Renewal Assessment Report

under Regulation (EC) 1107/2009

Zoxamide

Volume 3

Active substance
B.6 Toxicology and Metabolism data

Rapporteur Member State: Latvia
Co-Rapporteur Member State: France

Version history

Date	Subject
May 2001	Initial DAR. Draft Assessment Report (DAR) – prepared in the context of the application for the first inclusion of the a.s. in Annex I to Council Directive 91/414/EEC.
July 2016	Renewal Assessment Report (RAR) – prepared in the context of the application for renewal of approval of the a.s. according to Regulation (EC) No 1107/2009. Note: The RAR is a stand-alone document containing the evaluations already displayed in the original DAR dated May 2001 considered as relevant, as well as the new assessments submitted for the Renewal in 2014. These new studies are evaluated and summarized below, under the relevant points of this report, together with the previously evaluated studies.

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B. SUMMARY OF THE DATA AND INFORMATION

B.6 TOXICOLOGY AND METABOLISM DATA

Introduction

Zoxamide is a non-systemic fungicide belonging to the benzamide group of compounds. It is intended to protect against oomycete diseases such as Phytophthora infestans (late blight of potato) and Plasmopara viticola (downy mildew of grapevines). Zoxamide inhibits germ tube development and mycelium growth by inhibiting cell division. As a result, the fungal organism dies.

Zoxamide has previously been evaluated and was included in the Annex I of the Council Directive 2003/119/EC concerning placing of plant protection products on the market (91/414/EEC) in 2003. This document presents data and information on the metabolism and toxicology of zoxamide submitted in support of the renewal of approval of zoxamide under Regulation (EC) 1107/2009. Most of the data presented were also submitted to secure the first inclusion of zoxamide in Annex I to Directive 91/414/EEC. The evaluation of these data was presented in the Draft Assessment Report (DAR) for zoxamide (United Kingdom, 2001) and in 3 addenda. The critical endpoints for use in risk assessment were published in the Review Report for the active substance zoxamide (SANCO/10297/2003-Final).

In this report new data for the renewal of the approval of Zoxamide has been evaluated only. Studies and investigations already assessed within the EU DAR (2001) have been re-evaluated in this report. The conclusions have been updated to meet current scientific standards.

This document covers hazard and risk assessments which were not part of the original dossier and which are necessary to reflect changes

· in requirements under Annex II and III to Directive 91/414/EC;

· in scientific and technical knowledge since the first inclusion;

· to representative uses.

B.6.1 Absorption, distribution, metabolism and excretion in mammals

B.6.1.1 Absorption, distribution, metabolism and excretion by oral route

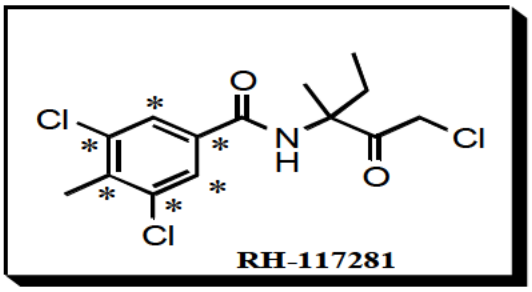
In DAR May (2001)

Reference:	CA 5.1.1/01, [redacted] (1998a)
Title:	^{14}C-RH-117,281: Pharmacokinetic and metabolism study in rats.
Report number:	No: 94R-235, ER Ref No: 24.1
Guidelines:	OECD 417 ≅ OPPTS 870.7485 ≅ JMAFF 4200
GLP:	Yes

ADME studies submitted for the original Annex I inclusion were conducted with zoxamide, labelled uniformly with 14C in the phenyl ring.

In a GLP compliant study (1998) the balance and excretion patterns of phenyl ring-labelled RH-7281 (Figure B.6.1-1), ^{14}C- and ^{13}C-labelled 3,5-dichloro-N-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-4-methylbenzamide (RH-7281) and the amount of residual radioactivity in blood, organs and tissues after oral administration to male and female Sprague-Dawley Crl:CDBR rats (220 – 426 g) was determined.

Figure B.6.1-1 Structure of RH-7281 (zoxamide, RH-117,281)



* - Uniformly labelled with ^{14}C in the phenyl ring

Twenty-one (21) groups of animals (3-6/sex/group) were administered by gavage ^{14}C-RH 7281, at nominal doses of 10 (low dose) or 1000 (high dose) mg/kg bw RH 7281. The test materials were prepared as suspensions in corn oil (5 ml/kg). For all groups, the ^{14}C-RH 7281 was combined with appropriate amounts (by weight) of nonradiolabelled RH 7281. In addition, ^{13}C-labelled RH 7281 was also added to appropriate low dose groups and the bile groups.

The test substances used in this study were:

1. ^{12}C-RH 7281: Lot No. DK2011, 94.2% a.i.
2. ^{12}C-RH 7281: Lot No. LG3517, 92.9% a.i.
3. ^{13}C-RH 7281: Lot No. CLM-3762/P-6909, 96.8% a.i.
4. ^{14}C-RH 7281: Lot No. 825.0102, Specific Activity 90.20 mCi/g, Radiopurity 98.7%
5. ^{14}C-RH 7281: Lot No. 891.0203, Specific Activity 52.87 mCi/g, Radiopurity 97.6%
6. ^{14}C-RH 7281: Lot No. 891.0402, Specific Activity 45.8 mCi/g, Radiopurity 99.5%

The following experiments were conducted to:

i) determine the excretion, distribution and mass balance of radioactivity (120 hr postdose) in Groups A & B (low dose) and C & D (high dose);

ii) evaluate the pharmacokinetics of radioactivity in blood (to determine the time to C_{max} and ½ C_{max}) in Groups 1, 2 (investigated for expired air) and 3,

iii) determine the tissue distribution of radioactivity at C_{max} and ½ C_{max} in Groups I - P,

iv) investigate repeat dose effects - animals in Groups E & F received a dietary treatment of 200 ppm nonradiolabelled RH 7281 technical for two weeks before receiving a single oral dose of 10 mg/kg bw ^{14}C-RH 7281, and a further set of animals, Groups G & H, received 5 consecutive daily doses of 10 mg/kg bw ^{14}C-RH 7281.

The excretion of radioactivity from a single oral dose of ^{14}C-RH 7281 followed similar patterns, independent of the administered dose level. Over 85 % of the administered radioactivity was excreted during the first 24 - 48 hours after dosing. The major route for the elimination of radiolabel was in the faeces, which contained 74 to 92 % of the administered dose. The remaining radioactivity (4 to 27 %) was excreted in the urine. Very little radioactivity remained in tissues (0.04 to 0.17 % of dose) or carcass (0.34 to 1.9 % of dose) at 5 days postdose indicating that RH-7281 does not accumulate. No radioactivity was recovered as either $^{14}CO_2$ or volatile organic compounds. Pre-treatment of animals with unlabeled RH-7281 in the diet for two weeks or five daily gavage ^{14}C-doses did not significantly alter the absorption or distribution of ^{14}C-RH 7281 compared to untreated, single ^{14}C-dosed animals (Table B.6.1.1-1).

Table B.6.1.1-1. Balance of the distribution of radiolabel in excreta, blood, tissues and residual carcass (% of Administered Dose Recovered)

Group	Dose (mg/kg)	Route[a]	Sex	Time of Sacrifice	Percent of Radioactive Dose					
					Urine[d]	Faeces	Blood	Tissues	Carcass	Total[e]
2	1000	Oral	M	7 Days	6.42	94.14	0.01	0.02	0.30	100.88[f]
2	1000	Oral	F	7 Days	11.18	84.44	0.01	0.02	0.33	95.98[f]
C	1000	Oral	M	5 Days	3.51	92.37	0.00	0.04	0.34	96.26
D	1000	Oral	F	5 Days	8.15	88.82	0.01	0.05	0.55	97.46
A	10	Oral	M	5 Days	10.29	87.78	0.01	0.16	1.86	100.10
B	10	Oral	F	5 Days	26.85	73.50	0.02	0.17	1.87	102.41
Q	10	Oral (Bile)	M	72 Hours	9.49	32.21	0.01	0.18	2.98	90.80[g]
R	10	Oral (Bile)	F	72 Hours	12.04	33.94	0.01	0.14	2.79	96.73[g]
E	10	Pulse[b]	M	5 Days	16.27	78.57	0.02	0.58	3.41	98.86
F	10	Pulse[b]	F	5 Days	28.72	71.05	0.02	0.19	1.55	101.53
G	10	Repeat[c]	M	C_{max} 5th dose	7.89	60.34	0.03	2.69	17.77	96.28[h]
H	10	Repeat[c]	F	C_{max} 5th dose	19.95	51.65	0.04	3.27	12.65	92.99[h]

[a] - All animals except those in Groups G and H were administered a single gavage dose (mg/kg bw) at a constant volume of 5 ml/kg bw.

[b] - These animals received 200 ppm nonradiolabeled RH 7281 in the diet for two weeks prior to receiving a single pulse oral dose.

[c] - These animals received 5 consecutive daily oral gavage doses and were sacrificed at the C_{max} time point (8 hrs postdose) on Day 5 of dosing.

[d] - Includes urine, urine funnel wash and urine cage wash.

[e] - Mean total percent of administered dose reflects the mean and standard deviation of the individual animals.

[f] - No radioactivity was recovered as either $^{14}CO_2$ or volatile organic compounds.

[g] - Bile accounted for 45.84 to 47.76% of dose for males and females, respectively. Stomach contents, stomach wash, and intestinal tract contents and wash accounted for 0.09 to 0.06% of dose for males and females, respectively.

[h] - Stomach contents, stomach wash, and intestinal tract contents and wash accounted for 7.55 to 5.42% of dose for males and females, respectively.

In pharmacokinetic studies, ^{14}C-RH 7281 was observed to be rapidly absorbed by rats. The maximum concentrations of radioactivity in plasma were observed at 8 hours postdose (C_{max} in plasma = 8 hrs; ½ C_{max} = 22 hrs). Peak blood and tissue levels were noted to be low. Elimination of radiolabel from plasma followed a biphasic pattern. The overall elimination half-life of ^{14}C-label in plasma was essentially similar (12 to 14 hrs) in both low and high dose animals of both sexes (Table B.6.1.1-2).

Table B.6.1.1-2 Pharmacokinetic Half-lives ($T_{1/2}$) of ^{14}C-Label. Elimination and Peak Concentration in Plasma for Rats Dosed Orally With ^{14}C-RH 7281 (Blood Pharmacokinetics Groups)[a]

Group	Dose (mg/kg)	Sex	Elimination Half-life[b] (hr)	Alpha-Phase Half-life[c] (hr)	Beta-Phase Half-life[d] (hr)	Peak Concentration[e] (ppm)	AUC[f] (ppm ∗ hr)
1	1000	**M**	11.7	5.5	100.5	31.67	1359.8
1	1000	**F**	13.8	6.3	106.6	43.34	1882.4
3	10	**M**	14.0	5.6	70.2	0.62	26.0
3	10	**F**	13.1	6.6	163.6	0.98	44.5

[a] - Elimination rates calculated for a two compartment pharmacokinetic model (PK Analyst® Model #13) with first order input and first order output (elimination):

$$Conc(Time) = A \bullet e^{-\alpha \bullet Time} + B \bullet e^{-\beta \bullet Time} + C \bullet e^{-K_{AB} \bullet Time}$$

[b] - Half-life of the systemic elimination rate from the body

[c] - Half-life of the initial rapid-phase distribution and elimination of plasma radioactivity

[d] - Half-life of the later slower elimination of plasma radioactivity

[e] - Maximum peak plasma concentrations occurred at the 8 hour time point

[f] - Area Under Curve (AUC)

The concentrations of radioactivity in the tissues were highest in the organs associated with oral absorption [liver, stomach, intestines, and carcass (which included the caecum)]. The tissue distribution results were consistent with the pharmacokinetic data and indicated that radioactivity was rapidly cleared from tissues (Table B.6.1.1-3).

Table B.6.1.1-3 Mean Concentration of Radioactivity in Blood, Carcass, Plasma and Tissues at Specified Times Postdose

	µg Equivalents ^{14}C-RH 7281/g tissue																	
	1000 mg/kg		**1000 mg/kg**		**1000 mg/kg**		**1000 mg/kg**		**10 mg/kg**		**10 mg/kg**		**10 mg/kg**		**10 mg/kg**		**10 mg/kg**	
	C_{max}		1/2 C_{max}		5 Days		7 Days		C_{max}		1/2 C_{max}		5 Days		5 Days		C_{max} - 8 Hrs	
	8 Hrs		22 Hrs		Postdose		Postdose		8 Hrs		22 Hrs		Postdose		Postdose[a]		5th Dose[b]	
Tissue	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Adrenals	59	185	16	33	0.88	1.4	NC	NC	0.51	0.66	0.87	0.93	0.03	0.05	0.13	0.08	1.2	1.8
Bone Marrow	14	22	6.5	7.5	0.28	1.0	5.3	0.85	0.19	0.20	0.13	0.17	0.01	0.02	0.09	0.07	0.22	0.39
Brain	2.5	4.2	0.48	1.2	0.17	0.21	NC	NC	0.04	0.03	0.01	0.02	0.00	0.00	0.00	0.00	0.04	0.07

Carcass (residual)	805	727	88	159	3.3	5.9	3.7	4.7	5.8	3.9	2.5	4.0	0.20	0.21	0.42	0.20	11	8.9
Fat	7.5	14	3.7	9.5	0.21	0.35	NC	NC	0.11	0.23	0.09	0.14	0.01	0.01	0.02	0.01	0.17	0.52
Heart	19	31	4.4	8.5	0.41	0.66	NC	NC	0.20	0.25	0.10	0.16	0.02	0.03	0.03	0.03	0.30	0.53
Intestinal Tract	1779	1826	257	375	7.9	15	12	10	33	33	6.4	6.1	0.36	0.44	2.8	0.65	29	20
Kidneys	120	177	18	31	1.3	2.5	3.0	4.7	1.7	2.1	0.41	0.59	0.06	0.09	0.13	0.13	1.9	4.2
Liver	879	1131	71	175	2.7	4.1	4.2	4.3	15	25	2.5	4.1	0.11	0.16	0.28	0.19	15	32
Lungs	29	41	6.4	11	0.55	0.90	NC	NC	0.29	0.39	0.13	0.19	0.02	0.03	0.05	0.04	0.41	0.69
Muscle (thigh)	6.7	9.6	2.0	3.5	0.18	0.33	NC	NC	0.08	0.07	0.04	0.06	0.01	0.01	0.01	0.01	0.12	0.18
Ovaries	NA	34	NA	12	NA	1.1	NA	NC	NA	0.65	NA	0.21	NA	0.04	NA	0.04	NA	0.56
Plasma	50	64	11	17	0.51	1.0	NC	NC	0.53	0.73	0.20	0.30	0.02	0.04	0.05	0.05	0.76	1.0
Spleen	21	28	5.3	8.2	0.49	1.0	0.88	1.5	0.19	0.21	0.09	0.16	0.02	0.03	0.05	0.05	0.28	0.53
Stomach	4846	1750	23	93	0.34	0.72	0.91	1.6	14	20	1.0	0.44	0.02	0.02	0.06	0.02	8.5	9.1
Testes	7.7	NA	2.1	NA	0.18	NA	NC	NA	0.09	NA	0.06	NA	0.01	NA	0.02	NA	0.16	NA
Thyroids	30	39	13	23	0.74	1.7	NC	NC	0.34	0.43	0.24	0.29	0.02	0.05	0.21	0.15	0.57	0.81
Whole Blood	38	49	12	18	1.6	2.3	5.3	4.6	0.49	0.55	0.22	0.31	0.05	0.08	0.12	0.11	0.73	1.0

Note: Values below 0.01 have been recorded as 0.00.

[a] - These animals received 200 ppm nonradiolabeled RH 7281 in the diet for two weeks prior to receiving a single pulse oral dose.

[b] - These animals received 5 consecutive daily oral gavage doses and were sacrificed at the C_{max} time point (8 hrs postdose) on Day 5 of dosing.

NA - Not Applicable
NC - Not Collected

Biliary excretion of radioactivity from a single oral dose of 10 mg/kg bw ^{14}C-RH 7281 was rapid. The majority of the dose was recovered within 12 hours postdose. Based on the recovery of radioactivity from the bile, blood, urine, tissues and carcasses, 59 – 63 % of the administered oral dose of ^{14}C-RH 7281 was considered to be systemically absorbed.

Metabolism

Urine, faeces and bile samples underwent metabolite identification and quantification. Urine, faeces, and bile samples from ^{14}C-RH 7281 dosed rats were pooled by gender and dose group to create composite samples containing greater than 90 % of the excreted radiolabel. In most cases this was accomplished by pooling the samples collected on days 1, 2, and 3 only. For bile, collection times up to 48 hours were pooled. A total of 6 urine, 6 faeces, and 2 bile samples were analyzed.

Faeces were studied by extracting homogenates with methanol, then analyzing the methanol fractions by reverse phase high performance liquid chromatography (RP-HPLC) and normal phase thin layer chromatography (NP-TLC). Urine samples were filtered and examined directly by RP-HPLC and NP-TLC. Bile was analyzed directly by RP-HPLC and liquid chromatography/mass spectroscopy (LC/MS). The amounts of metabolites were quantified using liquid scintillation counting (LSC) of collected HPLC fractions, except for two benzoic acid metabolites RH-141,455 and RH-141,452, which were quantified from the high dose group samples by GC/ECD using a non-radiolabelled method. Isolated metabolites were characterized and identified by TLC, gas chromatography/mass spectroscopy analyses (either as derivatised or underivatised metabolites), and/or LC/MS with electrospray (ESI) or atmospheric chemical ionization (APCI), and/or by comparison to authentic reference standards.

Within each dose group, the results for the excretion, pharmacokinetics, and tissue distribution of radioactivity were generally comparable for males and females, although female rats excreted a slightly higher proportion of the dose in the urine as compared to males. The overall mass balance ranged from 91 to 102 % of the administered dose in the main study groups used for metabolite identification.

RH 7281 was found to be extensively metabolised. Including parent compound, a total of 36 metabolites were found in the faeces and urine; 24 of these were identified. In bile, 17 products were detected and 13 were identified. Altogether 32 structures were determined. No single metabolite other than parent RH 7281 accounted for **more than 10 %** of the administered dose. RH 7281 was observed in faeces at 12 to 23 % of the 10 mg/kg dose and 72 to 74 % of the 1000 mg/kg dose (female versus male values, respectively).

The single most abundant metabolite in excreta, found at 1.0 to 7.4 % of the administered dose in the various groups, was **RH-141,643**, which is the product of hydrolysis and subsequent side chain oxidations (Table B.6.1.1- 4 a-d). Other major identified products found in the urine and faeces included parent RH 7281; hydrolysis product RH-141,288 and dehalogenation product RH-127,450; subsequent oxidation products of them: RH-141,454, M-10A, M-9, M-16; subsequent sugar conjugate of RH-141,454, M-18; glutathione derivatives M-5, M-8B, M-13, and M-15; and subsequent oxidation products of them: M-8A and M-10B. Altogether these major metabolites, some of them measured in mixtures, accounted for 64 % of the dose in the low dose male group and 59 % of the dose in the low dose female group.

In bile, the single most abundant derivative at 7.8 - 8.6 % of the administered dose was M-25, the glucuronic acid conjugate of hydrolysis product RH 141288 (M-4). Other major components were glutathione derivative metabolites M-13, M-14A, M-26, M-27, M-28, -M-29; glucuronides M-18, M-19,

and M-14A; and the oxidation product RH163353 (M-23). Together these accounted for 82 % and 85 % of the total radioactivity found in bile in female and male groups respectively and 39 % of the administered dose (Table B.6.1.1- 4d).

Each individual metabolite which accounted for 5 % or more of the administered dose was identified with the exceptions of two faecal unknowns in broad retention time fractions in the low and repeat low dose groups, and two unidentified metabolites M-11A and M-11B which combined accounted for 7.8 % of the administered dose in the repeat low dose males. These metabolites were not identified because there was insufficient sample available for analysis.

Table B.6.1.1- 4a. Distribution of the Metabolites after a low dose (10 mg/kg bw) as % of applied dose (Groups A and B)

	% of Dose			% of Dose		
	Low Dose Male - A			**Low Dose Female - B**		
Metabolite	Faeces	Urine	Subtotal	Faeces	Urine	Subtotal
M-1 (RH 7281)	22.96		22.96	12.10		12.10
M-2 (RH-127,450)	2.75		2.75	2.43		2.43
M-3 (RH-141,643)	7.44		7.44	4.93		4.93
M-4 (RH-141,288)	2.84		2.84	3.15		3.15
M-5	2.46		2.46	1.54		1.54
M-7 (RH-141,454)	5.74		5.74	3.73		3.73
M-8A, M-8B, and M-15	3.36	0.70	4.06	2.94	5.05	7.99
M-9	4.14		4.14	3.58		3.58
M-10A, M-10B, M-16, M-17 (RH-141,452), and M-18	7.47	4.30	11.77	9.14	4.93	14.07
M-12		0.11	0.11		1.09	1.09
M-13					5.06	5.06
M-14A and M-14B		0.55	0.55		1.27	1.27
M-19 and M-20		1.78	1.78		2.65	2.65
M-21A and M-21B		0.94	0.94		1.02	1.02
unknown faeces met 15-16 min	0.20		0.20	0.36		0.36
unknown faeces met 16-19.5 min[a]	4.78		4.78	3.96		3.96
unknown faeces met 29-30.5 min[b]	0.90		0.90	0.60		0.60
unknown faeces met 38-44.5 min	5.69		5.69	5.47		5.47

unknown faeces met 50.5-65 min	5.32		5.32	4.06		4.06
unknown urine met 10-14 min		0.13	0.13			
unknown urine met 14.5-15 min					0.13	0.13
unknown urine met 27-31 min		0.25	0.25		1.03	1.03
unknown urine met 31-33 min		0.07	0.07		0.33	0.33
unknown urine met 33-35.5 min					0.42	0.42
unknown urine met 40.5-42 min					0.20	0.20
Sum of identified metabolites	59.16	8.38	67.54	43.54	21.07	64.61
Total Metabolite Distribution	76.05	8.83	84.88	57.99	23.18	81.17
% of Administered Dose Submitted for Analysis	87.78	8.84	96.62	73.50	23.17	96.67
Total % of Administered Dose	87.78	10.29*	98.07	73.50	26.85*	100.35

* - This includes urine, urine funnel wash and urine cage wash. Urine funnel wash and urine cage wash were not analysed for metabolites.

