg/L. Analysing the increase of the cell density and the mean section-by-section specific growth rate, DS concluded that some of the control validity criteria were not met. Due to lack of reliable 72h endpoints, DS considered this study as supporting information only. The 96h endpoints used were re-calculated 96h E_bC_{50} and E_rC_{50} similar to these in the original report 8.99 and 10.4 mg/L, respectively (this is consistent with the conclusions in the dRAR, 2017). (157 PXA (1999c) and 157 PXA suppl.-1 (2016b).

Acute (short-term) toxicity to other aquatic organisms

No additional studies.

Long-term aquatic hazard

A summary of the suitable chronic aquatic toxicity studies for pethoxamid, as reviewed under EU Regulation 1107/2009, is presented in the following Table.

***Table**: Summary of relevant information on chronic aquatic toxicity*

Method	Species	Test material	Results[1]	Remarks	Reference
OECD TG 210	*Oncorhynchus mykiss*	Pethoxamid techn., Purity: 95.8 % w/w, re-analysed 96.2 %. Batch No.: P1351-JaK-T2-23-6	94 d (60 d post hatch) NOEC: **0.0924 mg/L** (mm)	Flow-through, fry survival	1451 PXA (2015)
OECD TG 211	*Daphnia magna*	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No.: TB-960306-C	21 d NOEC (survival, growth, repro): **2.8 mg/L** (mm)	Static renewal, survival, growth and reproduction	156 PXA (2000)
OECD TG 201	*Pseudokirchneriella subcapitata (Selenastrum capricornutum)*	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No.: TB-960306-C	72 h E_rC_{50}[2]: 0.00408 mg/L (mm) 72 h E_rC_{10}[2]: **0.00119 mg/L** (mm) 120 h NOEC[3] (biomass and growth ate): 0.0012 mg/L (mm)	Static, growth rate	158 PXA (1999b) and 158 PXA suppl.-1 (2016a) and 158 PXA suppl.-2 (2016c)
OECD TG 201	*Anabaena*	Pethoxamid techn. (TKC-94), Purity:	96 h E_rC_{50}[2]: 10.4 mg/L (mm)	Static, growth rate	157 PXA (1999c) and

Method	Species	Test material	Results[1]	Remarks	Reference
	flos-aquae	94.8 %. Lot-No.: TB-960306-C	96 h E_rC_{10}[2]: 8.39 mg/L (mm) 120 h NOEC[3] (biomass and growth ate): 3.8 mg/L (mm)		157 PXA suppl.-1 (2016b) and 157 PXA suppl.-2 (2016d)
OECD TG 221	*Lemna minor*	Pethoxamid techn. (TKC-94), Purity: 94.8 %. Lot-No.: TB-960306-C	14 d E_rC_{50}[2]: 0.0172 mg/L (mm) 14 d E_rC_{10}[2]: 0.0029 mg/L (mm) 14 d NOEC[3]: **0.001 mg/L** (mm)	Static renewal, growth rate	160 PXA (1999d) and 160 PXA suppl.-1 (2016e)

[1]

Endpoints in bold are the critical endpoints for that organism group. The letters to the right of the numbers refer to what concentration of the active substance the endpoint is based on: n – nominal mm – mean measured im – initial measured

[2]Endpoints included in the dRAR (2017) (based on re-calculation by Wenzel 2016b and 2016c, as indicated).

[3] Endpoints included in the original study report and reported in dRAR (2017).

Chronic toxicity to fish

The chronic toxicity study of pethoxamid to Rainbow trout was available. The fish species were exposed to nominal test concentrations 0.095, 0.19, 0.38, 0.75 and 1.5 mg/L, alongside a dilution control in a 94d test (60 days post-hatch) performed under flow-through conditions. Corresponding mean measured test concentrations were 0.0924, 0.177, 0.365, 0.722 and 1.44 mg/L. The validity criteria for this study were considered to have been met.

Statistically significant differences in post-hatch survival between the control and 0.177 and 0.365 mg/L concentrations were deemed to be not biologically relevant in the report because no significant effects were observed at 0.722 mg/L. However, in the dRAR it was proposed that these statistically significant effects should not be ignored. Therefore, based on mean measured concentrations and the most sensitive endpoint (post-hatch survival, before reduction) the chronic NOEC and LOEC for toxicity of pethoxamid to Rainbow trout were 0.0924 and 0.177 mg/L, respectively.

Chronic toxicity to aquatic invertebrates

The chronic toxicity of pethoxamid was studied for 21 days under static-renewal conditions. Groups of twenty-five *Daphnia Magna* (ten individually housed plus three groups of five) were exposed to nominal test concentrations 1.4, 3.1, 6.8, 15 and 32 mg/L, alongside a dilution control for. Corresponding mean measured test concentrations were 1.3, 2.8, 6.3, 13 and 29 mg/L). The validity criteria were met. Based on mean measured concentrations, the 21d NOEC for survival, growth and reproduction (used in the risk assessment) and EC_{10}, for reproduction, were 2.8 and 4.3 mg/L, respectively (156 PXA (2000)

Chronic toxicity to algae or other aquatic plants

There are three studies available on algae and aquatic plants.