[a] - Also designated, M-11A and M-11B.

[b] - Also designated, M-6.

Table B.6.1.1- 4b Distribution of the Metabolites in high dose (1000 mg/kg dose groups C and D) as percentage of applied dose

	% of Dose			**% of Dose**		
	High Dose Male - C			High Dose Female - D		
Metabolite	Faeces	Urine	Subtotal	Faeces	Urine	Subtotal
M-1 (RH 7281)	73.61		73.61	71.61		71.61
M-2 (RH-127,450)	1.21		1.21	1.16		1.16
M-3 (RH-141,643)	1.77		1.77	1.04		1.04
M-4 (RH-141,288)	0.87		0.87	1.88		1.88
M-5	0.79		0.79	0.51		0.51
M-7 (RH-141,454)	0.64		0.64	0.61		0.61
M-8A, M-8B, and M-15	0.93	0.21	1.14	0.54	1.17	1.71
M-9	0.99		0.99	0.50		0.50
M-10A, M-10B, M-16, M-17						

(RH-141,452), and M-18	0.97	1.49	2.46	1.51	1.44	2.95
M-12		0.02	0.02		0.16	0.16
M-13					2.17	2.17
M-14A and M-14B		0.16	0.16		0.39	0.39
M-19 and M-20		0.35	0.35		0.59	0.59
M-21A and M-21B		0.20	0.20		0.39	0.39
Unknown faeces met 15-16 min						
Unknown faeces met 16-19.5 min[a]	1.24		1.24	0.63		0.63
Unknown faeces met 29-30.5 min[b]	0.17		0.17	0.10		0.10
Unknown faeces met 38-44.5 min	1.66		1.66	1.38		1.38
Unknown faeces met 50.5-65 min	3.89		3.89	3.84		3.84
Unknown urine met 10-14 min		0.09	0.09		0.09	0.09
Unknown urine met 14.5-15 min		0.07	0.07		0.03	0.03
Unknown urine met 27-31 min		0.44	0.44		0.49	0.49
Unknown urine met 31-33 min		0.04	0.04		0.11	0.11
Unknown urine met 33-35.5 min					0.15	0.15
Unknown urine met 40.5-42 min		0.01	0.01		0.10	0.10
Sum of identified metabolites	81.78	2.43	84.21	79.36	6.31	85.67
Total Metabolite Distribution	88.74	3.08	91.82	85.31	7.28	92.59
% of Administered Dose Submitted for Analysis	92.37	3.07	95.44	88.82	7.27	96.09
Total % of Administered Dose	92.37	3.51*	95.88	88.82	8.15*	96.97

* - This includes urine, urine funnel wash and urine cage wash. Urine funnel wash and urine cage wash were not analyzed for metabolites.

[a] - Also designated, M-11A and M-11B.

[b] - Also designated, M-6.

Table B.6.1.1- 4c Distribution of Metabolites after repeat dietary dosing with 10 mg/kg bw (Groups E and F)

	% of Dose	**% of Dose**

	Pulse Dose Male - E			Pulse Dose Female - F		
Metabolite	Faeces	Urine	Subtotal	Faeces	Urine	Subtotal
M-1 (RH 7281)	5.56		5.56	5.84		5.84
M-2 (RH-127,450)	2.64		2.64	2.28		2.28
M-3 (RH-141,643)	5.30		5.30	5.37		5.37
M-4 (RH-141,288)	4.02		4.02	6.43		6.43
M-5	2.09		2.09	1.76		1.76
M-7 (RH-141,454)	6.19		6.19	2.79		2.79
M-8A, M-8B, and M-15	2.22	1.38	3.60	3.84	3.08	6.92
M-9	5.12		5.12	3.78		3.78
M-10A, M-10B, M-16, M-17 (RH-141,452), and M-18	9.42	5.90	15.32	8.12	3.64	11.76
M-12		0.75	0.75		1.58	1.58
M-13		0.32	0.32		9.64	9.64
M-14A and M-14B		1.67	1.67		1.71	1.71
M-19 and M-20		2.46	2.46		2.26	2.26
M-21A and M-21B		1.34	1.34		1.48	1.48
unknown faeces met 15-16 min	0.64		0.64	0.67		0.67
unknown faeces met 16-19.5 min[a]	7.79		7.79	4.78		4.78
unknown faeces met 29-30.5 min[b]	0.41		0.41	0.75		0.75
unknown faeces met 38-44.5 min	5.23		5.23	5.91		5.91
unknown faeces met 50.5-65 min	8.57		8.57	6.18		6.18
unknown urine met 10-14 min		0.19	0.19		0.64	0.64
unknown urine met 14.5-15 min		0.37	0.37		0.33	0.33
unknown urine met 27-31 min		0.74	0.74		0.88	0.88
unknown urine met 31-33 min		0.11	0.11		0.49	0.49
unknown urine met 33-35.5 min					0.40	0.40
unknown urine met 40.5-42 min		0.06	0.06		0.33	0.33
Sum of identified metabolites	42.56	13.82	56.38	40.21	23.39	63.60
Total Metabolite Distribution	65.20	15.29	80.49	58.50	26.46	84.96

% of Administered Dose Submitted for Analysis	78.57	15.30	93.87	71.05	26.45	97.50
Total % of Administered Dose	78.57	16.27*	94.84	71.05	28.72*	99.77

* - This includes urine, urine funnel wash and urine cage wash. Urine funnel wash and urine cage wash were not analysed for metabolites.

[a] - Also designated, M-11A and M-11B.

[b] - Also designated, M-6.

Table B.6.1.1- 4d Distribution of the Metabolites in Bile (Percent of Administered Dose) Bile - 10 mg/kg bw (Groups Q and R)

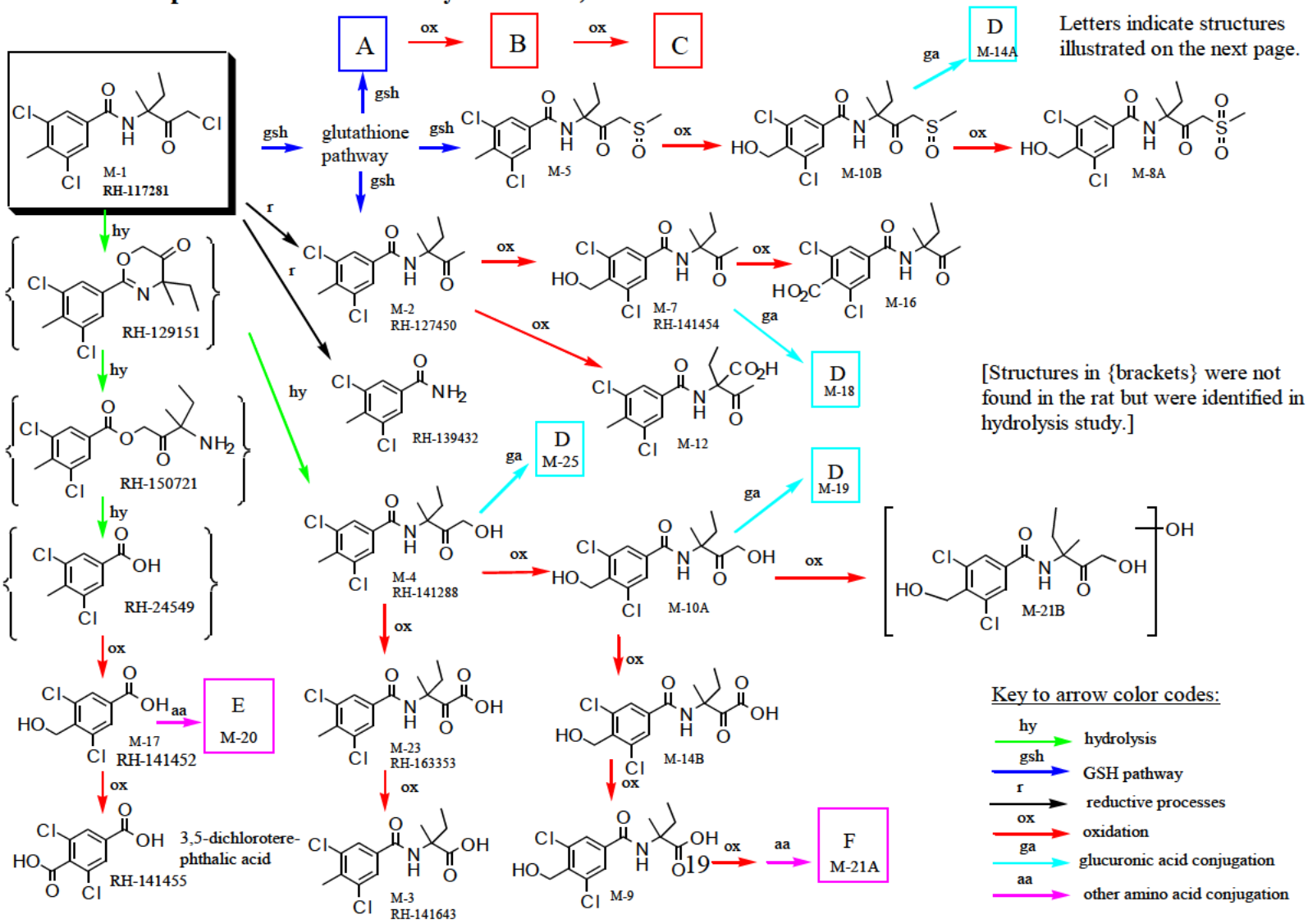
	% of Dose	
Metabolite	**Male – Q**	**Female - R**
M-12, M-22	0.87	1.51
M-13	4.94	3.51
M-14A, M-19, and M-27	10.65	11.38
M-18	3.73	4.36
M-23 (RH-163,353)	2.58	2.50
M-24	0.64	0.93
M-25	7.84	8.60
M-26	6.59	5.46
M-28 and M-29	2.75	3.56
Unknown bile met 14.5 - 16 min	0.61	0.59
Unknown bile met 32 - 34 min	2.23	1.46
Unknown bile met 45 - 49.5 min	1.52	2.61
Unknown bile met 49.5 - 59.5 min	0.89	1.29
Sum of identified metabolites	40.59	41.81
Total Metabolite Distribution	45.84	47.76
Total % of Administered Dose in Bile	45.84	47.76

Overall metabolism was considered to be similar irrespective of dose or gender. The metabolic profile of the high dose faeces was qualitatively similar to that of low dose animals except for the large amount of parent which was considered to be suggestive of incomplete absorption for the higher dose. The amount of parent found in repeat dietary dose animals (5.56 – 5.84 %) were reduced compared with

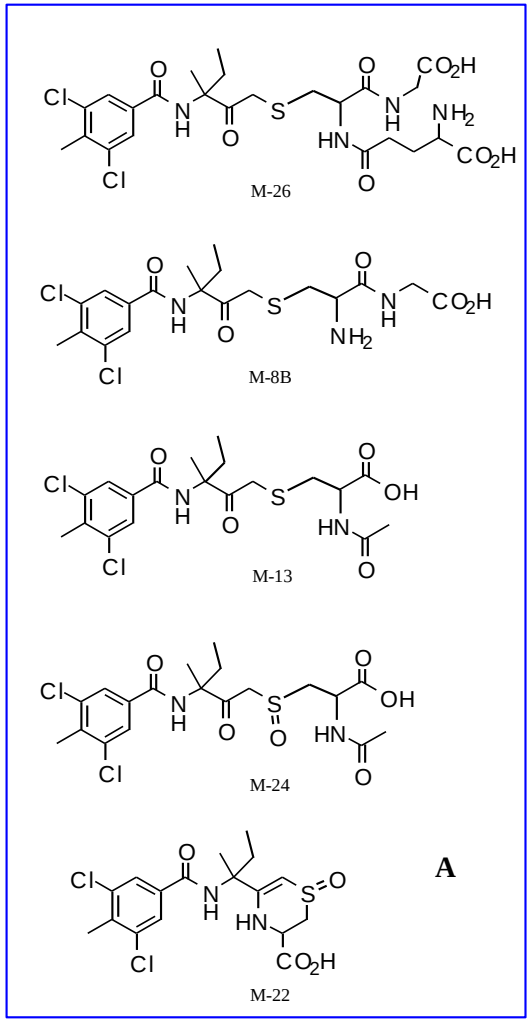
other low dose animals (12.10 – 22.96 %) suggesting possible enzyme (eg. Glutathione transferase) or co-factor (glutathione) induction following repeat dosing.

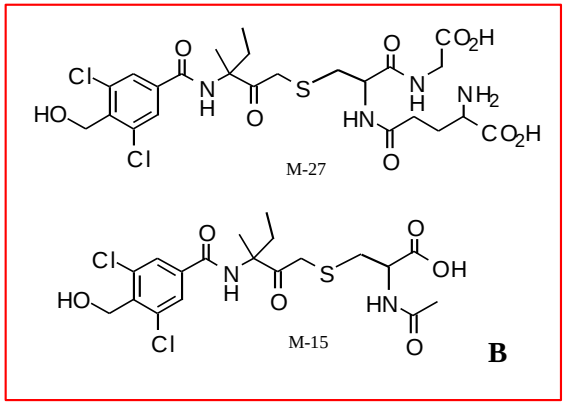
The structures of the individual metabolites and the proposed metabolic pathways of RH 7281 in rats are illustrated in Figure 5.2 and summarized in Figure 5.3. The metabolism of RH 7281 was extensive and was found to occur through a labyrinth of pathways involving primary hydrolysis, glutathione (GSH)-mediated reactions, and reductive dehalogenation; secondary oxidation on both the aromatic methyl and the aliphatic side chain; and terminal glucuronic acid and amino acid conjugations.

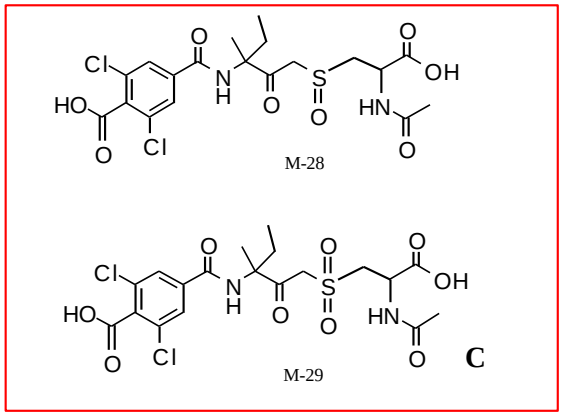
Revised Proposed Metabolic Pathway of RH-117,281 in the Rat

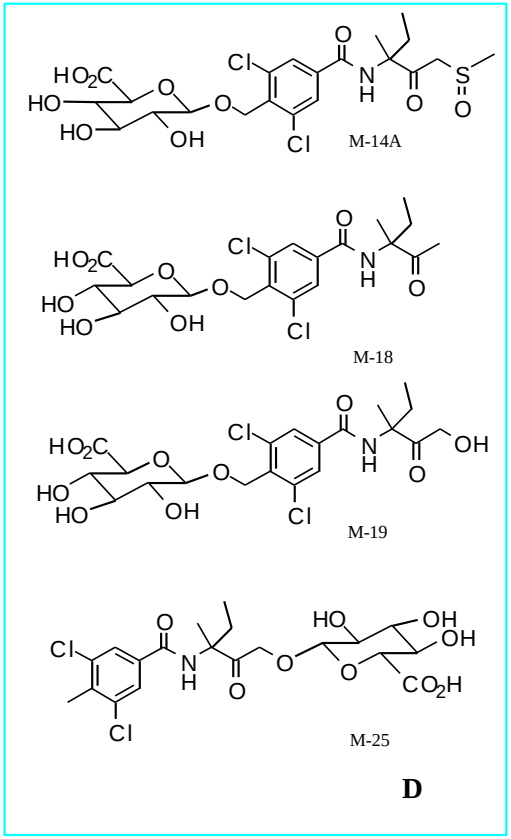


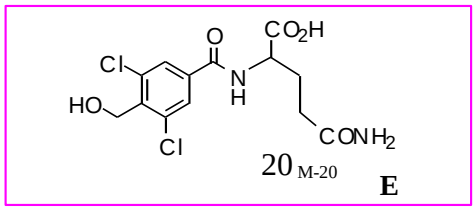
Page 2. Revised Proposed Metabolic Pathway of RH-117,281 in the Rat, cont.

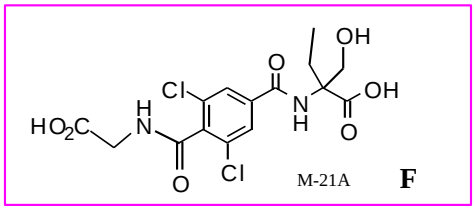












The study was submitted as a supplementary study.

Reference:	CA 5.1.1/03 Reibach P.H., Detweiler B.S. (2001)
Title:	Identification of RH-139432 from zoxamide (RH-117,281) Rat Pharmacokinetic Study Samples
Report number:	34-00-105, ER Ref No: 45.3
Guidelines:	OECD 417 ≅ OPPTS 870.7485 ≅ JMAFF 4200
GLP:	Yes

In a supplementary study, samples of male bile and female urine retained from the above metabolism study were analysed in order to determine whether the benzamide metabolite RH-139432 was present. Quantitative analysis was by 2-dimensional thin layer chromatography with both reverse phase and normal phase coatings. Radio analysis was performed prior to chromatography to allow for material balance calculations. For bile, RH-139432 was found to account for 0.209 to 0.252% of the radioactivity applied to the TLC plate, depending on the TLC phase used. For urine, RH-139432 accounted for 0.16 – 0.026% of the applied radioactivity depending on the TLC phase used. Following purification of the material obtained from the bile sample, the identity of RH-139432 was confirmed by both GC/ mass spectrometry and HPLC/ mass spectrometry.

Conclusion: For bile, RH-139432 was found to account for 0.209 to 0.252% of the radioactivity applied to the TLC plate, depending on the TLC phase used. For urine, RH-139432 accounted for 0.16 - 0.026% of the applied radioactivity depending on the TLC phase used.

RMS: The study is acceptable.

In DAR May (2001)

Reference:	CA 5.1.1/02 (5.4.2/03) [redacted] (1998b)
Title:	Distribution of ^{14}C-RH-117,281 to the bone marrow of mice.
Report number:	No: 97R-173, ER Ref No: 24.2
Guidelines:	OECD 417 ≅ OPPTS 870.7485 ≅ JMAFF 4200
GLP:	Yes

In an investigation of the distribution of RH-7281 in bone marrow (1997), a single oral dose of 2000 mg/kg bw of ^{14}C-RH 7281 was administered orally by gavage to groups of 4 male and 4 female mice. Mice were killed at 4, 8, 24 and 48 hours after administration of the test material and bone marrow tissue samples were collected and analysed for ^{14}C-content.

At all-time points (i.e., 4, 8, 24 and 48 hours), ^{14}C-label derived from ^{14}C-RH 7281 was present in the bone marrow of both male and female mice. Mean concentrations observed in male mice at 4, 8, 24

and 48 hours were 55.5, 34.1, 8.9 and 5.1 µg equivalents of ^{14}C-RH 7281 per gram bone marrow (ppm), respectively. Mean concentrations observed in female mice at 4, 8, 24 and 48 hours were 39.3, 25.0, 8.5 and 5.0 µg equivalents of ^{14}C-RH 7281 per gram bone marrow (ppm), respectively (Table B.6.1.1 - 5).

Table B.6.1.1- 5 Concentration of radioactivity in bone marrow and blood after oral dosing with 2000 mg ^{14}C-RH 7281/kg

	Hours postdose	**µg Equivalents ^{14}C-RH 7281/g tissue**	
		Bone marrow	**Whole Blood**
Males	**4**	55.5	47.6
	8	34.1	74.8
	24	8.9	24.1
	48	5.1	16.3
Females	**4**	39.3	61.1
	8	25.0	57.7
	24	8.5	32.0
	48	5.0	18.4

Findings: The findings demonstrate that ^{14}C-RH 7281 is distributed to bone marrow tissue following a single oral dose (2000 mg/kg) of the test material to adult male and female mice. The information is considered to provide support for the micronucleus study in mouse bone marrow cells (Micronucleus Assay in CD-1 Mouse Bone Marrow Cells) and determines the extent of RH 7281 distribution to the bone marrow of mice after oral administration.

Conclusion: The mean concentration of ^{14}C-RH 7281 in the bone marrow of mice ranged from 5.0 to 55.5 ppm depending on the time following dosing with comparable bone marrow concentrations observed irrespective of sex.

RMS: The study is acceptable.

B.6.1.2 Absorption, distribution, metabolism and excretion by other routes

In accordance with the data requirements for active substances of Commission Regulation (EU) No 283/2013 of 1 March 2013, a comparative in vitro metabolism study was conducted with zoxamide in cryopreserved human, dog, rat and mouse hepatocytes

Submitted for the purpose of renewal.

Reference: CA 5.1.1/04, Scullion, P., (2013)

Title: A study to compare the metabolite profile of ^{14}C-zoxamide in cryopreserved human, dog, rat and mouse hepatocytes

Report number: CXR1237

Guidelines: Not applicable

GLP: No

Executive Summary

A profile of the metabolites was generated by incubation of ^{14}C-Zoxamide with human, dog, rat and mouse hepatocytes *in vitro*. All metabolites detected in human, were also seen in other species apart from one transient metabolite (C10) seen at trace levels in the Human 60 min incubation by Radio-HPLC. Co-chromatography indicated that C5 is metabolite standard ZXM004 (RH-141454).

Analysis by TOF LC-MS detected a number of metabolites together with a proposed elemental composition. Comparison with Radio-HPLC results led to the tentative assignment of a number of the metabolites with a proposed formula.