DS concluded that the most sensitive aquatic species in chronic tests was the green algae (*Selenastrum capricornutum*, also known as *Pseudokirchneriella subcapitata*) with a reported 72h NOEC value of 0.0012 µg/L, based on biomass and growth rate, and the calculated 72h E_rC_{10} value of 0.00119 mg/L.

In this study the toxicity of pethoxamid to green alga was examined in a 120h acute toxicity test performed under static conditions with nominal pethoxamid exposure concentrations of 0.000625, 0.00125, 0.0025, 0.0050 and 0.010 mg/L alongside a culture medium control and a solvent control. Corresponding mean measured concentrations were 0.00058, 0.0012, 0.0024, 0.0046 and 0.0094 mg/L. Test conditions were controlled and acceptable. The validity criteria were met, according to the DS (158 PXA (1999b) and 158 PXA suppl.-1 (2016a)).

In the study on blue-green alga (*Anabaena flos-aquae*), the original 96h NOEC based on biomass and growth rate was 3.8 mg/L and the calculated 96h E_rC_{10} was 8.39 mg/L.

The chronic toxicity study of pethoxamid to freshwater aquatic plant *Lemna minor* was conducted for 14d under static renewal test system (draft OECD guideline (1984) **using 0.001**, 0.0032, 0.01, 0.032 and 0.1 mg/L, pethoxamid concentrations plus a dilution water and solvent control. Corresponding mean measured concentrations of pethoxamid were 0.001, 0.0029, 0.0091, 0.028 and 0.085 mg/L. Temperature and pH remained within acceptable levels throughout the test. The most sensitive parameter, the 14d NOEC and LOEC were determined as 0.001 and 0.0029 mg/L (root length), respectively. Experimental data were subsequently re-evaluated to determine EC_{10} and EC_{20} values for growth rate (frond number), yield and biomass. The 14 d E_rC_{10} for growth rate (the preferred endpoint for classification purposes) was 0.0029 mg/L (confidence limits 0.00203 – 0.0038 mg/L). However, in the study, the frond number increased 6.4 and 5.8-fold within days 0 to 7 respectively in the control and solvent control group, instead 7-fold within 7 days as recommended in the test guidelines. Although overall the growth reached >30-fold in 14 days, in the dRAR (2017) evaluation, this study was deemed 'borderline acceptable' (160 PXA (1999d) and 160 PXA suppl.-1 (2016e)).

Chronic toxicity to other aquatic organisms

No additional studies.

Comparison with the CLP criteria

The DS has concluded that relevant acute aquatic toxicity data on pethoxamid are available for all three trophic levels and among them algae is the most acutely sensitive trophic group. The lowest reliable acute value, the 72h mean measured EC_{50} of 0.00408 mg/L for *Selenastrum capricornutum (Pseudokirchneriella subcapitata* classification Aquatic Acute 1, with M-factor of 100.

The DS has considered pethoxamid as not rapidly degradable and with a low potential for bioaccumulation based on experimentally determined steady state BCF of 33 kg/L.

Chronic aquatic toxicity data on pethoxamid are available for all three trophic levels and among them algae and aquatic plants are the most sensitive group with lowest 14d NOEC of 0.001 mg/L (root length) for *Lemna minor* and 72h NOEC of 0.0012 mg/L (biomass and growth rate) for *Selenastrum capricornutum (Pseudokirchneriella subcapitata*); the lowest 14d E_rC_{10} for growth rate is 0.0029 mg/L for *Lemna minor and 72-h* E_rC_{10} for biomass and growth rate is 0.00119 mg/L for *Selenastrum capricornutum* (*Pseudokirchneriella subcapitata*).

The DS accepted that E_rC_{10} for growth rate is the preferred endpoint for classification purposes and concluded that based on the lowest 72-h E_rC_{10} for *Selenastrum capricornutum* (*Pseudokirchneriella subcapitata*) of 0.00119 mg/L pethoxamid should be classified as Aquatic Chronic 1 with a chronic M-factor of 10 ($0.01 \geq E_rC_{10} > 0.001$), for a non-rapidly degradable substance.

The DS concluded on the classification and labelling for environmental hazards as follows:

- Aquatic Acute 1; H400: Very toxic to aquatic life, M-factor = 100
- Aquatic Chronic 1; H410: Very toxic to aquatic life with long lasting effects, M-factor = 10

Comments received during consultation

Two MSs, one national authority and one company manufacturer supported the proposed aquatic hazard classification and the proposed M-factors. One national authority noted that pethoxamid undergoes significant primary degradation, and the ecotoxicity data are available for some of the obtained metabolites and asked DS to consider if this can impact hazard classification and associated M-factors. One MS propose to DS to add additional summary of the results of the OECD 308 study in the table for substance degradability. MS agreed that long-term aquatic hazard should be preferably based on the E_rC_{10} and support the proposed classification as Aquatic Chronic 1 with an M-factor of 10, as well as the classification as Aquatic Acute 1 with an M-factor of 100.

The DS noted the comments and considered the proposal on significant primary degradation of pethoxamid, however as the substance clearly does not meet the CLP-criteria of "rapidly degradable" due to environmental fate and behaviour, DS was of the opinion that the toxicity of metabolites would have no impact on the hazard classification and M-factor.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the DS to consider pethoxamid as 'not rapidly degradable', as based on the following information:

- A readily biodegradability study (144 PXA, 1999), conducted according to the OECD Test Guideline 301B, without identified major deficiencies.
- Pethoxamid does not hydrolyse at any pH.

Based on data for primary degradation from the surface water simulation study, the substance undergoes rapid primary degradation. However, as no adequate data are available for all hydrolysis products, it cannot be excluded that the criteria for classification as hazardous to the aquatic environment are not met for these hydrolysis products. Furthermore, the data for the metabolite MET-6 (7-d EC50= 0.778 mg/L) indicates that it is classifiable. Therefore, the substance cannot be regarded as rapidly degradable for classification via primary degradation.

Bioaccumulation

RAC support DS to consider pethoxamid as a substance with low potential for bioaccumulation based on experimentally determined BCFs - mean steady state whole-fish BCFs of 33 (Log Kow = 2.96 at 20°C (pH 5)).

Acute aquatic hazard

Acute aquatic toxicity data on pethoxamid are available for all three trophic levels and among them algae are the most acutely sensitive trophic group. The lowest reliable acute value, the 72h mean measured EC_{50} of 0.00408 mg/L for *Selenastrum capricornutum (Pseudokirchneriella subcapitata.* RAC support DS to classify pethoxamid in category Aquatic Acute 1, with M-factor of 100.

Chronic aquatic hazard

Pethoxamid is not rapidly degradable. Chronic aquatic toxicity data on pethoxamid are available for all three trophic levels and among them algae and aquatic plants are the most sensitive group with lowest 14d NOEC of 0.001 mg/L (root length) for *Lemna minor* and 72-h NOEC of 0.0012 mg/L (biomass and growth rate) for *Selenastrum capricornutum (Pseudokirchneriella subcapitata*); the lowest 14d E_rC_{10} for growth rate is 0.0029 mg/L for *Lemna minor and 72-h* E_rC_{10} for biomass and growth rate is 0.00119 mg/L for *Selenastrum capricornutum* (*Pseudokirchneriella subcapitata*).

In line with the current CLP Guidance, preference is given to EC_{10} values for the chronic hazard classification over NOEC values and so EC_{10} values have been used where appropriate.

RAC support DS to classify pethoxamid in category Aquatic Chronic 1 with M-factor 10. The lowest NOEC is 0.001 mg/L for *Lemna minor,* determining an M-factor 100 for chronic toxicity, but the reliability of the study is questionable. That is why RAC agree with DS for M-factor 10 based on 72-h EC_{10} of 0.00119 mg/L for (biomass and growth rate) for *Selenastrum capricornutum (Pseudokirchneriella subcapitata*).

Comparison with the criteria

In conclusion, RAC agrees with the DS that penthoxamid **warrants classification as Aquatic Acute 1; H400, M = 100 and Aquatic Chronic 1; H410, M = 10.**

RAC evaluation of hazards to the ozone layer

Summary of the Dossier Submitter's proposal

Short summary and overall relevance of the provided information on ozone layer hazard

Pethoxamid vapour pressure and Henry's law constant are 2.8 x 10^{-3} Pa at 25°C and 1.18 x 10^{-3} Pa m³/mol at 20°C respectively.

The potential for long-range transport of pethoxamid is limited based on the short atmospheric half-life of pethoxamid of 1.167 hours, estimated using the program AOPWIN (v 1.92) (1423 PXA, 201e). DS concluded that it is highly unlikely that pethoxamid can deplete the stratospheric ozone layer.

Pethoxamid is not listed in Annex I to Regulation (EC) No. 1005/2009 (recognising the Montréal Protocol) and no Ozone Depleting Potential (ODP) is reported.

Comparison with the CLP criteria

DS concluded that properties of pethoxamid such as short atmospheric half-life and low vapour pressure and Henry's law constant do not fulfil CLP guidance criteria to be classified as hazardous to the Ozone Layer in Category 1. The observed environmental fate and behaviour do not indicate that it may present a danger to the structure and/or the functioning of the stratospheric ozone layer.

Comments received during consultation

No comments have been received.

Assessment and comparison with the classification criteria

No classification, observed environmental fate and behaviour of pethoxamid do not indicate that it may present a danger to the structure and/or the functioning of the stratospheric ozone layer.

RAC supports the DS on the conclusion for n**o classification of pethoxamid as hazardous for the ozone layer.**

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).