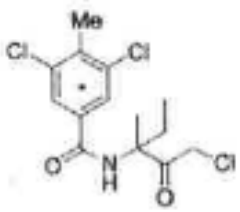
The results from the rat incubation are in agreement with the *in vivo* study (Swenson, R.E., 1998a) summarized in the 2001 DAR.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material

Zoxamide



Description: Not indicated

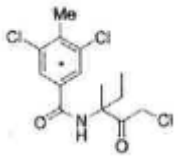
Lot/Batch: 1351400

Purity: 99.0% (analysed 04 January 2013)

CAS#: 156052-68-5

Stability: Expiration date on CoA 31 January 2019. Recommended storage condition on CoA: Cool dry place.

[Phenyl-UL-^{14}C]zoxamide



Description: Not indicated

Lot/Batch: 76045-4-30

Specific Activity: 49 mCi/mmol

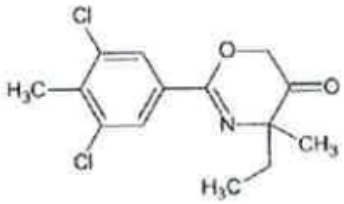
Radiochemical Purity: 99.6% (99.0% chemical purity)

CAS#: 156052-68-5

Stability: Prepared 18 March 2013. Recommended storage condition on CoA: Freezer -20ºC.

Reference substances

1. ZXM 002, RH-129151, 2-(3,5-Dichloro-4-methylphenyl)-4-ethyl-4-methyl-4*H*-1,3-oxazilin-5(6H)-one. CAS 209809-72-3

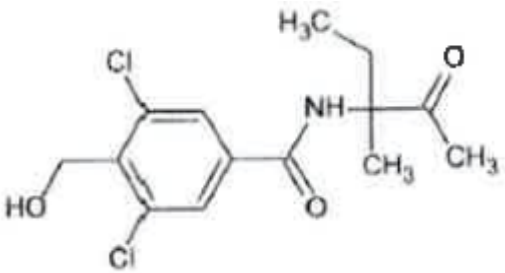


Lot/Batch: EPP /RH 1067.1,

Purity: 99.4%

Stability: Expiry date: 22 February 2014 when stored in a sealed amber glass bottle kept at room temperature.

2. ZXM 004, RH-141454, 3,5-Dichloro-4-(hydroxymethyl)-*N*-(3-methyl-2-oxopentan-3-yl)benzamide. CAS not listed

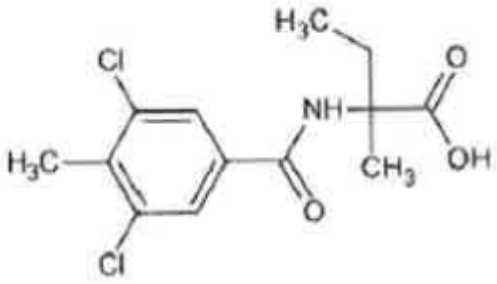


Lot/Batch: EPP / LEE 086.1

Purity: 99.2%

Stability: Expiry date: 28 March 2014 when stored in a sealed amber glass bottle kept at room temperature.

3. ZXM 005, RH-141643, *N*-(3,5-Dichloro-4-methylbenzoyl)isovaline, CAS 202126-92-9

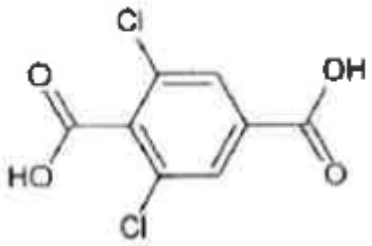


Lot/Batch: EPP / LEE 058.2

Purity: 99.3%

Stability: Expiry date: 2nd April, 2014 when stored in a sealed amber glass bottle kept at room temperature.

4. ZXM 006, RH-141455, 2,6-Dichloro-1,4-benzenedicarboxylic acid, CAS 116802-97-2



Lot/Batch: EPP / RH 1066.9

Purity: 99.1%

Stability: Expiry date: 3 March, 2014 when stored in a sealed amber glass bottle kept at room temperature.

3. Test System Cryopreserved male and female hepatocytes from each species (human, dog, rat and mouse) were obtained from In vitro Technologies GmbH (Leipzig, Germany). Hepatocytes were thawed and male and female pooled together for incubation with Zoxamide.

4. Preparation of Dose solutions

Preparation of Test Item stock solution and metabolite standards

The ^{14}C zoxamide was supplied as a weighed amount of 3.5 mg and an activity of 0.5 mCi. 1 mL of acetonitrile was added to give a ^{14}C concentration of 0.5 mCi/mL and 3.5 mg/mL.

A solution of unlabelled zoxamide was prepared at a concentration of 0.5 mg/mL in acetonitrile. 200 µL of the labelled solution was added to 800 µL of the unlabelled zoxamide to give a stock solution of 1.1 mg/mL of zoxamide (3.27 mM) and100 µCi/mL. To test the radiolabelled purity, 10 µL of the stock

solution was diluted to 1 mL with acetonitrile and injected on the Radio-HPLC system.

A positive control (7-Ethoxycoumarin) was prepared by dissolving 6.1 mg in 3.21 mL of Dimethylsufoxide (DMSO).

Metabolite standards were dissolved in either acetonitrile or DMSO before dilution with 50/50 Acetonitrile/water (ACN/H_2O) to give a final concentration of 50 µg/mL. Samples for co-chromatography were prepared by adding 100 µL of the appropriate metabolite standard to 100 µL of incubate.

B. STUDY DESIGN AND METHODS

Hepatocyte incubations

Hepatocyte suspensions of 10^6 viable cells/mL in Leibowitz CL15 culture medium containing 10% foetal calf serum were incubated with one concentration of zoxamide (nominal final concentrations of 30 µM and 1 µCi/mL). 7-Ethoxycoumarin (7-EC, 100 µM) was included as a positive control substrate and incubated for 60 minutes. The reactions were initiated by the addition of zoxamide and incubated in a shaking water bath at 37ºC. Samples were removed and any reaction terminated by addition to an equal volume of acetonitrile after 0, 15, 30, 60, or 120 minutes of incubation. Reaction mixtures were split in two, frozen and stored at –70°C approximately. One set of samples were used for this study and the other set stored at -70°C for possible future analysis. The samples were centrifuged to remove cell debris and an aliquot of supernatant removed and analysed.

Radio-HPLC conditions for the analysis of hepatocyte incubations

The HPLC column used was a Zorbax RX-C18, 5 µm, 250×4.6 mm from Agilent. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile at 1.2 mL/min flow rate. The HPLC system comprised a Gynkotek binary LC pump, Dionex ASI-100 autosampler, HPLC Technology column heater and Berthold LB509 radioflow detector fitted with a YG150-U4D cell. The LC gradient used is shown below.

Table 6.1.2-2

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
5	95	5
10	70	30
30	5	95
35	5	95
40	95	5
45	95	5

For co-chromatography, a Dionex UV340D detector was fitted in-line immediately after the column. Samples were prepared by adding 100 µL of a 50 µg/mL solution of the appropriate standard to 100 µL of incubate.

Mass spectrometer metabolite identification

Analysis of selected samples was performed on the LCT Premier mass spectrometer using accurate mass determination and Metabolynx software for identification of any Phase 1 and Phase 2 metabolites

produced (default masses scanned were included in Appendix 2 of the report). A Waters Alliance HPLC system with a Zorbax RX-C18, 5 µm, 250×4.6 mm column from Agilent was used with a modified LC gradient, detailed in the Table below. The flow rate was 0.5 mL/min.

Table 6.1.2-1

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
5	95	5
15	5	95
25	5	95
25.1	95	5
35	95	5

A total ion scan was performed with a mass range (m/z 100 – 1000). The neutral monoisotopic mass was 335.0246 and the +ve ion monoisotopic mass was 336.0325. A LockSpray electrospray ionization source was used with leucine enkephalin reference compound, +ve ion monoisotopic mass of 556.2771.

Samples analysed on the LCT Premier mass spectrometer were processed using Metabolynx software to determine the possible appearance of the available metabolite standards, using accurate mass determination.

II. RESULTS AND DISCUSSION

Analysis of the 7-Ethoxycoumarin samples from the four hepatocyte species was done by HPLC-UV and confirmed that metabolism had occurred.

No radiolabelled impurities were detected in the dilution of the ^{14}C-Zoxamide stock solution.

Comparison of metabolic profile from cryopreserved Human, Dog, Rat and Mouse hepatocytes

After 120 min incubation, all four species showed extensive metabolism with the parent compound accounting for <50% of the total recovered radioactivity (TRR).

Metabolite distribution: Total Recovered Radioactivity (% of administered dose) after 60 minutes of incubation.

Table 6.1.2-3

RT - min	Peak designation	Human	Dog	Rat	Mouse
10.67	ZXM 006, RH-141455				
13.33	C1			2.97	
16.18	C2	5.76	17.48	41.79	35.43
16.48	C3	3.44	BLQ	BLQ	
16.77	C4	BLQ	BLQ	BLQ	
17.88	C5 ZXM 004 RH-141454	BLQ		BLQ	

19.21	C6				BLQ
19.90	C7	14.78	12.39	15.77	7.73
21.22	C8 ZXM 005 RH-141643	10.72	BLQ		5.73
24.24	C9	16.99	5.25	7.01	10.30
25.50	Zoxamide	42.33	58.12	27.23	38.26
28.74	C10 ZXM 002 RH-129151	BLQ			
Total		94.02	93.24	94.77	97.45

BLQ: below limit of quantitation

Metabolite distribution: Total Recovered Radioactivity (% of administered dose) after 120 minutes of incubation.

Table 6.1.2-4

RT - min	Peak designation	Human	Dog	Rat	Mouse
10.67	ZXM 006, RH-141455				
13.33	C1			9.43	
16.18	C2 *GSH conjugate*	8.13	19.68	48.27	41.80
16.48	C3	4.51	BLQ	BLQ	
16.77	C4	BLQ	BLQ	BLQ	
17.88	C5 ZXM 004 RH-141454	6.09		6.56	
19.21	C6				
19.90	C7 *mono-oxidation product*	14.16	14.03	15.85	6.37
21.22	C8 ZXM 005 RH-141643	14.27	4.39		6.76
24.24	C9 *Dechlorination product*	31.49	7.30	9.45	13.14
25.50	Zoxamide	18.90	48.53	7.53	31.93
28.74	C10 ZXM 002				

	RH-129151 *hydrolysis product of Zoxamide*				
Total		97.55	93.93	97.09	100.00

BLQ: below limit of quantitation

A profile of the metabolites generated by incubation of ^{14}C-Zoxamide with four species of hepatocytes was generated and a comparison made. All metabolites detected in human hepatocytes, by Radio-HPLC, were also seen in other species apart from metabolite C10 detected at trace levels in the Human 60 min incubation only. C10 was identified by co-chromatography and Time-of-Flight mass spectrometry (TOF LC-MS) using an authentic reference standard (ZXM002 – RH-129151). ZXM002 has been postulated as an intermediate metabolite in a previous pharmacokinetic and metabolism study in rat and is a known hydrolysis product of Zoxamide. Although C10 was a transient metabolite, not detected in the human 120 min incubation, it was however seen in all samples by TOF LC-MS which were analysed at a later date than the Radio-HPLC analysis. Therefore it is proposed that C10 is mainly a hydrolysis product of Zoxamide.

The metabolites identified from the rat incubation were in agreement with previous studies (see the 2001 DAR (UK RMS), Swenson, R.E., 1998a; Oesch, F. *et al*., *Xenobiotica*, 2010, 40(1), 78-82). One metabolite (C5) was identified using an authentic reference standard (ZXM004 - RH-141454) by both co-chromatography and analysis by TOF LC-MS. Comparison of the TOF LC-MS and Radio-HPLC chromatograms led to the tentative assignment of four other metabolites, corresponding to oxidation, de-chlorination plus oxidation, dechlorination plus conjugation by glutathione (GSH) and de-chlorination plus glucuronidation.

III. CONCLUSIONS

A profile of the metabolites generated by incubation of ^{14}C-Zoxamide with four species of hepatocytes was generated. All metabolites detected in human, were also seen in other species apart from one transient metabolite (C10) seen at trace levels in the Human 60 min incubation by Radio-HPLC. All samples analysed at a later date than the Radio-HPLC analysis revealed the presence of C10 and it is therefore proposed that this represents mainly a hydrolysis product of zoxamide.

Co-chromatography indicated that C5 is metabolite standard ZXM004.

Analysis by TOF LC-MS detected a number of metabolites together with a proposed elemental composition. Comparison with Radio-HPLC results led to the tentative assignment of a number of the metabolites with a proposed formula.

The results from the rat incubation are in agreement the *in vivo* study (Swenson, R.E., 1998a) summarized in the 2001 DAR.

RMS: The study is acceptable. All metabolites detected in human, were also seen in other species apart from (C10) seen at trace levels in the Human 60 min incubation by Radio-HPLC. All samples analysed at a later date than the Radio-HPLC analysis revealed the presence of C10 and it is therefore proposed that this represents mainly a hydrolysis product of zoxamide.

Submitted for the purpose of renewal.

Reference: CA 5.1.1/05, Powrie, R., (2014)

Title: A study to investigate the presence of a specific metabolite of zoxamide, RH-150721, in analytical data from previous study CXR1237

Report number: CXR1416

Guidelines: Not applicable

GLP: No

Study was submitted as supplementary for investigation of presence of specific metabolite RH-150721.

Summary/Conclusion:

In Study CXR1237 the samples from cryopreserved hepatocyte incubations from four species were analysed to determine the appearance of possible metabolites using accurate mass determination. A total ion scan was performed with a mass range (m/z 100 – 1000). A LockSpray electrospray ionization source was used with leucine enkephalin reference compound, +ve ion monoisotopic mass of 556.2771. The data collected from CXR1237 was re-processed using Metabolynx software to determine the appearance, or not, of a specific metabolite, RH-150721, using accurate mass determination, as shown below:

Table 6.1.2.-5

Name	Molecular formula	Neutral Monoisotopic mass	+ve ion Monoisotopic mass
RH-150721	$C_{14}H_{17}Cl_2NO_3$	318.0664	319.0742

The results from the re-processing of the original data from Study CXR1237 using MetaboLynx software have shown that no peaks with the associated mass or isotope pattern of RH-150721 could be detected in the incubated hepatocyte samples. It can be concluded from these results that RH-150721 metabolite was not produced in the original incubations of Zoxamide with cryopreserved human, dog, rat and mouse hepatocytes in Study CXR1237.

RMS: The study is acceptable. RH-150721 metabolite was not produced in the original incubations of Zoxamide with cryopreserved human, dog, rat and mouse hepatocytes in Study CXR1237.

One open literature study was submitted as potential relevant.

Oesch, F; Metzler, M; Fabian, E; Kamp, H; Bernshausen, T; Damm, G; Triebel, S; Döhmer, J; Landsiedel, R; Van Ravenzwaay, B, 2010

Study indicates that using zoxamide as a prototype, in principle, mammalian metabolism and its relationship to mammalian detoxication of fungicidal mitosis inhibitors may be reasonably anticipated from in vitro studies. In addition, the results provide a rational for the observed absence of typically mitosis inhibition-associated toxicities of zoxamide in mammals in vivo. No impact on human health assessment.

B.6.2 Acute toxicity

For the first inclusion of zoxamide in Annex I to Directive 91/414/EEC, a complete data set on acute toxicity was evaluated. The data is considered satisfactory, and sufficient for the purposes of reregistration. Changes to the original conclusions are proposed regarding eye irritation study results. In addition because of the UV absorbing properties of zoxamide a phototoxicity study was conducted. Zoxamide was not phototoxic.

B.6.2.1 Oral

B.6.2.1.1 Rat

In DAR May (2001)

Reference: CA 5.2.1/01 [redacted] (1996a)

Title: RH-117,281 Technical: Acute oral toxicity study in male and female rats.

Report number: 95R-268, ER Ref No.1.3

Guidelines: OECD 401 ≅ OPPTS 870.1100/FIFRA 81-1 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.1

GLP: Yes

In a study (1996) six male and 6 female fasted Crl:CD®BR rats were each administered by gavage a limit dose of 5000 mg/kg bw (20 ml/kg) RH-7281 (lot no: DSR-9510, purity 92.3 %) in corn oil). Mortality, body weight gain and signs of toxicity were recorded during the 14-day observation period. All surviving animals were killed at the end of the observation period and grossly examined at necropsy. At the start of treatment, the rats were 52 (males) or 64 (females) days old and average body weight was 209 ± 3 g in males and 201 ± 6 g in females.

Findings: No mortalities were observed during the 14-day observation period. A slight decrease in body weight gain was noted in females only (20 %) compared to historical controls. Red stained eyes and/or muzzle and gastrointestinal effects were observed up to day 1. Necropsy revealed no gross changes in organs and tissues.

Conclusion: The acute oral LD_{50} for RH-7281 in male and female rats was > 5000 mg/kg bw.

RMS: The study is acceptable. According Regulation EC No 1272/2008 classification is not required for RH-7281.

B.6.2.1.2 Mice

In DAR May (2001)

Reference: CA 5.2.1/02, [redacted] (1998a)

Title: RH-117,281 Technical: Acute oral toxicity study in male and female mice.

Report number:	98R-165, ER Ref No. 24.3
Guidelines:	OECD 401 ≅ OPPTS 870.1100/FIFRA 81-1 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.1
GLP:	Yes

In a study (1998) six male and 6 female fasted Crl:CD-1®(ICR)BR mice were each administered by gavage a dose of 5000 mg/kg bw (20 ml/kg) RH-7281 (lot no: DSR-9510, purity 94.35 %) in corn oil). Mortality, body weight gain and signs of toxicity were recorded during the 14-day observation period. All surviving animals were killed at the end of the observation period and grossly examined at necropsy. At the start of treatment, the rats were 7 (males) or 9 (females) weeks old and average fasted body weight was 28.6 ± 1.5 g in males and 26.6 ± 0.9 g in females.

Findings: There were no mortalities, clinical signs of toxicity. Body weight was not affected compared with historical control data. Necropsy revealed no gross changes in organs and tissues.

Conclusion: The acute oral LD50 for RH-7281 in male and female mice was > 5000 mg/kg bw.

RMS: The study is acceptable. According Regulation EC No 1272/2008 classification is not required.

B.6.2.2 Dermal

In DAR May (2001)

Reference:	CA 5.2.2/01, [redacted] (1996b)
Title:	RH-117,281 Technical: Acute dermal toxicity study in male and female rats
Report number:	95R-269, ER Ref No. 1.4
Guidelines:	OECD 402 ≅ OPPTS 870.1200/FIFRA 81-2 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.3
GLP:	Yes

In a study (1996) a single dose of 2000 mg/kg bw RH-7281 (lot no: DSR-9510, purity 92.3 %) in corn oil was administered under an occlusive dressing to the clipped dorsal skin of groups of 6 male and 6 female Crl:CD®BR rats. The area of application was approximately 20 % of the body surface area. Mortality, body weight and signs of toxicity or abnormal behaviour were recorded during the 14-day observation period. All surviving animals were killed at the end of the observation period and grossly examined at necropsy. At the start of treatment, the rats were 7 (males) or 9 (females) weeks old and average fasted body weight was 243 ± 4 g in males and 216 ± 10 g in females.

Findings: There were no mortalities or clinical signs of toxicity. Body weight was not affected compared with historical control data. Necropsy revealed no gross changes in organs and tissues.

Conclusion: The acute dermal LD50 for RH-7281 in male and female mice was > 2000 mg/kg bw.

RMS: The study is acceptable. According Regulation EC No 1272/2008 classification is not required.

B.6.2.3 Inhalation

In DAR May (2001)

Reference: CA 5.2.3/01, [redacted] (1996)

Title: RH-117,281 Technical: Acute inhalation toxicity study in rats

Report number: 95R-266, ER Ref No. 2.2

Guidelines: OECD 403 ≅ OPPTS 870.1300/FIFRA 81-3 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.2

GLP: Yes

In a study (1996), groups of 6 male and 6 female Crl:CD®BR rats were exposed (nose-only) for four hours to a dust aerosol of RH-7281 (lot no: DSR-9510, purity 92.3 %) at analytical concentrations of 0 (air control), 1.9 or 5.3 mg/l. All animals were observed daily for signs of ill health or reaction to treatment for 14 days following the exposure. Body weights were recorded weekly during the observation period and all survivors were necropsied after 14 days. At the start of treatment, average body weight ranged from 209 - 229 g in males and 221 - 230 g in females.

Table 6.2.3 - 1 Summary of exposure conditions

	Exposure Values	
Mean aerosol concentration (analytical)	5.3 mg/L	1.9 mg/L
Mean mass aerodynamic diameter (MMAD)	4.3µm	4.0µm
Mean geometric standard deviation (GSD)	2.1	2.2
Mean respirable aerosol fraction	41.5 %	44.5 %

Findings: There were no mortalities, clinical signs of toxicity or effects on body weight gain. Necropsy did not reveal gross changes in organs and tissues.

Conclusion: The acute inhalation LC_{50} for RH-7281 in male and female rats was > 5.3 mg/l.

RMS: The study is acceptable. According Regulation EC No 1272/2008 classification is not required.

B.6.2.4 Skin irritancy

In DAR (2001)

Reference: CA 5.2.4/01, [redacted] (1996c)

Title: RH-117,281 Technical: Skin irritation study in rabbits

Report number: 95R-270, ER Ref No. 1.5

Guidelines: OECD 404 ≅ OPPTS 870.2500/FIFRA 81-5 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.4

GLP: Yes

In a study (1996), 0.5 g of RH-7281 (lot no: DSR-9510, purity 92.3 %) moistened with 1 ml of distilled water (1:2 weight/volume), was applied topically to the intact shaven skin of 6 male rabbits under a semi-occluded dressing. After the 4 hr exposure period, the application site was wiped with paper towels saturated with tap water and blotted dry with paper towels. Skin irritation was evaluated according to Draize criteria at approximately 1, 24, 48 and 72 hrs after patch removal.

Findings: No mortality or clinical signs of systemic toxicity were observed during the study. No skin irritation (i.e., no erythema or edema) was observed during the study. The mean 24 - 72 h scores were for erythema 0 and oedema 0.

Table 6.2.4 - 1 Summary of skin irritation scores (Draize Criteria)

	Time after patch removal			
Erythema				
Animal No	**1hr**	**24hr**	**48hr**	**72hr**
95-27221	0	0	0	0
95-27219	0	0	0	0
95-27206	0	0	0	0
95-27190	0	0	0	0
96-27005	0	0	0	0
96-27006	0	0	0	0
Mean score:	**0.0**	**0.0**	**0.0**	**0.0**
Edema	**1hr**	**24hr**	**48hr**	**72hr**
Animal No	0	0	0	0
95-27221	0	0	0	0
95-27219	0	0	0	0
95-27206	0	0	0	0
95-27190	0	0	0	0
96-27005	0	0	0	0
96-27006	**0.0**	**0.0**	**0.0**	**0.0**
			Erythema sums:	**0.0**
			Edema sums:	**0.0**

Conclusion: RH-7281 was not irritating to the skin of the rabbit and is not classified as a skin irritant in accordance with EC criteria.

RMS: The study is acceptable. According Regulation EC No 1272/2008 classification is not required.

B.6.2.5 Eye irritancy

In DAR May (2001)

Reference:	CA 5.2.5/01, [redacted] (1996d)
Title:	RH-117,281 Technical: eye irritation study in rabbits.
Report number:	95R-271, ER Ref No. 1.6
Guidelines:	OECD 405 ≅ OPPTS 870.2400/FIFRA 81-4 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.5
GLP:	Yes

In a study (1996), 0.1 g of undiluted RH-7281 (lot no: DSR-9510, purity 92.3 %) was applied into the conjunctival sac of one eye of six male rabbits. The untreated eye served as control. Eye irritation was evaluated according to Draize criteria at approximately 1, 24, 48, and 72 hrs and at 7 days after dosing. After the 24 hr observation period, each eye (treated and control) was irrigated with 0.9 % saline for approximately 60 seconds.

Findings: No mortality or clinical signs of systemic toxicity were observed during the study. Transient slight irritation was observed in all animals and included: corneal effects at 24, 48 and 72 hrs; very slight iritis in one animal only at 24 hrs and conjunctival effects at 1, 24 and 48 hrs. Ocular effects were completely resolved by day 7. The mean 24 - 72 h scores were as follows; corneal opacity 1.1, iritis 0.1, conjunctival redness 1.3 and oedema 1.4.

Table 6.2.5 - 1 Summary of eye irritation scores (Draize Criteria)

Animal numbers:	**96-27001**			**96-27002**			**96-27007**			**96-27009**			**96-27012**			**96-27010**			Mean*
Time (hr):	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	
Corneal Opacity	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	**1.1**
Iris Lesions	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	**0.1**
Conjunctival Redness	2	2	0	2	1	0	2	2	1	2	1	1	2	1	0	2	2	1	**1.3**
Conjuctival Chemosis	2	1	0	2	1	1	2	1	1	2	1	1	2	2	1	2	2	1	**1.4**

* Mean of 24,-, 48,-, 72,-hr Draize scores fore all six animals.

Conclusion: According Regulation EC No 1272/2008 classification category 2 is required if the corneal opacity mean score of 24,-, 48,-, 72,-hr is ≥ 1. Mean score of corneal opacity in this study is 1,1, so RH-7281 is irritating to the eye of the rabbit and is classified as a eye irritant with **hazard class category 2, H319.**

RMS comment: The study is acceptable. According Regulation EC No 1272/2008 classification hazard class category 2, H319 is required.

B.6.2.6 Skin sensitisation

6.2.6.1 M&K maximization test

In DAR May (2001)

Reference:	CA 5.2.6/01, [redacted] (1997)
Title:	Dermal sensitization study of RH-117,281 Technical in guinea pigs - maximization test.
Report number:	95RC-170, ER Ref No. 4.2
Guidelines:	OECD 406 ≅ OPPTS 870.2600/FIFRA 81-6 ≅ 92/69/EEC B.6 - GPMT
GLP:	Yes

In a study (1996; experimental phase 1995) of the skin sensitisation potential of RH-7281 (lot number: LG3517, purity: 92.9 %) by the Magnusson and Kligmann (M&K) maximisation method, groups of 20 (test compound group) or 10 (control group) young adult albino *Dunkin Hartley* Hra: (DH) fBR guinea pigs were tested accordingly to the following dosing regimen.

On day 1, intradermal induction consisting of 6 injections was administered pairwise to anterior, middle and posterior sites of the clipped shoulder area (4 cm x 5 cm) of 20 animals in the treatment group as follows:

0.1 ml of 1:1 dilution of Freund's Complete Adjuvant (FCA) in sterile water;

0.1 ml of 5 % w/v RH-7281 in mineral oil;

and 0.1 ml of 5 % w/v RH-7281 in FCA in sterile water.

For the vehicle control group of 10 animals, the injections were as follows,

0.1 ml of 1:1 dilution of FCA in sterile water;

0.1 ml of mineral oil;

and 0.1 ml of 1 : 1 dilution of vehicle in FCA

Six days later, all test animals were pre-treated with 10 % w/w sodium lauryl sulfate in petrolatum applied topically at the injection sites. On Day 8, a 25 % w/w mixture of either RH-7281 or sulfathiazole (positive controls) in petrolatum, or petrolatum alone (solvent controls) was applied over the injection sites of the animals in the respective groups. All induction sites were then occluded for 48 hours.

Two weeks after the topical application, all animals received a challenge dose. A 25 % w/w mixture of the RH-7281 in petrolatum was applied to the right side of the test group animals and the control material (petrolatum) was applied to the left side of each animal in test compound group and its control group. A 10 % w/w mixture of sulfathiazole in petrolatum was applied to the right side and petrolatum alone was applied to the left side of each animal in the sulfathiazole positive test group and its solvent control groups. All test and control sites were occluded for 24 hours and then wiped clean. The challenge sites were examined for dermal reactions at 24 and 48 hours after patch removal.

The test concentrations used were determined in preliminary screening tests. One group of 4 male guinea pigs was administered dermally under an occlusive dressing RH-7281 in petrolatum at concentrations of 1 %, 10 %, 15 %, and 25 % w/w with each animal receiving two different concentrations of the test material (two test sites per animal). The test sites were occluded for 24 hours and dermal irritation was evaluated at 24 and 48 hours after removal of the patches. In a second group of 4 male guinea pigs were each administered intradermally 0.1ml of RH-7281 in mineral oil at concentrations of 1 %, 5 %, 10 %, and 25 % w/v in mineral oil (four injection sites per animal). These injection sites were evaluated for dermal reactions at 24 and 48 hours after injection. Based on the results of the irritation screening study, the test material was administered as 5 % w/v mixtures for the intradermal injection phase and as a 25% w/w mixture in petrolatum for the topical induction application and for the challenge phase in the definitive study.

At the start of the study, the guinea pigs were young adults and body weights were 354 – 480 g for both sexes.

Findings: All 20 animals in the RH-7281 test group exhibited moderate to intense dermal reactions at the treated skin site but only a mild dermal reaction was exhibited by two animals of the control group, treated with the petrolatum alone at challenge. None of the animals in RH-7281 and sulfathiazole irritation control groups exhibited a dermal reaction to the challenge application of the test substance (RH-7281) or positive control (sulfathiazole). All animals in the sulfathiazole positive control exhibited sensitisation reactions on the test sites treated with sulfathiazole and no dermal reactions on the test sites treated with petrolatum alone (Table B.6.2.6.1-1).

Table B.6.2.6.1-1 Summary of findings of the maximisation test in guinea pigs at 24 and 48 h after challenge

Group	Number of animals showing an allergenic response following challenge	
	24-hours	48-hours
RH-7281 Test	20/20	20/20
RH-7281 Irritation Control	0/10	0/10
Sulfathiazole Positive Control Test	10/10	9/10
Positive Test Irritation control	0/5	0/5

Conclusion: RH-7281 was sensitising to skin in the M & K test in guinea pigs.

RMS: The study is acceptable. According Regulation EC No 1272/2008 classification hazard class category 1A, H317 is required.

6.2.6.2 Buehler test in guinea pigs

In DAR May (2001)

Reference: CA 5.2.6/02, [redacted] (1998a)

Title: RH-117,281 Technical: Delayed contact hypersensitivity study in guinea pigs

Report number: No. 97R-074, ER Ref No. 23.2

Guidelines: OECD 406 ≅ OPPTS 870.2600/FIFRA 81-6 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.6

GLP: Yes

In a study (1998), the skin sensitisation potential of RH-7281 (lot no: R63240, purity 93.83 %) was investigated in young adult female Hartley guinea pigs. One group of 20 guinea pigs (Group 2) received three 6-hr induction doses (1 dose/week for 3 consecutive weeks) of 0.4 g of RH-7281 wetted with approximately 0.7 ml of distilled water. An additional 5 groups of 10 guinea pigs each (Groups 3-7) received three 6-hr induction doses of 0.4 ml of 25 %, 7.5 %, 2.5 %, 0.75 %, and 0.25 % (w/w) RH-7281 in 80 % (v/v) aqueous ethanol, respectively. A day later, a vehicle control group of 5 guinea pigs (Group 8) received three 6-hr induction doses of 0.4 ml of 80 % (v/v) aqueous ethanol. The test substance was applied to a modified "Hill Top" patch, which was placed onto the clipped dorsal skin and kept under an occlusive dressing during the exposure period. After the exposure period, the treated skin site was washed with lukewarm tap water and dried with paper tissues. Twelve days after the last induction dose, Groups 2-8 were challenged with an additional dose of the same volume, vehicle, and concentration as the induction doses. A naive control group of 10 guinea pigs (Group 1) was challenged at separate sites with 0.4 g RH-7281 wetted with approximately 0.7 ml distilled water; 0.4 ml of 25 % and 7.5 % (w/w) RH-7281 in 80 % (v/v) aqueous ethanol; and with 80 % (v/v) aqueous ethanol. Dosing sites were evaluated for erythema response at 24 and 48 hrs after challenge.

Findings: One test animal in the 100 % RH-7281 dose group (Group 2) died. No significant erythema was observed in the non-induced (naive) control group at any site challenged with either RH-7281 or 80 % (v/v) aqueous ethanol. A 20 % incidence of erythema (1/5) was observed in the vehicle control guinea pigs induced and challenged with 80 % (v/v) aqueous ethanol. The incidences of erythema observed in guinea pigs induced and challenged with the indicated concentrations of RH-7281 are shown in Table B.6.2.6.2-1

Table B.6.2.6.2-1 Summary of the findings of the Buehler test in guinea pigs

Group	RH-7281 (%)		No. of positive responses at scoring time after challenge treatment	
	Induction treatment	Challenge treatment	24-hours	48-hours
1	Naive Control: Blank patch	100% (wetted with water)	0/10	0/10
		25% in ethanol	0/10	0/10
		7.5% in ethanol	0/10	0/10

		80% ethanol	0/10	0/10
2	100% (wetted with water)	100% (wetted with water)	2/19	10/19
3	25% in ethanol	25% in ethanol	8/10	7/10
4	7.5% in ethanol	7.5% in ethanol	4/10	6/10
5	2.5% in ethanol	2.5% in ethanol	7/10	6/10
6	0.75% in ethanol	0.75% in ethanol	9/10	9/10
7	0.25% in ethanol	0.25% in ethanol	6/10	7/10
8	Vehicle control: 80% ethanol	80% ethanol	1/5	1/5

Conclusion: RH-7281 showed delayed contact hypersensitivity at all test concentrations in the Buehler test.

RMS: The study is acceptable. According Regulation EC No 1272/2008 classification hazard class category 1, H317 is required.

Reference: CA 5.2.6/03, [redacted] (1998b)

Title: RH-117,281 Technical: Delayed contact hypersensitivity (dilution) study in guinea pigs.

Report number: No: 98R-154, ER Ref No: 2.3

Guidelines: OECD 406 ≅ OPPTS 870.2600/FIFRA 81-6 ≅ 59 Nohsan No. 4200 ≅ 92/69/EEC B.6

GLP: Yes

In a study (1998), 6 groups of 10 young adult male Hartley guinea pigs (groups 2 – 7) received three 6-hr induction doses (1 dose/week for 3 consecutive weeks) of 0.4 ml of 0.25 %, 0.25 %, 0.1 %, 0.025 %, 0.0025 %, and 0.00025 % (w/w) RH-7281 (lot no: T66246D, purity 97.85 %) in 80 % (v/v) aqueous ethanol, respectively. The test substance was applied to a modified "Hill Top" patch, which was placed onto the clipped dorsal skin and kept under an occlusive dressing during the exposure period. After the exposure period, the treated skin site was washed with lukewarm tap water and dried with paper tissues. Twelve days after the last induction dose, Group 2 guinea pigs were challenged with 0.4 ml of 0.25 % (w/w) RH-7281 in 80 % (v/v) aqueous ethanol. Groups 3-7 guinea pigs were challenged with 0.4 ml of 0.25 %, 0.1 %, 0.025 %, 0.0025 % and 0.00025 % (w/w) RH-7281 in acetone respectively. A naive control group of 10 guinea pigs (Group 1) was challenged at separate sites with 0.4 ml RH-7281 as dosed in the six treated groups. The treated skin sites were evaluated for erythema response at 24 and 48 hrs after challenge.

No erythema was observed in the non-induced (naive) control group at any site challenged with RH-7281. The incidences of erythema observed in guinea pigs induced with the indicated concentrations of RH-7281 in 80 % aqueous ethanol and challenged as indicated below is shown in Table B.6.2.6.2-2

Table B.6.2.6.2-2 Number of animals showing a positive dermal response on challenge in Buehler test

Group	RH-7281 (%)		Time after challenge	
	Induction treatment	Challenge treatment	24-hours	48-hours
1	Naive Control: Blank patch	0.25% in ethanol	0/10	0/10
		0.25% in acetone	0/10	0/10
		0.1% in acetone	0/10	0/10
		0.025% in acetone	0/10	0/10
		0.0025% in acetone	0/10	0/10
		0.00025% in acetone	0/10	0/10
2	0.25% in ethanol	0.25% in ethanol	6/10	7/10
3	0.25% in ethanol	0.25% in acetone	1/10	1/10
4	0.1% in ethanol	0.1% in acetone	1/10	1/10
5	0.025% in ethanol	0.025% in acetone	0/10	0/10
6	0.0025% in ethanol	0.0025% in acetone	0/10	1/10
7	0.00025% in ethanol	0.00025% in acetone	0/10	0/10

Challenge with 0.25 % RH-7281 in 80 % (v/v) aqueous ethanol produced slight to moderate erythema in 8/10 animals and was considered to be sensitising to the skin of the guinea pig under the conditions of this study. No skin sensitisation was observed when animals were challenged with RH-7281 in acetone at concentrations up to and including 0.25 %. When acetone was used as the challenge vehicle, the incidences of erythema at concentrations of 0.25 % and lower were minimal (1/10) at most.

Conclusion: RH-7281 was considered to be sensitising to the skin of the guinea pig under the conditions of this study

RMS comment: The study is acceptable. According Regulation EC No 1272/2008 classification hazard class category 1A, H317 is required.

B.6.2.7 Phototoxicity

Since zoxamide absorbs electromagnetic radiation in the range 290-700 nm at over 10 L/mol/cm, a phototoxicity study is required. Absorption was <1000 L/mol/cm, hence photomutagenicity studies will not be required.

Reference:	CA 5.2.7/01, A. Heppenheimer (2014)
Title:	Zoxamide: Cytotoxicity assay in vitro with BALB/c 3T3 cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight.
Report number:	1641200
Guidelines:	OECD 432
GLP:	Yes

Submitted for the purpose of renewal.

The study was performed to assess the phototoxic potential of Zoxamide. The test was performed using BALB/c 3T3 cells clone 31.

The experiment was performed two times. The first experiment served as a range finding experiment (RFE), the second experiment was the main experiment (ME). 62.5 µg/mL of the test item, dissolved in DMSO (final concentration of DMSO in EBSS: 1% (v/v)), was used as the highest concentration, and was determined by the solubility of the test item.

The following concentrations of the test item were tested with and without irradiation in both experiments:

0.49, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5 µg/mL

EBSS containing 1% (v/v) DMSO was used as solvent control.

Chlorpromazine was used as positive control. The following concentrations were applied:

without irradiation	6.25, 12.5, 25, 37.5, 50, 75, 100, 200 µg/mL
with irradiation	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0 µg/mL

One test group of cells treated with the test item was irradiated with artificial sunlight for 50 minutes with 1.65 mW/cm^2 UVA, resulting in an irradiation dose of ~ 5 J/cm^2 UVA. Another test group of test item treated cells were kept in the dark for 50 minutes.

Evaluation of Results:

Based on the results obtained, the test item is evaluated as follows:

If **PIF < 2 or MPE < 0.1**: no phototoxic potential predicted.

If **PIF > 2 and < 5 or MPE >0.1 and <0.15** a probable phototoxic potential is predicted.

If **PIF > 5 or MPE > 0.15** a phototoxic potential predicted

Acceptability of the Assay:

The assay meets the acceptance criteria:

- if after irradiation with a UVA dose of ~ 5 J/cm² the cell viability of the solvent control is > 80% of non irradiated cells;
- if for the positive control CPZ the factor (PIF) between the two ED_{50} values is > 6; and
- if the mean OD_{540} of solvent controls is > 0.4.

Historical Data

Historical Data of the Positive Control Chlorpromazine and the Solvent Control Table 6.2.7-1

	Positive Control					**Solvent Control**	
	EC_{50} +UV [µg/mL]	**EC_{50} -UV [µg/mL]**	**L_{+UV} of L_{-UV}**	**PIF**	**MPE**	**OD irradiated cultures**	**OD non irradiated cultures**
Mean	0.48	14.74	94.32	44.73	0.595	0.707	0.754
Std. Dev.	± 0.28	± 5.58	± 7.62	± 35.78	± 0.116	± 0.171	± 0.178
Ranges	0.07 - 1.65	0.45 - 40.65	80.1 - 118.1	7.80 - 212.96	0.245 - 0.906	0.338 - 1.214	0.373 - 1.279

Data of 258 studies performed from April 2006 until March 2014

Treatment of BALB/c 3T3 with Zoxamide Table 6.2.7-2

With artificial sunlight				**Without artificial sunlight**			
Conc. [µg/mL]	**$O.D._{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**$O.D._{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.7186*	0.0814	100.00	**Solvent Control**	0.7396*	0.0340	100.00
0.49	0.7243	0.0273	100.79	**0.49**	0.7847	0.0189	106.10
0.98	0.7194	0.0088	100.11	**0.98**	0.7698	0.0435	104.09
1.95	0.7388	0.0499	102.81	**1.95**	0.7138	0.0211	96.52
3.91	0.6562	0.0226	91.32	**3.91**	0.6392	0.0196	86.43
7.81	0.6039	0.0167	84.04	**7.81**	0.5376	0.0247	72.69
15.6	0.5830	0.0113	81.14	**15.6**	0.4903	0.0213	66.29
31.3	0.5177	0.0250	72.04	**31.3**	0.4547	0.0112	61.48
62.5	0.4937	0.0157	68.70	**62.5**	0.4135	0.0131	55.91

* mean $O.D._{540\ nm}$ out of 12 wells

µg/mL ED_{50} values = could not be determined, since the viability of the cells was not reduced with and without irradiation

PIF = could not be determined, since no ED_{50} values could be calculated

MPE = -0.044

Mean $OD_{540\ nm}$ solvent control value (≙ viability) irradiated versus non-irradiated group: 97.2%

Treatment of BALB/c 3T3 with the Positive Control (chlorpromazine) table 6.2.7-3

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.$_{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.$_{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.7168*	0.0860	100.00	**Solvent Control**	0.7314*	0.0226	100.00
0.125	0.7001	0.0462	97.67	**6.25**	0.7280	0.0226	99.52
0.250	0.0693	0.0058	9.67	**12.50**	0.4670	0.0281	63.84
0.500	0.0712	0.0031	9.94	**25.00**	0.1789	0.0559	24.46
0.750	0.0730	0.0065	10.18	**37.50**	0.0678	0.0057	9.27
1.000	0.0701	0.0049	9.78	**50.00**	0.0751	0.0063	10.26
1.500	0.0677	0.0053	9.45	**75.00**	0.0569	0.0050	7.77
2.000	0.0682	0.0048	9.51	**100.00**	0.0564	0.0045	7.72
4.000	0.0727	0.0086	10.15	**200.00**	0.0584	0.0046	7.98

* mean O.D.$_{540\ nm}$ out of 12 wells

ED_{50} value (with artificial sunlight) = 0.18 µg/mL

ED_{50} value (without artificial sunlight) = 14.83 µg/mL

PIF = 83.63

MPE = 0.787

Mean OD_{540} nm solvent control value (≙ viability) irradiated versus non-irradiated group: 98.0%

Treatment of BALB/c 3T3 with Zoxamide Table 6.2.7-4

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.$_{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.$_{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.6805*	0.0757	100.00	**Solvent Control**	0.7451*	0.0538	100.00
0.49	0.6673	0.0675	98.06	**0.49**	0.7776	0.0345	104.36

0.98	0.7299	0.0471	107.25	**0.98**	0.7283	0.0461	97.74
1.95	0.7102	0.0389	104.37	**1.95**	0.7278	0.0544	97.67
3.91	0.6819	0.0243	100.21	**3.91**	0.6196	0.0515	83.15
7.81	0.5691	0.0416	83.63	**7.81**	0.5196	0.0756	69.73
15.6	0.5464	0.0343	80.29	**15.6**	0.4968	0.0457	66.67
31.3	0.4543	0.0613	66.76	**31.3**	0.4644	0.0187	62.32
62.5	0.4206	0.0562	61.81	**62.5**	0.4051	0.0040	54.37

* mean $O.D._{540\ nm}$ out of 12 wells

µg/mL ED_{50} values = could not be determined, since the viability of the cells was not reduced with and without irradiation

PIF = could not be determined, since no ED_{50} values could be calculated

MPE = -0.055

Mean $OD_{540\ nm}$ solvent control value (≙ viability) irradiated versus non-irradiated group: 91.3%

Treatment of BALB/c 3T3 with the Positive Control (chlorpromazine) Table 6.2.7-5

With artificial sunlight				**Without artificial sunlight**			
Conc. [µg/mL]	**$O.D._{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**$O.D._{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.7433*	0.0430	100.00	**Solvent Control**	0.7407*	0.0694	100.00
0.125	0.6690	0.0661	90.01	**6.25**	0.7476	0.0727	100.92
0.250	0.1044	0.0676	14.05	**12.50**	0.5106	0.0435	68.94
0.500	0.0683	0.0053	9.18	**25.00**	0.1071	0.0183	14.46
0.750	0.0706	0.0067	9.50	**37.50**	0.0741	0.0079	10.00
1.000	0.0744	0.0074	10.01	**50.00**	0.0666	0.0074	8.99
1.500	0.1002	0.0595	13.48	**75.00**	0.0678	0.0105	9.16
2.000	0.0719	0.0052	9.68	**100.00**	0.0755	0.0217	10.19
4.000	0.0721	0.0055	9.69	**200.00**	0.0810	0.0475	10.93

* mean $O.D._{540\ nm}$ out of 12 wells

ED_{50} value (with artificial sunlight) = 0.18 µg/mL

ED_{50} value (without artificial sunlight) = 14.53 µg/mL

PIF = 80.84

MPE = 0.739

Mean OD_{540} nm solvent control value (≙ viability) irradiated versus non-irradiated group: 100.3%4.1

Discussion: The study was performed to assess the phototoxic potential of Zoxamide. The test was performed using BALB/c 3T3 cells clone 31.

Two experiments were performed, the first one served as range finding experiment (RFE), the second one was the main experiment (ME). 62.5 µg/mL of the test item, dissolved in DMSO (with a final concentration of 1% in EBSS), was used as the highest concentration, and was determined by the solubility of the test item.

One test group of cells treated with the test item was irradiated with artificial sunlight for 50 minutes. Another test group of test item treated cells was kept in the dark for 50 minutes.

Slight toxicity was observed in the absence of irradiation and in the presence of irradiation with artificial sunlight. The viability of the cells did not decrease below 50%, therefore ED_{50} values could not be determined. Consequently, PIF values could not be calculated. The resulting MPE values were -0.044 and -0.055, and therefore, the test item is classified as not phototoxic.

Conclusion: It can be stated that in this study and under the experimental conditions reported, the test item Zoxamide **does not** possess any phototoxic potential.

RMS: The study is acceptable. Zoxamide does not possess phototoxic potential

B.6.3 Short-term toxicity

B.6.3.1 Oral 28-day study

B.6.3.1.1 Four-week range-finding toxicity study in dogs

Studies already peer-reviewed and presented in the original Annex II Dossier: All information presented in the original Annex II Dossier and evaluated by European authorities is summarized hereafter.

In DAR (2001)

Reference:	CA 5.3.1/01 [redacted] (1996)
Title:	RH-117,281 Technical: four-week range-finding toxicity study in dogs.
Report number:	No: 94R-234, ER Ref No: 2.3
Guidelines:	None Stated (Range finding study), but satisfied essential criteria of OECD guideline 407.
GLP:	Yes, with the exception that dietary concentrations were not verified analytically.

In a range-finding study (1996, conducted 1995), groups of 2 male and 2 female Beagle dogs were administered in the diet RH-7281 (lot no: LG3517, purity 92.9 %) at dietary concentrations of 0, 500, 5000, 15,000 and 30,000 ppm (equivalent to approximately 0, 20, 175, 542 and 1045 mg/kg bw/day for males and 0, 20, 191, 579 and 1085 mg/kg bw/day for females, respectively) for 4 weeks. All dogs were observed daily for signs of ill health or reaction to treatment. Treatment was preceded by a two-week pretest period. During pretest and throughout treatment, feed consumption was monitored daily, physical examinations were performed and body weights were determined weekly. During the first week of pretest and after two and four weeks of treatment, blood samples were collected from all dogs (fasted) for haematology and clinical chemistry analysis. After four weeks on test, all dogs were killed and organs and tissues were grossly examined. Selected organ weights were recorded at necropsy. Microscopic examination was conducted on all gross lesions and on all tissues and organs collected from all animals in the highest dose group and controls. At the start of treatment, the dogs were 5 - 6 months old and weighed.

Findings: There were no deaths during the study. There were no treatment-related clinical signs during the study at doses ≤ 5000 ppm except for soft faeces in 1/4 dogs at 5000 ppm and in both sexes at ≥ 15,000 ppm (Table B.6.3.1.1-1).

Table B.6.3.1.1-1 Summary of the main clinical signs findings in the 4-week Range-Finding Study in Dogs

Dose Level (ppm)	**0**	**500**	**5000**	**15000**	**30000**
Interval					
Week 1					
Animal Incidence*	0/4	0/4	1/4	4/4	4/4
Total incidence*	0/28	0/28	2/28	17/28	17/28
Week 2					
Animal Incidence*	0/4	0/4	1/4	4/4	4/4
Total incidence*	0/28	0/28	5/28	19/28	16/28
Week 3					
Animal Incidence*	0/4	0/4	0/4	4/4	3/4
Total incidence*	0/28	0/28	0/28	20/28	15/28
Week 4					
Animal Incidence*	0/4	0/4	1/4	3/4	3/4
Total incidence*	0/28	0/28	1/28	16/28	13/28
Weeks 1-4					
Animal Incidence*	0/4	0/4	1/4	4/4	4/4
Total incidence*	0/112	0/112	7/112	72/112	61/112

*Number of animals per group (males and females combined) for which soft faeces were recorded, and the incidence of the observation during the treatment period. Only the results of

morning observations are included since soft faeces were almost exclusively observed at that time.

There were no treatment-related effects on body weight, body weight gain or feed consumption after four weeks at doses ≤ 30,000 ppm. There were no treatment-related haematology or clinical chemistry changes after two and four weeks of treatment at doses ≤ 30,000 ppm. No treatment-related changes in absolute or relative organ weights were observed after four weeks of treatment. Gross examination at necropsy and histopathological examination of organs and tissues revealed no treatment-related changes.

Conclusion: The NOAEL in the 4-week dietary study in dogs was 30000 ppm (equivalent 1045 or 1085 mg/kg bw/day). The only finding was the occurrence of a minimal incidence of soft stools at 5000 ppm (175 or 191 mg/kg bw/day) and an increased incidence at dose levels of ≥ 15000 ppm, however, soft faeces was not reported in other studies in dogs with a longer duration.

RMS: The study is acceptable. The NOAEL in study is 30000 ppm (equivalent 1045 or 1085 mg/kg bw/day).

B.6.3.2. Oral 90-day study and 1 year study

B.6.3.2.1 three-month dietary toxicity study in mice.

In DAR May (2001)

Reference:	CA 5.3.2/01, [redacted] (1996)
Title:	RH-117,281: three-month dietary toxicity study in mice.
Report number:	No: 94R-075, ER Ref No: 5.1
Guidelines:	OECD 408
GLP:	Yes

In a study (1996), groups of 10 male and 10 female Crl:CD-1 (ICR) BR mice were administered RH-7281 (lot no: DK2011; 94.2% active ingredient) in the diet for 3 months at concentrations of 0 (control), 70, 700, 2500 and 7000 ppm (equivalent to 0, 12, 123, 436 and 1212 mg/kg bw/day respectively in males, and 0, 17, 174, 574, 1666 mg/kg bw/day in females. All mice were observed daily for signs of ill health or reaction to treatment. Physical examinations were performed weekly. Body weight and feed consumption were determined weekly. At the end of the study period, all surviving mice were bled for haematology and clinical chemistry investigations, killed and necropsied. Selected organ weights were recorded and tissues were collected for histopathological evaluation.

Findings: No treatment-related deaths or clinical signs of toxicity were observed. There were no treatment-related effects on body weight or body weight change in males at any dose level. After 4 weeks of treatment, there was a persistent and apparently treatment-related but statistically non-significant decrease in body weight and cumulative body weight gain in females at 7000 ppm compared with concurrent controls (Table B.6.3.2.1-1). In male animals a number of statistically significant decreases in mean body weight and cumulative body weight gain were observed in the 700 ppm and 2500 ppm dose groups throughout the treatment period but these decreases were not considered treatment-related due to the absence of a dose-response. There was no treatment-related effect on feed consumption in either sex

at any dose level. However, in females there was a statistically non-significant but dose-related reduction in body weight gain (34 %) and in body weight in females at the 7000 ppm dose level compared with controls. It is noted that the achieved dosage in females was relatively much greater than that of top dose males.

Table B.6.3.2.1-1Summary of body weights and cumulative body weight gain in mice.

Dose Level (ppm)	0	70	700	2,500	7,000
Parameter/Interval					
Males					
Body Weight					
Week 1 (g)	30.8	30.4	30.2	29.2	29.8
Week 4	34.6	33.7	**33.2***	32.0	32.8
Week 8	37.9	36.3	**35.8***	**34.3***(↓10.5%)	35.4
Week 13	41.1	38.5	**38.0***	36.7	38.3
Cumulative Body Weight Gain Week 13 (g)	12.3	9.5	**9.1***	**8.9***	10.0
Females					
Body Weight					
Week 1 (g)	24.2	24.3	23.7	23.1	22.4
Week 4	26.8	26.8	25.6	25.1	**23.6**(↓13.5%)
Week 8	28.1	28.9	27.3	26.8	**25.0**(↓12.4%)
Week 13	30.6	31.1	29.3	28.7	**25.8**(↓18.6%)
Cumulative Body Weight Gain Week 13 (g)	7.4	7.7	7.0	5.9	4.9

* Significant difference from control ($p<0.05$); ANOVA Dunnett's t-test

Haematology and clinical chemistry parameters did not reveal any significant treatment-related differences.There were no treatment-related effects on absolute or relative organ weights in either sex at any dose level. No treatment-related gross pathological changes or histopathological findings were observed in any tissues.

Conclusion: The NOAEL in the 90-day dietary study in mice was 2500 ppm (equivalent 574 mg/kg bw/day) based on reduction in body weight gain and in overall body weight in female mice at 7000 ppm (1666 mg /kg bw/day).

RMS comment: The study is acceptable. The NOAEL in the 90-day dietary study in mice was 2500 ppm (equivalent 574 mg/kg bw/day.

B.6.3.2.2 Three-month dietary toxicity/neurotoxicity study in rats

In DAR (2001)

Reference:	CA 5.3.2/02 (5.7.1/02), [redacted] (1996a)
Title:	RH-117,281: three-month dietary toxicity/neurotoxicity study in rats.
Report number:	No: 94R-233, ER Ref No: 3.1
Guidelines:	Not stated, but satisfied the essential criteria of OECD guideline # 408
GLP:	Yes

In a study (1996, in-life 1995) groups of 15 male and 15 female Crl:CD®BR rats were administered RH-7281 (Lot no: LG3517; purity 92.9 %) in the diet for three months at concentrations of 0 (control), 1000, 5000, and 20,000 ppm (equivalent to 0, 74, 372, and 1509 mg/kg bw/day respectively in males, and 0, 80, 401, and 1622 mg/kg bw/day in females). All rats were observed daily for signs of ill health or reaction to treatment. Physical examinations were performed weekly. Body weight and feed consumption were monitored weekly. Of the 15 rats/sex/group, 10 were randomly selected for in-life neurotoxicity testing via a standard battery of behavioural observations (Functional Observational Battery) and motor activity testing during pretest and at weeks 4, 8, and 13 of dosing. From the ten rats/sex/group, five were randomly selected prior to terminal necropsy for whole body perfusion and special neuropathology evaluation. Hematology and clinical chemistry tests were conducted on blood samples taken from the remaining surviving rats. The rats were then killed and necropsied. Selected organ weights were recorded and tissues were collected for routine histopathological evaluation. Urinalysis was performed on these animals during week 13 of treatment. All animals were given ophthalmologic exams prior to pretest and during week 13 of the study.

Findings: No treatment-related mortalities or clinical signs of toxicity were observed during the study period. Body weight gain and feed consumption showed not treatment-related intergroup differences. There were no treatment-related effects on any hematology, clinical chemistry, or urinalysis parameters. The function observation battery and motor activity assessments for evidence of neurotoxicity did not show any treatment-related abnormalities. No treatment-related effects on organ weights or ophthalmologic changes were observed. There were no observations of treatment-related macroscopic or histopathological changes in either the routine necropsy or perfusion neuropathology animals of either sex.

Conclusion: The NOAEL for RH-7281 in the 3-month dietary study in rats was 20000 ppm (equivalent to 1509 or 1622 mg/kg bw/day in males or females respectively).

RMS comment: The study is acceptable. The NOAEL for RH-7281 in the 3-month dietary study in rats was 20000 ppm (equivalent to 1509 or 1622 mg/kg bw/day in males or females respectively

B.6.3.2.3 90-day oral study in dogs

Reference:	CA 5.3.2/03, [redacted] (1997)

Title: RH-117,281 Technical: three-month dietary toxicity study in dogs.

Report number: No: 96R-030 ER Ref No: 9.1

Guidelines: OECD 409 ≅ OPPTS 870.3150

GLP: Yes

In a study (1997, in-life 1996) groups of 4 male and 4 female Beagle dogs were each administered in the diet RH-7281 (lot no: DSR-9510, purity 92.3 %) for at least 13 weeks at concentrations of 0, 1500, 7500 or 30,000 ppm (equivalent to 0, 54.5, 281.1, and 1139.3 mg/kg bw/day respectively in males, and 0, 61.8, 321.6 and 1054.5 mg/kg bw/day respectively in females). One female dog in the 1500 ppm dose group was replaced with another dog during the third week of dosing, hence the treatment period was extended to 16 weeks.

All dogs were observed daily for signs of ill health or toxicity. Feed consumption was determined daily for all animals beginning two weeks prior to treatment (i.e., pretest) and throughout the treatment period. Physical examinations were performed and body weights were determined weekly. Urinalysis was conducted on all dogs during pretest and on all dogs surviving to necropsy. Blood samples were collected from all dogs for haematology and clinical chemistry analyses during pre-test, and after 8 and 16 weeks of treatment. Ophthalmology examinations were performed on all dogs during pre-test and after the treatment period. After the treatment period, all dogs were anaesthetised, killed and necropsied. Selected organs were weighed and tissues were collected for histopathological evaluation.

Findings: There were no treatment-related deaths or clinical signs indicative of systemic toxicity through the treatment period. There was a treatment-related decrease in mean body weight, cumulative body weight gain, and feed consumption in males and females in the 30,000 ppm group (Table B.6.3.2.3-1).

Table Table B.6.3.2.3-1Summary of the changes in body weight and feed consumption in 3-month dog study

Dose Level (ppm)	**0**	**1500**	**7500**	**30,000**
Parameter/Interval				
Males				
Body Weight (g)				
Week 1	8,339	8,091	8,118	7,515
Week 8	9,861	9,714	9,584	7,803*
Week 16	11,207	11,111	10,773	8,833*
Body Weight Gain (g)				
Week 1-16	3,296	3,290	2,718	1,296*
Food Consumption (g)				
Week 1				

Dose Level (ppm)	**0**	**1500**	**7500**	**30,000**
Parameter/Interval				
Week 8	290	300	285	226*
Week 16	364	366	343	297*
	369	351	381	348
Females				
Body Weight (g)				
Week 1	6,885	7,116	6,753	6,529
Week 8	8,043	7,749	7,728	6,669
Week 16	8,876	8,561	8,559	7,289
Body Weight Gain (g)				
Week 1-16	2,314	1,658	1,899	824
Food Consumption (g)				
Week 1	270	278	280	187*
Week 8	330	330	341	243*
Week 16	319	324	340	246*

* Significant difference from control ($p<0.05$), ANOVA, Dunnett's t-test

Haematology investigations did not reveal any treatment-related changes in either sex at dose levels of ≤ 7500 ppm. However, some statistically significant changes including decreased red blood cell count, increased mean cell haemoglobin, and increased mean cell haemoglobin concentration were seen in the 30,000 ppm females. A similar but statistically non-significant trend was seen in males. Decreased lymphocytes and increased segmented neutrophils were also observed in males at the 30000 ppm dose level in the differential white blood cell counts but the total white blood cell count did not show any treatment-related differences. The investigators considered the toxicological significance of these haematological changes to be unclear. It is noted that the haematological findings were not repeated in the one-year dog study (Table B.6.3.2.3-2)

Table B.6.3.2.3-2 Summary of haematological changes in 3-month dietary study in dogs

Dose Level (ppm)	**0**	**1500**	**7500**	**30,000**
Males				
RBC (10^6/CMM)				
Week 8	5.92	6.05	6.11	5.72
Week 16	6.61	6.75	6.45	5.88

Dose Level (ppm)	**0**	**1500**	**7500**	**30,000**
MCH (pg)				
Week 8	21.4	22.0	21.3	22.1
Week 16	21.5	21.9	21.6	22.6
MCHC (%)				
Week 8	34.0	34.2	34.3	34.7
Week16	34.2	34.1	34.4	34.9
Segmented Neutrophils (%)				
Week 8	64	62	64	69
Week 16	56	71	77	83*
Lymphocytes (%)				
Week 8	31	35	30	30
Week 16	38	26	20	14*
Females				
RBC (10^6/CMM)				
Week 8	6.33	6.07	5.89	5.71*
Week 16	7.01	6.57	6.63	5.98
MCH (pg)				
Week 8	21.7	22.1	22.0	23.5*
Week 16	21.7	22.3	22.2	24.3*
MCHC (%)				
Week 8	34.0	34.3	34.2	35.1
Week16	33.7	34.6	34.5	35.8*

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Clinical chemistry parameters did not show any changes in either sex at dose levels of ≤ 7500 ppm. At 30000 ppm, apparent treatment-related decreases in albumin and albumin/globulin ratio were seen in both sexes after 8 and 16 weeks of treatment (Table B.6.3.2.3-3).

Table B.6.3.2.3-3 Summary of clinical chemistry changes in 3-month dietary study in dogs

Dose Level (ppm)	0	1500	7500	30,000

Dose Level (ppm)	0	1500	7500	30,000
Males				
Albumin (g/dl)				
Week 8	3.4	3.4	3.4	3.0
Week 16	3.6	3.8	3.6	3.1*
Albumin/globulin ratio				
Week 8	1.5	1.6	1.5	1.2*
Week 16	1.5	1.7	1.4	1.1*
Females				
Albumin (g/dl)				
Week 8	3.6	3.5	3.5	3.2
Week 16	3.9	3.8	3.7	3.3
Albumin/globulin ratio				
Week 8	1.8	1.5	1.6	1.4
Week 16	1.7	1.6	1.6	1.3

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Urinalysis parameters did not show any treatment-related differences. Ophthalmology at 16 weeks showed no treatment related effects. Organ weights showed a treatment-related increase in the absolute and relative liver weights in females at ≥ 7500 ppm and in males at 30,000 ppm. The increase in absolute (≥ 23 %) and relative liver weight (29 %) of the 7500 ppm females was considered to be treatment-related but not an adverse effect since no corresponding clinical pathology or histopathological findings were observed at the 7500 ppm dose (Table B.6.3.2.3-4). There were no treatment-related gross findings. Treatment-related microscopic changes were observed in the 30000 ppm dose group and consisted of a diffuse hepatocellular hypertrophy affecting all male and female dogs, and hypertrophy of the thyroid follicular epithelium in one male and one female dog. Although the microscopic changes in the thyroid were at a low incidence, this change was considered treatment-related since this effect is frequently associated with hepatocellular hypertrophy and microsomal enzyme induction (Table B.6.3.2.3-4).

Table B.6.3.2.3-4 Summary of the organ weight and histopathological findings in the 3-month dog study

Dose Level (ppm)	**0**	**1500**	**7500**	**30,000**
Males				

Absolute Liver (g)	304.350	319.000	309.500	370.895
Relative Liver (%)	2.664	2.804	2.801	4.141*
Females				
Absolute Liver (g)	244.275	268.950	299.725	328.600
Relative Liver (%)	2.678	3.111	3.444*	4.375*
Histopathology				
	# Affected / # Examined			
Males				
Liver Hypertrophy	0/4	0/4	0/4	4/4
Thyroid Hypertrophy	0/4	0/4	0/4	1/4
Females				
Liver Hypertrophy	0/4	0/4	0/4	4/4
Thyroid Hypertrophy	0/4	0/4	0/4	1/4

*Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

During the study, three dogs (one 7500 ppm male; two 30,000 ppm dogs, 1 male and 1 female) treated with RH-7281 showed symptoms and/or pathologic evidence of canine juvenile polyarteritis syndrome. It was stated by the investigators that this is an idiopathic syndrome considered a latent condition whose clinical, clinical pathology, and histopathological manifestations may be precipitated in predisposed dogs by exposure to various xenobiotics. The manifestations of this syndrome were therefore not considered direct toxic effects of RH-7281, but were an indirect expression of an underlying condition. Furthermore, this disease was noted to be specific to dogs, most commonly beagle dogs, and therefore not considered relevant to human health. Therefore, the effects observed in the dogs diagnosed with CJPS were not considered adverse with respect to humans.

Conclusion: The NOAEL in the dog was considered to be 7500 ppm (equivalent to 281 or 321 mg/kg bw/day in males and females respectively) based on treatment-related changes including the reduction in body weight and in body weight gain in both sexes, haematological changes in red blood cell parameters in females, increase in absolute and relative liver weights of the liver accompanied by evidence of liver hypertrophy and an incidence of thyroid hypertrophy in both sexes at 30 000 ppm (equivalent 1054 or 1139 in males or females respectively. An increase in absolute liver weight and a statistically significant increase in relative liver weights were noted at the 7500 ppm dose level but the changes in organ weights were not accompanied by any histopathological change.

RMS comment:

In ECCO meeting the NOAEL value for this study was changed.
From ECCO 118 meeting:
The meeting considered the 3 short-term studies in dogs as the most critical studies, as the dog seems to be the most sensitive species concerning the short-term toxicity of zoxamide. Some Member States were concerned with the rapporteur Member State's (UK) proposal for the lowest relevant oral NOAEL at 7500 ppm. The experts had a long discussion whether more than 20 % increase of the relative liver weight in

the 90-day and 1-year dog study is an adverse effect or not, as there were no changes in clinical chemistry and histopathology at the dose level of 7500 ppm. The meeting concluded that the liver is the main target and that a significant reduction in bodyweight gain only occurred at higher dose levels; therefore, the increased liver weights at 7500 ppm should be considered as an adverse effect.

The meeting agreed on a NOAEL of 50 mg/kg bw/d (1500 ppm) based on the 90-day and 1-year dog study.

The RMS agreed with a NOAEL of 50 mg/kg bw/d (1500 ppm).

Because thyroid is one of organs related to endocrine disruptors, RMS asked for clarification regarding effects seen on thyroid both in 90-day and 1year study.

The applicant response:
With respect to any thyroid effects in the 3-month dog study, hypertrophy of the thyroid follicular epithelium (thyroid hypertrophy) was only seen at doses in excess of 1000 mg/kg bw/day (30000 ppm), and only in one out of the four animals available for each sex in that dose group. In contrast, diffuse hepatocellular hypertrophy (liver hypertrophy) was present in all animals at this dose level. Liver weights were also increased at the top dose (relative to body weight increased by 55% in males and 63% in females) and there was an effect on liver weight at the mid dose in females (ca. 280-321 mg/kg bw/day, 7500 ppm; relative to body weight increased by 28%). Thyroid effects were absent at the mid dose. In the top dose group bodyweight gain was under half that in other groups at the end of the study. See Table 1:

Table 1. Summary of the body weight gain, organ weight and histopathological findings in the 3-month dog study

Dose Level (ppm)	0	1500	7500	30,000
Males				
Body Weight Gain (g) Week 1-16	3296	3290	2718	1296*
Females				
Body Weight Gain (g) Week 1-16	2314	1658	1899	824
Males				
Absolute Liver (g)	304.350	319.000	309.500	370.895
Relative Liver (%)	2.664	2.804	2.801	4.141*
Females				
Absolute Liver (g)	244.275	268.950	299.725	328.600
Relative Liver (%)	2.678	3.111	3.444*	4.375*
Histopathology				
	# Affected / # Examined			
Males				
Liver Hypertrophy	0/4	0/4	0/4	4/4
Thyroid Hypertrophy	0/4	0/4	0/4	1/4
Females				
Liver Hypertrophy	0/4	0/4	0/4	4/4
Thyroid Hypertrophy	0/4	0/4	0/4	1/4

*Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

The 1-year dog study was conducted at the same dietary dose levels, resulting in very similar body burdens over the course of the study to those in the 3-month study (255-280 mg/kg bw/day at 7500 ppm and ca. 1000 mg/kg bw/day at 30000 ppm). At the top two dose levels in both sexes, thyroid hypertrophy

was not seen, but increases in thyroid weight were seen at the top dose and potentially at the mid dose in males and females. Body weight gain at the top dose was ca. 80% of controls in males and ca. 65% of controls in females.

Table 2. Summary of the body weight, organ weight and histopathological changes in 1-year dietary study in dogs

Dose Level (ppm)	0	1500	7500	30,000
Males				
Body Weight Gain (g)				
Week 4	1093	1058	799	254*
Week 13	2794	2737	2773	1584*
Week 25	3777	4027	4357	2836
Week 52	3888	4283	4672	3159
Females				
Body Weight Gain (g)				
Week 4	895	877	382*	-141*
Week 13	2238	2227	1567	1056*
Week 25	3172	2730	2425	1523
Week 52	3167	2795	2538	2052
Males				
Absolute Liver (g)	293.050	330.250	353.575	412.750*
Relative Liver (%)	2.501	2.735	2.911	3.712*
Absolute Thyroid (g)	0.811	0.920	1.067	1.044
Relative Thyroid (%)	0.007	0.007	0.009	0.010
Females				
Absolute Liver (g)	278.075	276.350	309.500	341.033
Relative Liver (%)	2.748	2.915	3.367*	3.822*
Absolute Thyroid (g)	0.730	0.749	0.810	0.983*
Relative Thyroid (%)	0.007	0.008	0.009	0.011*
Histopathology				
	# Affected / # Examined			
Males				
Liver Hypertrophy	0/4	0/4	0/4	2/4
Females				
Liver Hypertrophy	0/4	0/4	0/4	1/4

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

It is possible that thyroid hypertrophy may have been due to liver enzyme induction in dogs, although there is no T3/T4 data to corroborate this. Clearance of thyroid hormone via enzymic degradation in the liver will occur in all mammals, although the exact hepatic enzymes responsible, or isoforms thereof, may vary from species to species. In the case of the 3-month study, thyroid hypertrophy as a result of enhanced clearance of thyroid hormone might be considered unlikely, because it was seen in only 1/4 animals/sex at the top dose, where liver hypertrophy was already present in 4/4 animals/sex at the same dose.

It may be that the thyroid hypertrophy in the 3-month study was a chance incidental finding. It was not seen in any other study with zoxamide.

Another hypothesis for the increased thyroid weights in the 1-year dog study at least (but possibly also the thyroid hypertrophy in the 3-month study), is that it may be connected to the reduced rates of body weight gain[1],[2]. In the absence or any other thyroid pathology, it may be a secondary adaptive response connected

[1] Cabello, G., & Wrutniak, C. (1989). Thyroid hormone and growth: relationships with growth hormone effects and regulation. *Reproduction Nutrition Development*, *29*(4), 387-402.

in some way to the reduced body weight gain and not a direct adverse effect of treatment. The increases in weight were not accompanied by any histopathology finding.
If the thyroid hypertrophy in the 3-month study is treatment related, then its amelioration to simple increased weights at 12 months indicates it is regressing, rather than progressing in severity/ adversity. Much of the bodyweight deficit at 3 months had also been re-gained by 12 months.
Overall, the thyroid effects in dogs are most likely a secondary adaptive effect, rather than a direct adverse effect of treatment. Not least because it is only seen or most pronounced at/above the limit dose of 1000 mg/kg bw/day, and there were not corroborating thyroid effects seen in the rodent studies (see below). The 2-year rat study also being conducted up to the limit dose of 1000 mg/kg bw/day.
Given the extremely high dosages required to produce any thyroid effects in dogs, it is considered unlikely that these findings have any relevance for human exposure.

RMS: comment: RMS partly agrees with this justification because direct adverse effect of treatment could not be excluded as no hormone levels have been measured.

As there is well known mode of action that could explain non relevance for humans, this mode of action is not proved by the applicant, as no hormone levels have been measured. Also this mode of action is specific to the rodents not dogs. RMS believes that taking into account all data zoxamide is not an endocrine disruptor as there were no effects e.g. effects on endocrine organs weights (except liver in 2 studies) or pathology, reproduction, time to reach developmental miles stones, oestrus cycling, or gamete production or function that would indicate zoxamide possessed any endocrine activity.

Zoxamideis considered not to have endocrine disrupting properties on the basis that it is not or has not to be classified in accordance with the provisions of Regulation (EC) No. 1272/2008 as carcinogen category 2 and / or toxic for reproduction category 2. Furthermore, the available toxicity data on zoxamide demonstrate that there are no adverse effects on endocrine tissues or any evidence of perturbation of endocrine function. Therefore, it is concluded that zoxamide is not likely to have endocrine disrupting properties.

B.6.3.3 .2.4 One-year chronic dietary toxicity study in dogs

In DAR (2001)

Reference:	CA 5.3.2/04, [redacted] (1998c)
Title:	RH-117,281 Technical: one-year chronic dietary toxicity study in dogs.
Report number:	No: 95R-277, ER Ref No: 25.1
Guidelines:	OECD 452
GLP:	Yes

In a study (1998, in-life 1996/97), groups of 4 male and 4 female beagle dogs were administered in the diet RH-7281 ((lot no: DSR-9510, purity 92.3%) for one year at concentrations of 0, 1500, 7500 or 30,000 ppm (0, 50, 255, and 1016 mg/kg bw/day respectively in males, and 0, 48, 278 and 994 mg/kg/day

[2] Decuypere, E., Buyse, J., Scanes, C. G., Huybrechts, L., & Kühn, E. R. (1987). Effects of hyper-or hypothyroid status on growth, adiposity and levels of growth hormone, somatomedin C and thyroid metabolism in broiler chickens.*Reproduction Nutrition Développement*, *27*(2B), 555-564.

respectively in females). All dogs were observed daily for signs of ill health or reaction to treatment. Feed consumption was determined daily for all animals beginning two weeks before treatment (i.e., pretest) and continued until the end of week 13 of treatment. Thereafter, feed consumption was measured for a one-week period every four weeks until the end of the study. Body weights were determined weekly for all animals beginning two weeks before treatment (i.e., pretest) and continued until the end of the thirteenth week of treatment. Thereafter, body weights were measured once every four weeks until the end of the study. Physical examinations were performed weekly beginning two weeks prior to treatment. Clinical chemistry, haematology, and urinary parameters were evaluated on all dogs during pretest, after 3, 6, and 9 months of treatment, and on all dogs surviving to necropsy. Ophthalmologic examinations were performed on all dogs during pretest and just before the end of treatment. After the treatment period, all dogs were anaesthetized, killed, and necropsied. Selected organs were weighed and tissues were collected for histopathological evaluation.

Findings: There were no treatment-related deaths or clinical signs indicative of systemic toxicity during the study. There was a treatment-related reduction in mean body weight, cumulative body weight gain, and feed consumption in both sexes at 30000 ppm compared with controls. In females there was treatment-related transient reduction in body weight and cumulative body weight gain early during treatment with recovery by the end of the study (Table B.6.3.2.4-1).

Table B.6.3.2.4-1 Summary of changes in body weight and feed consumption in 1-year dietary study in dogs.

Parameter/Interval	Dose Level (ppm)			
	0	1500	7500	30,000
Males				
Body Weight (g)				
Week 4	8,939	9,094	8,374	8,136
Week 13	10,640 11,623	10,773	10,349	9,466
Week 25	11,734	12,063	11,933	10,718
Week 52		12,318	12,247	11,041
Body Weight Gain (g)				
Week 4	1,093	1058	799	254*
Week 13	2,794	2737	2773	1584*
Week 25	3,777	4027	4357	2836
Week 52	3,888	4283	4672	3159
Food Consumption (g)				
Week 1	290.1	285.7	241.2	217.1
Week 25	324.4	343.1	330.8	359.8
Females				

Parameter/Interval	Dose Level (ppm)			
	0	1500	7500	30,000
Body Weight (g)				
Week 4	7,898	7,658	7,137	6,839*
Week 13	9,242	9,008	8,322	8,035
Week 25	10,176	9,511	9,179	8,503*
Week 52	10,171	9,576	9,293	8,912
Body Weight Gain (g)				
Week 4	895	877	382*	-141*
Week 13	2,238	2,227	1,567	1056*
Week 25	3,172	2,730	2,425	1,523
Week 52	3,167	2,795	2,538	2,052
Food Consumption (g)				
Week 1	259.9	254.4	250.5	196.4*
Week 25	281.6	252.6	313.5	274.2

Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Haematology and urinalysis parameters did not reveal any treatment-related intergroup differences. Clinical chemistry parameters showed treatment-related decreases in albumin and increases in alkaline phosphate in both sexes after 3, 6, 9, and 12 months of treatment at 30000 ppm (Table Table B.6.3.2.4-2).

Table Table B.6.3.2.4-2 Summary of clinical chemistry changes in 1-year dietary study in dogs

Dose Level (ppm)	0	1500	7500	30,000
Males				
Albumin (g/dL)				
Pretest	3.7	3.3	3.4	3.6
Day 93	3.7	3.7	3.5	3.4*
Day 183	3.7	3.5	3.6	3.3*
Day 274	3.7	3.4	3.4	3.2*
Day 365	3.8	3.7	3.7	3.5
Alkaline Phosphatase (U/L)				

Dose Level (ppm)	0	1500	7500	30,000
Pretest				
Day 93	260	402	498	325
Day 183	190	241	388	367
Day 274	122	168	275	434*
Day 365	117	174	236	412*
	97	159	200	414*
Females				
Albumin (g/dL)				
Pretest	3.4	3.6	3.5	3.3
Day 93	3.6	3.8	3.6	3.2*
Day 183	3.6	3.8	3.5	3.1
Day 274	3.6	3.8	3.5	3.3
Day 365	3.7	3.6	3.6	3.3*
Alkaline Phosphatase (U/L)				
Pretest	283	314	311	290
Day 93	208	177	258	311*
Day 183	170	116	242	297*
Day 274	176	109	219	304*
Day 365	152	108	183	330*

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Organ weights showed a treatment-related increase in absolute and relative liver weights in both sexes at 30,000 ppm and in females at 7500 ppm. A treatment-related increase in absolute and relative thyroid weights was also observed in the females at 30,000 ppm (Table Table B.6.3.2.4-3).

Ophthalmology at termination did not reveal any treatment-related ocular changes. No treatment-related gross findings were made at necropsy. Treatment-related microscopic changes were observed in the 30,000-ppm dose group and consisted of diffuse hepatocellular hypertrophy in two males and one female dog (Table Table B.6.3.2.4-3).

Table Table B.6.3.2.4-3 Summary of the organ weigh and histopathological changes in 1-year dietary study in dogs

Dose Level (ppm)	0	1500	7500	30,000
Males				
Absolute Liver (g)	293.050	330.250	353.575	412.750*
Relative Liver (%)	2.501	2.735	2.911	3.712*
Absolute Thyroid (g)	0.811	0.920	1.067	1.044
Relative Thyroid (%)	0.007	0.007	0.009	0.010
Females				
Absolute Liver (g)	278.075	276.350	309.500	341.033
Relative Liver (%)	2.748	2.915	3.367*	3.822*
Absolute Thyroid (g)	0.730	0.749	0.810	0.983*
Relative Thyroid (%)	0.007	0.008	0.009	0.011*
Histopathology				
	# Affected / # Examined			
Males				
Liver Hypertrophy	0/4	0/4	0/4	2/4
Females				
Liver Hypertrophy	0/4	0/4	0/4	1/4

Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

During this study, two dogs (one 30,000 ppm female, one 1500 ppm male) treated with RH-7281 displayed symptoms and/or pathologic evidence of canine juvenile polyarteritis syndrome (CJPS). The investigators submitted that this is an idiopathic syndrome which is considered a latent condition whose clinical, clinical pathological, and histopathological manifestations may be precipitated in predisposed dogs by exposure to various xenobiotics. Therefore the investigators did not consider the manifestations of this syndrome to be direct toxic effects of RH-7281 but that they were an indirect expression of an underlying condition. Furthermore, this disease was noted to be specific to dogs, most commonly beagle dogs, and therefore, was not considered relevant to human health. Therefore, the effects observed in the dogs diagnosed with CJPS were not considered adverse with respect to humans.The applicant provided detailed comments on the observed changes in the liver and the thyroid. These discussions are noted to be well established scientific observations but there is limited mechanistic data on RH-7281 in support of the conclusions.

Conclusion: To conclude, the overall NOAEL in the one-year dietary study in the dog was 7500 ppm (equivalent 255 or 278 mg/kg bw/day in males or females respectively). This was based on reduction in body weight gain, clinical chemistry changes including reduction in albumin levels and increased alkaline phosphatase; increase in the absolute and relative liver and thyroid weights and increase incidence of liver hypertrophy in both sexes at 30000 ppm (equivalent 1016 or 994 mg/kg bw/day).

RMS comment: In ECCO meeting the NOAEL value for this study was changed.

From ECCO 118 meeting:
The meeting considered the 3 short-term studies in dogs as the most critical studies, as the dog seems to be the most sensitive species concerning the short-term toxicity of zoxamide. Some Member States were concerned with the rapporteur Member State's (UK) proposal for the lowest relevant oral NOAEL at 7500 ppm. The experts had a long discussion whether more than 20 % increase of the relative liver weight in the 90-day and 1-year dog study is an adverse effect or not, as there were no changes in clinical chemistry and histopathology at the dose level of 7500 ppm. The meeting concluded that the liver is the main target and that a significant reduction in bodyweight gain only occurred at higher dose levels; therefore, the increased liver weights at 7500 ppm should be considered as an adverse effect.

The meeting agreed on a NOAEL of 50 mg/kg bw/d (1500 ppm) based on the 1-year dog study.

The RMS agreed with a NOAEL of 50 mg/kg bw/d (1500 ppm).

B.6.3.3 Other routes

B.6.3.3.1 28-day dermal study in the rat

In DAR (2001)

Reference:	CA 5.3.3/01, [redacted] (1998d)
Title:	RH-117,281 Technical: twenty-eight day dermal toxicity study in rats
Report number:	No: 97R-075, ER Ref No: 23.3
Guidelines:	OECD 410
GLP:	Yes

In a study (1998), a dose of 0 (control), 150, 400 or 1000 mg/kg bw/day RH-7281 (lot no: R63240, 93.83 % purity), was moistened with tap water (1:2 w/v) and applied topically under an occlusive dressing to the shaved intact skin (an area approximately 10% of the total body surface area) of groups of 10 male and 10 female Crl:CD®BR rats for at least 6 hrs/day, 5 days/week over at least a 28 day period resulting in a total of at least 22 daily applications. After the 6 hr exposure, the occluded dressing was removed and the exposure site of each animal was washed with a 1% Ivory® Soap solution. The exposure sites were wiped with paper towels saturated with tap water and gently blotted dry with paper towels. The 0 mg/kg (control) group was treated in a manner which was identical to animals in all other groups except they were dosed with tap water only (based on the same volume of water used in the 1000 mg/kg group).

All animals were observed at least once daily for mortality, signs of ill health or reaction to treatment. A more detailed clinical exam was performed weekly beginning 1 week prior to the start of dosing. Application sites were evaluated for skin irritation effects prior to each daily dose and the terminal sacrifice. Body weight (weekly), feed consumption (weekly), haematology and clinical chemistry (terminal sacrifice) parameters were measured. An indirect ophthalmoscopic examination was performed prior to initiation of dosing and at the end of the dosing period. At the end of the 28-day treatment period,

all rats were anaesthetized, exsanguinated, and necropsied. Selected organ weights were weighed and tissues were collected for histopathological evaluation.

Findings: There were no mortalities or clinical signs indicative of systemic toxicity in any of the treatment groups. Body weight and feed consumption were not affected by the treatment. No treatment-related effects in mean body weight or feed consumption were noted. However, the treated skin sites showed a dose-related increase in the incidence of reddening and scabbing with the duration of treatment (Table B.6.3.3.1-1)

Table B.6.3.3.1-1Summary of the findings of irritancy to the skin in the 28-day dermal toxicity study in rats.

Dose Level (mg ai/kg bw/day)		**0**	**150**	**400**	**1000**
		# Affected (10 animals per group)			
Males					
Reddened Areas:	Day 7	0	0	0	0
	Day 14	0	0	0	0
	Day 30	0	1	3	2
Scabbed Areas:	Day 7	0	0	1	0
	Day 14	0	2	5	6
	Day 30	0	3	8	9
Females					
Reddened Areas:	Day 7	0	0	0	0
	Day 14	0	0	0	0
	Day 30	2	7	5	7
Scabbed Areas:	Day 7	0	0	1	4
	Day 14	2	6	9	9
	Day 30	1	8	9	10

There were no treatment-related ocular abnormalities. No treatment-related changes in any haematology parameters were observed. Clinical chemistry tests showed statistically significant decreases in albumin and increases in globulin in females at 400 and 1000 mg/kg bw/day with corresponding decreases in the albumin/globulin ratio at 150, 400 and 1000 mg/kg bw/day. These were considered by the investigators to be secondary to the skin irritation seen in all dose groups. It is noted that a decrease in albumin was also reported in oral studies in the dog and hence the suggested explanation for the change in albumin is not considered to be conclusive. However, it is noted that there was no further evidence of target organ toxicity to suggest a treatment-relationship. (Table B.6.3.3.1-2)

B.6.3.3.1-2 Summary of clinical chemistry changes in 28-day dermal toxicity study in rats:

Dose Level (mg ai/kg bw/day)	0	150	400	1000
Females				
Albumin (g/dL)	4.2	3.9	3.7*	3.7*
Globulin (g/dL)	1.7	1.9	2.0*	2.1*
Albumin/Globulin ratio	2.5	2.1*	1.9*	1.8*

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Organ weight measurements did not reveal any significant treatment-related differences in absolute or relative organ weights. Treatment-related gross and histopathological findings were restricted to the treated skin of male and female rats at all dose levels. Microscopic changes included hyperplasia and hyperkeratosis of the epidermis with occasional areas of superficial necrosis and exudate formation and multifocal spongiosis (interepidermal edema). Changes in the dermis included enlarged (hyperplastic) sebaceous glands, mixed inflammatory cell infiltrations in the dermis and vasculitis/perivasculitis affecting small to medium-sized vessels in the deeper dermis (B.6.3.3.1-3).

Table B.6.3.3.1-3Summary of histopathological changes in the skin in 28-day dermal toxicity study in rats

Dose Level (mg/kg bw/day)	0	150	400	1000
	# Affected / # Examined			
Males				
Skin: Exudate, surface	0/10	3/10	4/10	7/10
Skin: Epidermis				
Hyperplasia/hyperkeratosis	10/10	10/10	10/10	10/10
Necrosis, focal/multifocal	0/10	3/10	3/10	4/10
Spongiosis, focal/multifocal	0/10	3/10	3/10	8/10
Skin: Dermis				
Hyperplasia, sebaceous glands	7/10	10/10	10/10	9/10
Inflammation, multifocal	0/10	10/10	10/10	10/10
Vasculitis/perivasculitis	0/10	3/10	8/10	7/10

Dose Level (mg/kg bw/day)	0	150	400	1000
	# Affected / # Examined			
Females				
Skin: Exudate, surface	0/10	8/10	3/10	8/10
Skin: Epidermis				
Hyperplasia/hyperkeratosis	9/10	10/10	10/10	10/10
Necrosis, focal/multifocal	0/10	8/10	6/10	6/10
Spongiosis, focal/multifocal	0/10	7/10	6/10	8/10
Skin: Dermis				
Hyperplasia, sebaceous glands	3/10	9/10	8/10	7/10
Inflammation, multifocal	0/10	9/10	9/10	6/10
Vasculitis/perivasculitis	0/10	8/10	9/10	10/10

Conclusion: To conclude, RH-7281 produced significant dermal effects at dose levels of ≥ 150 mg/kg bw/day. The only evidence of apparent systemic toxicity were changes in albumin and globulin which is of uncertain significance in the absence of any further evidence of target organ toxicity. The overall NOAEL was 1000 mg/kg bw/day based on the absence of any clear evidence of systemic toxicity at the highest test dose.

RMS comment: The study is acceptable. The overall NOAEL was 1000 mg/kg bw/day based on the absence of any clear evidence of systemic toxicity at the highest test dose.

B.6.4 Genotoxicity

For the first inclusion of zoxamide in Annex I to Directive 91/414/EEC, genotoxicity data was evaluated and is summarised below. The data is considered satisfactory, and sufficient for the purposes of reregistration. No changes to the original conclusions are proposed.

In the *in vitro* chromosome aberrations assay (CHO cells), mitotic accumulation was observed at concentrations inhibiting cell growth in tests with and without metabolic activation. A statistically significant increase in the frequency of cells with numerical aberrations at the delayed sampling time (44 hours) was observed in the repeat test, Experiment 2, in tests both with and without metabolic activation and exceeded the normal or historical control range. Sporadic frequencies of cells with numerical aberrations exceeding the normal range were also observed in cultures from other treatments. The increases observed in all cultures were predominantly due to increases in the frequency of cells with polyploidy.

It is noted that the 44 hour test was conducted only in Experiment 2 and the increases were present in a single sample each which leaves a degree of uncertainty in the reproducibility of the findings. However, given that the mode of action of RH-117,281 involves an antitubulin activity (see CA 5.4.2/02 (5.8.2.2/01)) this finding was considered to be positive. The *in vivo* mouse bone marrow micronucleus

assay however did not corroborate any activity *in vivo*, therefore, overall RH-117,281 is not considered to be a genotoxic compound.

B.6.4.1 In vitro studies

In DAR (2001)

Reference:	CA 5.4.1/01, Sames, J.S., Ciaccio, P.C. (1996a)
Title:	RH-117,281 Technical: Salmonella Typhimurium gene mutation assay (Ames test)
Report number:	No: 95R-262, ER Ref No: 2.7
Guidelines:	OECD 471
GLP:	Yes

In a bacterial gene mutation assay (1996), histidine-dependent TA98, TA100, TA1535, TA1537, and TA102 strains of *Salmonella typhimurium* were exposed to RH-7281 (lot no: DSR-9510, purity 92.3%) dissolved in acetone at concentrations of 0 (solvent control) to 5000 μg/plate in the presence and absence of a metabolic activation system (S-9 liver fraction from Aroclor 1254 treated rats).

The positive controls in the tests with metabolic activation was 2-anthramine. In the absence of metabolic activation, the positive controls used were: 2- nitrofluorene (TA98), sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), and mitomycin-c (TA102).

Findings: The test article did not induce an increase in revertants when compared to solvent controls in tests with and without metabolic activation. In the definitive assay, a precipitate was observed at 200 μg/plate and greater in strains TA98, TA1535 and TA1537 without metabolic activation and at 500 μg/plate and greater in strains TA98 and TA1537 with metabolic activation and TA100 with and without metabolic activation. A precipitate was also observed at 2000 μg/plate and greater in TA102 with and without metabolic activation and in TA1535 with metabolic activation. The assay was repeated at concentrations ranging from 16 to 300 μg/plate. In the confirmatory assay, a precipitate was observed at 300 μg/plate in strains TA98 and TA1537 with metabolic activation and at 160 μg/plate in strains TA98, TA1535 and TA1537 without metabolic activation.

Conclusion: RH-7281 was not mutagenic in the reverse mutation assay in strains of *S. Typhimurium* under the conditions of the assay

RMS: The study is acceptable.

In DAR (2001)

Reference:	CA 5.4.1/02, Riley, S. (1998)
Title:	RH-117,281: Test for chemical induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells.
Report number:	No: 96RC-125, ER Ref No: 23.6

Guidelines: OECD 473

GLP: Yes

In an *in vitro* cytogenetics assay (1998, in-life 1997), the potential for RH-7281 (lot no: DSR-9510, purity 92.3 %) to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells in the presence or absence of metabolic activation with Aroclor 1254 induced rat liver post-mitochondrial fraction (S-9). The test article was dissolved in acetone. The highest dose level used, 100 μg/mL, was close to but in excess of the solubility of the test article in medium.

In the first experiment, treatment in the test without metabolic activation was continuous for 20 hours (20+0). Treatment in the test with metabolic activation was 3 hours followed by a 17-hour recovery period before harvest (3+17). The test article dose levels for chromosome analysis were selected by evaluating the effect of RH-7281 on cell number and mitotic index. Chromosome aberrations were analysed at three dose levels. The highest concentrations chosen for analysis, 4.035 and 16.81 μg/mL, induced approximately 54 % and 57 % reduction in cell number in the absence and presence of S-9 respectively.

In the second experiment, the treatment regimens used in Experiment 1 were repeated and included a delayed (44 hour) sampling time. Chromosome aberrations were analysed in cells receiving 20+0 hour treatments in the absence of S-9 and 3+17 hour treatments in the test with metabolic activation at three dose levels. The highest concentrations chosen for analysis, 5.77 and 49 μg/mL, induced approximately 55 % and 26 % reduction in cell number respectively after 20 hours. The next highest dose in the test with metabolic activation, 70 μg/mL, gave a 90 % reduction in relative mitotic index. Analysis of chromosome aberrations was also investigated at three dose levels at the delayed sampling time. The highest concentrations analysed were 2.83 and 16.81 μg/mL in the absence (44 + 0) and presence (3 + 41) of metabolic activation respectively. The concentrations induced 56 % and 55 % reduction in cell number.

Appropriate negative (solvent) and untreated control cultures were included in the test system in both experiments under each treatment condition. The proportion of cells with structural aberrations in these cultures fell within historical solvent control ranges. Positive controls used were 4-nitroquinoline 1-oxide in the test without metabolic activation and cyclophosphamide in the tests with metabolic activation. Cells receiving these were sampled in each experiment, 20 hours after the start of treatment; both compounds induced statistically significant increases in the proportion of cells with structural aberrations.

The number of cells with structural aberrations was similar to those found in concurrent negative control cultures in the tests with and without metabolic activation. Mitotic accumulation was observed at concentrations, which inhibited cell growth in tests with and without metabolic activation. A statistically significant increase in the frequency of cells with numerical aberrations at the delayed sampling time (44 hours) was observed in Experiment 2 in tests both with and without metabolic activation and was observed to exceed the normal or historical control range (Table B.6.4.1-1). Sporadic frequencies of cells with numerical aberrations exceeding the normal range were also observed in cultures from other treatments. The increases observed in all cultures were noted to be predominantly due to increases in the frequency of cells with polyploidy.

RH-7281 did not produce any consistent evidence of structural aberrations in cultured Chinese hamster ovary cells when tested to the limit of toxicity in the absence and presence of S-9. Positive evidence of a potential to cause aberrations was limited to one sample each in the 44-hour exposure test which was not confirmed by a repeat assay. Mitotic accumulation and isolated incidences of increased

levels of numerical aberrations were observed following treatment with RH-7281 in both the absence and presence of S-9. RH-7281 was equivocal in this *in vitro* assay for the potential to cause chromosomal aberrations.

Table B.6.4.1-1 In vitro cytogenetics assay in CHO cells, Experiment 1

Conditions	-S-9 (20 hours)				+S-9 (3+17 hours)			
Treatment (μg/mL)	**RCG**	**RMI**	**%SA**	**%NA (%HEP)**	**RCG**	**RMI**	**%SA**	**%NA (% HEP)**
Untreated control[a]	122	86	0.00	0.5 (0, 0, 0.50)	100	106	2.25	4.5 (0, 1.43, 3.10)
Solvent control	100	100	2.00	2.4 (0.49, 0, 1.95)	100	100	0.75	3.6 (0.24, 1.45, 1.93)
0.9689	95	88	3.00	1.5 (0, 0, 1.48)	NT	NT		
1.384	81	96			NT	NT		
1.977	51	57	4.00	2.0 (0,0,1.96)	99	102	3.00	2.9 (0.49, 0.97, 1.46)
2.825	NR	287			85	116		
4.035	46	278	1.50	1.0 (0.5, 0, 0.5)	88	113		
5.765	40	283			73	107	2.00	6.5 (0, 0.93, 5.61)
8.235	43	211			81	92		
11.76	41	9			51	97		
16.81	32	16			43	262	5.50*	8.3 (0, 5.96, 2.29)*[a]
24.01	36	9			42	263		
34.3	25	0			44	306		
49	27	0			42	315		
70	NT	NT			44	196		
100	NT	NT			41	10		

Historical solvent control data for CHO cell cultures (defined normal range of aberrant cells per 100 scored) –S9, +S9: structural aberrations excluding gaps: 0-4, 0-5.

Numerical aberrations: 0-6, 0-6

Results for positive controls %SA=24, -S9, NQO; 92, +S9, CPA.

*Statistically significant differences using Fisher's exact test with Bonferroni adjustment;

*[a]significant difference ascribed to total (H+E+P) numerical aberrations

**Note that this is the repeat definitive assay; the initial definitive assay was rejected because of a higher than normal level of endoreduplication in the control samples.

Abbreviations: RCG, relative cell growth; RMI, relative mitotic index, SA, structural aberrations excluding gaps; NA, numerical aberrations; H, hyperdiploid; E, endoreduplication; P, Polyploidy; NT, not tested; NR, not reported

[a]Doses selected for scoring aberrations are highlighted in bold.

Table B.6.4.1-1 (cont.) *In vitro* cytogenetics assay on RH-7281 in CHO Cells, Experiment 2

Conditions	-S-9 (20 hours)				+S-9 (3+17 hours)			
Treatment (µg/mL)	RCG	RMI	%SA	%NA (%HEP)	RCG	RMI	%SA	%NA (% HEP)
Untreated control[a]	105	126	1.0	1.5 (0, 0, 1.5)	146	120	0.0	7.8 (0.23, 5.3, 2.3)
Solvent control	100	100	0.5	2.9 (0, 0, 2.9)	100	100	3.0	11.9 (0.44, 9.3, 2.2)
0.9689	80	91	1.5	2.9 (0, 0, 2.9)	NT	NT		
1.384	95	84			NT	NT		
1.977	86	65	0.0	1.5 (0.5, 0, 1.0)	112	101	2.0	12.7 (0, 11.8, 0.87)
2.825	74	115			117	64		
4.035	54	382			106	71		
5.765	45	415	0.0	1.0 (0, 0, 1.0)	112	85		
8.235	50	415			81	86		
11.76	41	18			80	107		
16.81	41	0			72	243	5.0	1.0 (0, 0.5, 0.5)
24.01	53	0			67	231		
34.3	46	0			65	274		
49	43	0			74	129	3.0	2.0 (0, 0, 2.0)
70	NT	NT			62	10		

100	NT	NT			48	0		

Historical solvent control data for CHO cell cultures (defined normal range of aberrant cells per 100 scored) –S9, +S9:

structural aberrations excluding gaps: 0-4, 0-5.

Numerical aberrations: 0-6, 0-5.

Results for positive controls %SA = 23, -S9, NQO; 62, +S9, CPA

Abbreviation: RCG, relative cell growth; RMI, relative mitotic index; SA, structural aberrations excluding gaps; NA, numerical aberrations; H, hyperdiploid; E, endoreduplication; P, Polyploidy; NT, not tested.

[a] Doses selected for scoring aberrations are highlighted in bold.

Table B.6.4.1-1continued *In vitro* cytogenetics on RH-7281 in CHO Cells, Experiment 2

Conditions	**-S-9 (44 hours)**				**+S-9 (3+41 hours)**			
Treatment (µg/mL)	RCG	RMI	%SA	%NA (%HEP)	RCG	RMI	%SA	%NA (% HEP)
Untreated control[c]	151	130	0.0	0.5 (0, 0, 0.5)	106	97	0.0	2.0 (0, 0, 2.0)
Solvent control	100	100	1.0	1.5 (0, 0, 1.5)	100	100	0.5	5.2 (1.9, 0, 3.3)
0.9689	73	93	1.5	2.9 (0.5, 0, 2.4)	NT	NT		
1.384	77	64			NT	NT		
1.977	43	43	2.0	**16.7 (0.8, 0, 15.8)**[ab]	104	93	2.0	3.8 (0, 0, 3.8)
2.825	44	55	2.5	**29.8 (0.7, 0, 29.1)**[ab]	88	86		
4.035	31	59			92	119		
5.765	26	100			75	97	1.5	3.4 (1.0, 0, 2.4)
8.235	21	34			71	120		
11.76	14	0			49	74		
16.81	6	0			45	66	1.0	55.8 (4.0, 0.4, 51.3)[ab]
24.01	14	0			34	84		
34.3	14	0			53	97		
49	11	0			35	149		
70	NT	NT			21	119		

100	NT	NT			11	0		

Historical solvent control data for CHO cell cultures (defined normal range of aberrant cells per 100 scored) –S9, +S9:

structural aberrations excluding gaps: 0-4, 0-5.
Numerical aberrations: 0-6, 0.6.

Results for positive controls %SA=24, -S9, NQC; 92, +S9, CPA, with 20 hour harvest only

*Statistically significant differences using Fisher's exact test with Bonferroni adjustment:

[a]significant difference ascribed to polyploidy (P);

[b]significant difference ascribed to total (H+E+P) numerical aberrations.

Abbreviations: RCG, relative cell growth; RMI, relative mitotic index; SA, structural aberrations excluding gaps; NA, numerical

aberrations; H, hyperdiploid; E, endoreduplication; P, Polyploidy; NT, not tested.

[c]Doses selected for scoring aberrations are highlighted in bold.

Conclusion: RH-7281 did not produce any consistent evidence of structural aberrations in cultured Chinese hamster ovary cells when tested to the limit of toxicity in the absence and presence of S-9. Positive evidence of a potential to cause aberrations was limited to one sample each in the 44-hour exposure test which was not confirmed by a repeat assay. Mitotic accumulation and isolated incidences of increased levels of numerical aberrations were observed following treatment with RH-7281 in both the absence and presence of S-9. RH-7281 **was equivocal** in this in vitro assay for the potential to cause chromosomal aberrations.

RMS comment:

RMS comment: Even though the experiment was considered as equivocal in this in vitro assay for the potential to cause chromosomal aberrations, RMS concluded that study is considered to be positive taking into account ECCO 118 meeting (summary see below).

It was an open point at ECCO 118 meeting.
From ECCO 118 meeting:
There were two positive results from the in vitro tests (CHO chromosome aberration test; CHO HPRT test). The result from the in vivo study in mice (micronucleus test) was negative. The experts stated that with this type of compound which inhibits microtubule assembly they would normally also expect positive in vivo results. The rapporteur Member State stated that this study has been conducted in accordance with the

guidelines. The meeting agreed that they have to be sure that there is no potential for induction of chromosome non-disjunction in vitro. This cannot be concluded from the studies which have been conducted so far; therefore, a data requirement was agreed on. Preferably, an in vitro micronucleus test in human lymphocytes using a combination of the cytokinesis-block micronucleus test with fluorescence in situ hybridization (FISH) is required, to demonstrate dose relations and threshold values for aneugenic effects including non-disjunction. Also, an in vivo test in a second species was requested.
In the peer review meeting considered:
During the peer review process (ECCO 121) requested the following two points concerning genotoxicity to be addressed.
It was concluded that applicant should submit:
4.1 *In vitro* micronucleus test in human lymphocytes using a combination of the cytokinesis-block micronucleus test with fluorescence *in situ* hybridisation

The ECCO Overview meeting (ECCO 121) agreed that an *in vitro* test should only be done IF the *in vivo* new study requested to address 4.2 produced an adverse result.

4.2 An *in vivo* test in a second species is required (chromosome aberration test or micronucleus test).

An evaluation of this second study is given below :

Summary of the evaluation of the new *in vivo* micronucleus study and risk assessment

The findings of the new in vivo micronucleus study with kinetechore analysis now confirm that zoxamide has no clastogenic, aneugenic or genotoxic potential *in vivo*.

In DAR (2001)

Reference: CA 5.4.1/03, Pant, K. (1994)

Title: RH-117,281: Test for chemical induction of gene mutation at the HGPT locus in cultured Chinese hamster ovary cells with and without metabolic activation

Report number: No: 94RC-077, ER Ref No: 23.4

Guidelines: OECD 476

GLP: Yes

In a study (1994), RH-7281 (lot no. DK2011, purity 94.2 %) was tested for its potential to induce gene mutations at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus in cultured CHO cells with and without exogenous metabolic activation. Two range finding tests were performed to assess the test article's toxicity to CHO cells within this test system. The test article concentration was corrected for purity. In the first range finding test, 10 concentrations ranging from 0.05 - 50 µg/mL RH-7281 were tested with and without metabolic activation. Because this range of concentrations did not produce toxicity greater than 50 %, a second range finding test was performed with concentrations ranging from 2.6 - 667 µg/mL. The concentrations of 83, 167, 333 and 667 µg/mL formed a white precipitate in the culture medium indicating that the limit of solubility of the test article in the culture medium had been exceeded.

Toxicity was assessed by measuring the reduction in Relative Cloning Efficiency (RCE). The doses for the mutation assays were then selected based on 0-100 % cytotoxicity. The osmolality of the highest concentration tested (65 µg/mL) in the mutation assay was less than 500 mOsmol/kg in the culture medium.

The study was certified to be GLP compliant and was satisfied the essential criteria of OECD guideline # 476.

The Mutation Assay was performed in two independent trials: A definitive and a confirmatory assay. The doses for the definitive assay were selected based on results of the second RFT. The cultures were treated at 5.0, 10, 20, 30, 40, 50 and 65 µg/mL without activation and at 2.0, 5.0, 10, 20, 30, 40 and 55 µg/mL with activation. The concentrations of 50, 55 and 65 µg/mL formed a precipitate in the culture medium. After a 9-day period of expression, the concentrations of 5.0, 10, 20, 30, 40 and 50 µg/mL without activation and 5.0, 10, 20, 30, 40 and 55 µg/mL with activation were cloned.

The range of RCEs observed at these doses were as follows: 96-49 % at doses of 5.0-65 µg/mL without activation, and 126-64 % at doses of 2.0-55 µg/mL with activation (Table B.6.4.1-2).

Table B.6.4.1-2 Summary of the findings in the definitive HGPRT locus assay - cloning efficiency and mutation frequency.

Without Activation				**With Activation**			
RH-7281 Conc; µg/ml	% RCE*	% C.E.**	Mutants/ 10^6 surviving cells [@]	Test Article Conc; µg/ml	% RCE*	C.E.**	Mutants/ 10^6 surviving cells[@]
Acetone A	100	105	4	Acetone	100	106	5
B		91	4	A		111	5

						B			
5.0	A	96	86	12	5.0		96	106	7
	B		92	16		A		95	7
						B			
10	A	76	89	14	10		126	103	6
	B		81	3		A		99	7
						B			
20	A	75	93	4	20		95	104	4
	B		88	9		A		115	4
						B			
30	A	60	131	5	30		101	100	8
	B		89	5		A		99	9
						B			
40	A	49	100	7	40		105	105	6
	B		102	9		A		103	9
						B			
50	A	54#	41	4#	55		64#	80	5#
	B		61	10#		A		113	10#
						B			
EMS Sol.	A	100	71	5					
Control	B		90	10					
EMS	A	81	74	193	DMBA		36	68	326
0.5 µl/mL	B		86	187	5 µg/mL			54	556

* RCE = $\frac{\text{Average number of colonies in test plates}}{\text{Average number of colonies in control plates}}$ X 100

** CE = Cloning efficiency

= This concentration formed a white precipitate in the culture medium at the time of treatment

@ = Mutants/10^6 = $\frac{100}{\text{Survivors}}$ X Total Mutants $\frac{}{\text{CE x 2.4}}$ X $\frac{\text{Mutant Plates Seeded per Dose}}{\text{Mutant Plates Counted per Dose}}$

The confirmatory mutation assay was performed at the concentrations of 20, 25, 30, 35, 40, 45 and 50 µg/mL without activation and at 25, 30, 35, 40, 45, 50 and 55 µg/mL with activation. The concentrations of 50 and 55 µg/mL formed a white precipitate in the culture medium. The concentration of 50 µg/mL without activation could not be cloned after the 9-day expression period due to toxicity. The range of RCEs observed at these doses were as follows: 81-8 % at doses of 20-50 µg/mL without activation, and 78-20 % at doses of 25-55 µg/mL with activation (Table B.6.4.1-3).

Table B.6.4.1-3 Summary of the findings in the confrimatory assay of the HGPRT locus gene mutation assay in CHO cells - cloning efficiency and mutation frequency.

Without Activation					**With Activation**			
RH-117281 Conc; µg/ml		% RCE*	% C.E.**	Mutants/ 10^6 surviving cells @	Test Article Conc; µg/ml	% RCE*	C.E.**	Mutants/ 10^6 surviving cells@
Acetone	A	100	93	9	Acetone	100	99	8
	B		102	0			100	5
					A			
					B			
20	A	81	75	11	25	78	79	6
	B		78	0			95	4
					A			
					B			
25	A	69	76	0	30	74	69	0
	B		97	19			85	7
					A			
					B			
30	A	21	64	0	35	59	82	9
	B		73	1			66	4
					A			
					B			
35	A	16	71	0	40	79	76	7
	B		77	2			87	9
					A			
					B			
40	A	12	58	25***	45	80	90	4
	B		63	17***			89	9
					A			
					B			
45	A	12	59	0	50	20#	70	17#
	B		58	0			68	7#
					A			
					B			

EMS Sol.	A	100	87	9	55	36#	79	2#
Control	B		85	4	A		91	6#
					B			
EMS	A	65	75	213	DMBA	23	62	182
0.5 µl/mL	B		55	270	5 µg/mL		61	282

* RCE = $\frac{\text{Average number of colonies in test plates}}{\text{Average number of colonies in control plates}}$ X 100

** CE = Cloning efficiency

*** = Statistically significant ($p<0.05$), and the average number of mutants per $1x10^6$ surviving cells > 15

= This concentration formed a white precipitate in the culture medium at the time of treatment

@ = Mutants/10^6 = $\frac{100}{\text{Survivors}}$ X Total Mutants $\frac{}{\text{CE x 2.4}}$ X $\frac{\text{Mutant Plates Seeded per Dose}}{\text{Mutant Plates Counted per Dose}}$

At the end of the expression period, the cultures were cloned in medium containing 6-thioguanine to select HGPRT enzyme-deficient mutants. These cultures were then incubated for 7 days. After 7 days, the cloning efficiency and mutant frequencies were calculated for each culture by counting the number of colonies (Tables 5.32 and 5.33).

Conclusion: RH-7281 did not cause a significant increase in the mutant frequency at the HGPRT locus among the RH-7281-treated cultures either in the presence or absence of exogenous metabolic activation. The mutant frequencies of the solvent controls were within the investigating laboratory's historical negative control range. The positive controls caused a significant increase in the mutant frequencies. To conclude, RH-7281 was considered to be not genotoxic in the HGPRT assay in CHO cells under the conditions of the study.

RMS comment: The study is acceptable.

RMS comment: It's not clear why ECCO 118 meeting decided that test for chemical induction of gene mutation at the HGPT locus in cultured Chinese hamster ovary cells with and without metabolic activation is considered as positive, because statistical significant increase in mutant frequency is only observed in the second test without metabolic activation at 40 µg/ml (not observed at 45 µg/ml. RMS is on the opinion that RH-7281 was considered to be not genotoxic in the HGPRT assay in CHO cells under the conditions of the study.

In addition:
In the addendum 1 (June, 2002) of the DAR RMS stated that Gene mutation assay at the HGPRT locus in CHO cells was not genotoxic.

From addendum 1 (June, 2002):
RH-7281 was not genotoxic in the following in vitro studies.
Reverse mutation assay in strains of S. typhimurium
Gene mutation assay at the HGPRT locus in CHO cells

B.6.4.2 In vivo studies in somatic cells
In DAR (2001)

Reference:	CA 5.4.2/01, Sames, J.S., Vandenberghe, Y.L. (1996b)
Title:	RH-117,281 Technical: micronucleus assay in CD-1 mouse bone marrow cells
Report number:	No: 95R-264, ER Ref No: 1.9
Guidelines:	OECD 474
GLP:	Yes

Micronucleus study in the mouse

In a study (1996), RH-7281 (lot no: DSR-9510, purity 92.3 %) was evaluated for its potential to induce chromosomal damage *in vivo*, using the micronucleus assay in mouse bone marrow cells. Groups of 5 male and 5 female adult CD-1 mice received a single oral dose of RH-7281 in corn oil at concentrations of 0 (vehicle control) 200, 1000 or 2000 (the limit dose) mg/kg bw. The 2000 mg/kg bw dose group consisted of 7 animals/sex/dose group. Positive control animals received a single oral dose of corn oil (vehicle control), or an intraperitoneal injection of 0.35 or 2.0 mg/kg mitomycin-C (MMC). Animals from test article and solvent control groups were killed at 24 or 48 hours after treatment. Animals from the positive control group were killed 24 hours after treatment. Bone marrow slides were prepared and the incidence of micronucleated polychromatic erythrocytes was measured as an indicator of cytogenetic damage. For each animal, a total of at least 2000 polychromatic erythrocytes were scored for the presence or absence of micronuclei. In addition, the ratio of polychromatic/normochromatic (PCE/NCE) cells was measured to evaluate the cytotoxicity of the test agent.

Male and female mice in the corn oil vehicle control and in all RH-7281-treated dose groups exhibited signs of yellow/ brown anogenital staining and diarrhoea within 4 hours after treatment.

Conclusion: RH-7281 did not induce an increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow cells of male or female mice when compared to the solvent controls. There was no statistically significant change in the PCE/NCE ratio. A dose related increase in the frequency of micronucleated polychromatic erythrocytes was observed in the bone marrow cells of male and female mice treated with 0.35 and 2.0 mg/kg of the positive control, MMC. This response was a greater than 2 fold increase when compared to the solvent controls, and confirms the sensitivity of the assay to detect induced genetic damage.

RMS comment: The study is acceptable.

In Addendum 3 to DAR (2001)

Reference:	CA 5.4.2/03 (5.8.2.2/01), Gudi, R and Krsmanovic, L . (2002)
Title:	Zoxamide: Mammalian Erythrocyte Test with the Kinetochore Analyses
Report number:	No: AA65WR.126.BTL, ER Ref No: 46.7
Guidelines:	OECD 474
GLP:	Yes

In a study (Gudi and Krsmanovic, 2002), the clastogenic and aneugenic potential of zoxamide (batch no: T66246D, purity 97.4%) was investigated in the micronucleus assay with kinetochore analysis in rat bone marrow cells. Groups of 6 male adult Crl:CD BR rats were each administered zoxamide orally, by gavage, in corn oil at dose levels of 0 (vehicle control) 500, 1000 or 2000 (the limit dose) mg/kg bw in a volume of 10 ml/kg/day) on two consecutive days. Positive control animals received a single intraperitoneal dose of 40 mg/kg bw cyclophosphamide or 6 mg/kg bw vinblastine in a volume of 10 ml/kg bw. Animals from test article and solvent control groups were killed 24 hours after the second treatment. Animals from the positive control group were killed 24 hours after the treatment.

Bone marrow slides (two slides per dose group) were prepared and stained with acridine orange, a nucleic acid-specific stain. Slides from the first five animals of each dose group were subjected to micronucleus analysis. The incidence of micronucleated polychromatic erythrocytes was measured as an indicator of cytogenetic damage. For each animal, a total of at least 2000 polychromatic erythrocytes were scored for the presence or absence of micronuclei. In addition, the ratio of polychromatic erythrocytes/total erythrocytes was recorded per 1000 erythrocytes as a measure of erythropoiesis to evaluate the cytotoxicity of the test agent.

For kinetochore analysis, cell suspensions from five animals from the vehicle control, 2000 mg/kg bw and vinblastine-treated dose groups were processed for kinetochore staining[3] using centromere positive control primary antibodies and anti-human IgG (γ)-FITC and counterstained with propidium iodide. The procedure allowed the differentiation of the PCE and NCE population as well as the visualisation of micronuclei and kinetochores. At least 20 micronucleated polychromatic erythrocytes per animal were scored for the presence or absence of kinetochores.

The laboratory criteria for a positive in this assay was a dose-related and statistically significant increase in the number of micronucleated polychromatic erythrocytes.

Increase in the proportion of cells containing micronuclei in excess of both the values of the concurrent negative control range and the negative historical control range has also been investigated.

Homogeneity and stability of test formulations were investigated and found to be acceptable. The stability of zoxamide in corn oil and the concentrations in the vehicle were analytically confirmed to be within ±10% of the target concentrations except the low dose administration that was 114% of the target dose). It was reported that in a previous toxicokinetic study examining the distribution of radiolabelled zoxamide, a single oral dose of 2000 mg/kg bw ^{14}C-zoxamide was administered to groups of 4 male and 4 female rats. The rats were killed after 4, 8, 24 and 48 hours and bone marrow samples were analysed for radiolabel content. Radiolabelled zoxamide was found at all time points and the mean radiolabel content in bone marrow ranged from 5 - 55.5 ppm. Other evidence from toxicokinetic studies in rats (Table B6.3, Volume 3, Annex B) confirm the above report on exposure to bone marrow.

The administration of zoxamide, corn oil or cyclophosphamide did not result in any clinical signs of toxicity or deaths. However animals treated with vinblastine showed signs of lethargy, piloerection and in one rat prostration. No reduction in body weights was observed in vehicle control or zoxamide-treated rats. However slight reductions in body weight were observed for cyclophosphamide- (0.3 %) and vinblastine- (12 %) treated rats.

There was no clear treatment-related inhibition of erythropoiesis induced by the treatment of rats with zoxamide even though the ratio of polychromatic erythrocytes to total erythrocytes was significantly reduced

[3] Gudi, R et al, 1992. *Environ. Mutagen*. 20: (2), 106 - 116

(Tukey's pairwise comparison) in the low and mid-dose groups (Table 1). However, the incidence for the top dose (2000 mg/kg bw) was comparable to that of the vehicle control and the observations for the low and middose are noted to be well within the historical control range. There was no increase or remarkable difference in the number of micronucleated polychromatic erythrocytes (Table 6.4.2-1). The positive control cyclophosphamide produced the expected increase in the rate of polychromatic erythrocytes containing micronuclei, confirming the reliability of the test method.

Table 6.4.2-1 Summary of the bone marrow analysis in the rat micronucleus study

Parameter	Dose Group					
	0 (vehicle control)	500	1000	2000	Cyclo-phosphamide	Historical negative control males
PCE/Total Erythrocytes Mean ± SD (% Change)	0.647 ± 0.02 (-)	0.574 ± 0.04 (-11.3**)	0.571 ± 0.02 (-11.7**)	0.618 ± 0.03 (-4.5)	0.606 ± 0.06 (-6.3)	0.57 ± 0.10 Range: 0.38 - 0.83
Micronucleated PCEs /1000 PCEs	0.9 ± 0.65	0.9 ± 0.22	1.3 ± 0.27	1.0 ± 0.35	40.1 ± 9.29	0.40 ± 0.46 Range: 0 - 3
Micronucleated PCEs /PCEs scored	9/10000	9/10000	13/10000	10/10000	*401/10000	

* Statistically significant p<0.05 (Dunnett's t-test);

** Statistically significant p<0.01 05 (Dunnett's t-test, Tukeys pairwise comparison)

There was also no statistically significant increase (p >0.05, Fisher's exact test) in the proportion of micronucleated polychromatic erythrocytes with kinetochores in the 2000 mg/kg bw dose group compared with the vehicle control (Table 2).Vinblastine, the positive control for aneugenicity induced a statistically significant increase in the number of micronucleated polychromatic erythrocytes with kinetochores compared with the vehicle control (Table B.6.4.2-2).

Table B.6.4.2-2 Induction of micronucleated PCEs with kinetochores in rats after treatment with zoxamide

Dose Group	Animal Number	Micronucleated Polychromatic Erythrocytes (MPCEs)	
		K-	K+
Corn Oil	1	23	2
	2	24	1
	3	23	2
	4	25	0

	5	24	1
Total/Group		**119**	**6**
% Total MPCEs scored		**95**	**5**
Zoxamide (2000 mg/kg bw)	19	23	2
	20	22	3
	21	24	1
	22	22	3
	23	24	1
Total/Group		**115**	**10**
% Total MPCEs scored		**92**	**8**
Vinblastine (6 mg/kg bw)	31	4	21
	32	3	22
	33	5	20
	34	4	21
	35	4	21
Total/Group		**20**	**105****
% Total MPCEs scored		**16**	**84**

** Statistically significant p≤0.05 (Fisher's exact ratio test)

Conclusion: Zoxamide was not clastogenic or aneugenic in the rat bone marrow micronucleus study with kinetochore analysis in rats at dose levels of ≤ 2000 mg/kg.

RMS comment: The study is acceptable. It can be concluded that zoxamide do not have genotoxic potential.

B.6.4.3 In vivo studies in germs cells

No studies are submitted.

B.6.5 Long-Term Toxicity and Carcinogenicity

B.6.5.1 Oncogenicity study in rats

In DAR May (2001)

Reference: CA 5.5/01 and 02, [redacted] (1998a, 1998b)

Title: RH-117,281 Technical: 24-month dietary chronic/oncogenicity study in rats.

RH-117,281 Technical: 24-month dietary chronic/oncogenicity study in rats.

Photomicrographs on Selective Tissues.

Report number: No: 94RC-236, ER Ref No: 21.1

No: 94RC-236A, ER Ref No: 21.1

Guidelines: OECD 453

GLP: Yes

In a study (1998, inlife 1996-98), groups of 70 male and 70 female Sprague-Dawley Crl:CD®BR rats were administered RH-7281 (lot no: DSR-9511; purity 92.0 %) in the diet for 2 years at concentrations of 0 (control), 1000, 5000, and 20,000 ppm (corresponding to achieved daily intakes of 0, 51, 260, and 1058 mg/kg bw/day in males and 0, 65, 328, and 1331mg/kg bw/day in females). Ten rats/sex/group were randomly selected prior to treatment for interim sacrifice after 52 weeks of treatment.

Diet and water were provided *ad libitum*. The animals were observed twice daily (a.m. and p.m.) for mortality and moribundity. At least once each week, each animal was removed from its cage and examined for abnormalities and signs of toxicity. Body weights and food consumption data were collected weekly for 14 weeks and once every fourth week thereafter. During Weeks 13, 26, 52, 78, and 104, blood samples were collected for haematology and clinical chemistry tests from 20 animals/sex/group, and urine samples were collected from 10 animals/sex/group. The same animals were bled at each interval when possible. After either 52 (10/sex/group) or 104 (all survivors) weeks of treatment, the animals were weighed, anaesthetised, killed, and necropsied. At necropsy, macroscopic observations were recorded, selected organs were weighed, and selected tissues were collected and preserved. Animals that died during test or were sacrificed at an unscheduled interval were also necropsied, however organs were not weighed. Microscopic examinations were done on tissues from each animal in the control and high-dose groups and from each animal that died or was sacrificed at an unscheduled interval. The lung, liver, kidney, and any gross lesions were also examined microscopically from each animal in the low- and mid-dose groups.

There were no test material related effects on survival or in clinical signs in either male or female animals at doses up to and including 20,000 ppm. Survival rates for the 0, 1000, 5000, and 20,000 ppm dose groups at week 78 were 83, 82, 83, and 90 % for the males and 88, 85, 87, and 85 % for the females, respectively. At week 104 the corresponding survival rates in were 50, 44, 60, and 63 % for the males and 51, 49, 45, and 50 % for the females, respectively.

There were no test-material-related effects on mean body weights or body weight gain in either sex at any dose level. Mean male body weights were comparable throughout the study. Females body weights in all groups were noted by the investigators to be within the range of normal values in the laboratory and did not exhibit a dose response. Therefore, statistically significant reductions in female body weight were considered incidental and unrelated to treatment. (Table B.6.5.1-1). Food consumption and food efficiency were generally comparable across the dose groups within each sex.

Table B.6.5.1-1Summary of female body weights in 24-month dietary chronic/ oncogenicity study in rats

Dose Level (ppm)	0	1000	5000	20,000

Dose Level (ppm)	0	1000	5000	20,000
Females				
Body Weight (g)				
Week1	170	174	170	171
Week 13	303	298	288	296
Week 26	355	346	**332*** ↓(6.5 %)	340
Week 54	412	398	**372*** ↓(9.7%)	400
Week 78	449	**420*** ↓(6.5 %)	**400*** ↓ (11.0%)	**417*** ↓(7.2%)
Week 105	425	415	402	411

* Significant difference from control ($p<0.05$), ANOVA, Dunnett's t-test

Clinical chemistry and haematology did not reveal any treatment-related differences. Observed changes in the haematology and serum biochemistry parameters were considered incidental to the administration of the test material due to the lack of dose-response, the low magnitude of the change, the lack of histopathological correlation, or the inconsistent occurrence of the differences at the different sampling times. There were no treatment-related effects in any urinalysis parameter or in ophthalmic findings in either sex at any dose.

Organ weights at week 53 showed an increase in relative liver weight at 5000 ppm and 20,000 ppm in females only. This finding was not observed at the end of treatment and it was observed by the study investigators to be equivocal and not an adverse finding, as it was not accompanied by any histopathological changes (Table B.6.5.1-2). There were no test material-related effects on absolute and relative organ weights in either male or female animals at termination of treatment at doses up to and including 20,000 ppm.

Table B.6.5.1-2 Summary of changes in organ weights observed in 24-month dietary chronic/ oncogenicity study

Dose Level (ppm)	0	1000	5000	20,000
Females				
Week 53 (Interim sacrifice)				
Absolute Liver Weight (g)	9.50	10.03 ↑(5.6%)	10.14 ↑(6.7%)	11.06 ↑(16.4%)
Relative Liver Weight (%)	2.383	2.639 ↑(10,7%)	2.860*↑(20,0%)	2.906* ↑(21.9%)
Week 105 (Terminal sacrifice)				
Absolute Liver Weight (g)	11.46	11.66	11.86	11.80 ↑
Relative Liver Weight (%)	2.901	3.018	3.149	3.060

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Gross and histopathological examinations of organs and tissues did not reveal any treatment-related abnormalities. To conclude, RH-7281 was not carcinogenic and did not produce any evidence of significant systemic toxicity at dose levels of ≤ 20000 ppm (1058 mg/kg bw/day) in this 2-year dietary study in rats.

Conclusion: The NOAEL in the 2-year dietary study in the rat was 20000 ppm (1058 mg/kg bw/day) based on the absence of toxicity at the highest test dose.

RMS comment: The study is acceptable.

During ECCO –Peer review meeting 118 it was agreed to changes **NOAEL for this study from 20000 ppm (1058 mg/kg bw/day) to 50 mg/kg bw/d (1000 ppm) based same effects as in short term studies (increased liver weight).**

From ECCO –Peer review meeting 118 "Long-term effects were the same as the short-term effects: increased liver weight. For the lowest relevant NOAEL, the experts agreed on 50 mg/kg bw/d (1000 ppm) from the 2-year rat study, as proposed by some Member States.

There was no evidence for carcinogenic potential of zoxamide."

RMS comment: RMS supports Conclusion of ECCO –Peer review meeting 118 regarding NOAELof 50 mg/kg bw/d (1000 ppm) for this study, based on increased liver weight at week 53.

RMS asked for clarification regarding increased number of „C" cell adenomas in thyroid.

Thyroid

	Male	Female

Dose level	*0*	*1000*	*5000*	*20000*	*0*	*1000*	*5000*	*20000*
Number examined	**68**	**33**	**23**	**68**	**68**	**31**	**33**	**70**
Hyperplasia „C" cell	6	2	0	8	9	2	4	5
„C" cell adenoma	2	2	2	6	4	2	3	5

Applicant's response:

With respect to the rat historical control data, it has not been possible to procure this to date from [redacted].
In the meantime however, a comparison for the incidence of any type of tumour can be made against publically available [redacted] Historical Control data for Sprague Dawley rats[4] ([redacted] Sprague Dawley were used in the study in question, performed between January 1996 and January 1998) collected from control groups used in 2-year studies run in the period between 1994 and 2002 (the majority being in the five years prior to 1998).
A total of 30 studies for male rats (for a total of 2141 thyroids examined), and 31 studies for female rats (for a total of 2343 thyroids examined) are included in this data set. Table 3 presents a summary of data for spontaneous thyroid tumours.

Summary of incidence of spontaneous thyroid tumours in [redacted] Sprague-Dawley rats

Type of thyroid tumour	Male			Female		
	Percent of total	Minimum %	Maximum %	Percent of total	Minimum %	Maximum %
Follicular cell, adenoma	2.90	1.67	12.00	1.15	1.11	6.12
Follicular cell, carcinoma	0.89	0.87	3.85	0.60	0.87	3.33
C-cell, adenoma	7.85	1.43	14.77	7.21	2.86	16.67
C-cell, carcinoma	1.40	0.56	14.81	0.85	0.56	11.43
Ganglioneuroma	-	-	-	0.04	1.43	1.43

Concerning the alleged dose-dependent increase in follicular cell adenomas in male rats, the incidences for both the control and the top dose groups were low, exactly the same (2/68, Table 13D of the report) and at the same percentage incidence reported in the large [redacted] historical control data set (2.80%). Although an apparent increase was observed at the low dose level (2/33 – 6%), the percentage incidence is still well within the spontaneous incidence range (maximum 12%) and no tumours of this type were observed in the 23 rats of the mid-dose group.
Rather than the follicular cell adenomas it may be the VAAD mean the C-cell adenomas for which a greater number was observed at the top dose over the controls.
Concerning C-cell adenomas, a possible increase would not appear to be dose-related (2/68 – 2.90%, 2/33 – 6%, 2/23 – 8.7% and 6/68 – 8.8%, respectively, in order of ascending dosages). In addition, all the percentages, including at the top dose, are well within the spontaneous incidence range (maximum 15%).
The following observations are also considered pertinent:
Statistical analysis revealed no significantly positive finding for any of the tumours observed in either sex in this study. There also was no thyroid hyperplasia or increase in thyroid weights in any of the rat or mouse studies with zoxamide, which would usually accompany a genuine treatment-related increase in thyroid tumours. There was also no similar increase in females.
The available historical control data, and other evidence from existing zoxamide studies therefore suggest that the apparent increase in C-cell adenomas is not treatment related.

RMS comment: Applicant's justification is acceptaable. RH-117281 was not considered as carcinogenic in rats.

[4]Compilation of Spontaneous Neoplastic Lesions and Survival in Crl:CD (SD) Rats from Control Groups March 2004 http://www.criver.com/files/pdfs/rms/cd/rm_rm_r_lesions_survival_crlcd_sd_rats.aspx

In DAR May (2001)

Reference: CA 5.5/03, [redacted] (1998c)

CA 5.5/04, [redacted] (1998)

Title: RH-117,281 Technical: Eighteen-month dietary oncogenicity study in mice.

Report number: No: 96R-094, ER Ref No: 20.1

Guidelines: OECD 451

GLP: Yes

In a carcinogenicity study (1998, inlife 1996-97), groups of 60 male and 60 female Crl:CD-1 (ICR) BR (VAF/+) mice were administered RH-7281 (lot number: DSR-9510, purity 92.3 %) for eighteen months at dietary concentrations of 0 (control), 350, 1750, and 7000 ppm (equivalent to achieved concentrations of 0, 51.1, 251 or 1021 mg /kg bw/day and 0, 60.4, 326 or 1289 mg/kg bw/day in males and females respectively). All mice were observed daily for signs of moribundity, mortality, ill health or reaction to treatment. Physical examinations were performed weekly. Body weights and feed consumption were monitored weekly beginning one week prior to initiation of treatment through week 13 and then every fourth week thereafter through the duration of the study. After twelve months, all mice were bled via tail clip for white blood cell differential counts. After eighteen months, all mice were bled for white blood cell differential counts, killed, and organs and tissues grossly examined at necropsy. Selected organ weights were recorded and histopathological examination was conducted on tissues.

Findings: There were no effects on survival in mice at any dose level. No treatment-related deaths or clinical signs indicative of systemic toxicity were observed in any of the treatment groups. There were no treatment-related effects on body weight, cumulative body weight gain, or feed consumption in males or females at any dose level.

Haematology, limited to white blood cell differential counts from all high dose animals compared with controls at 12 or 18 months of treatment did not reveal any significant differences.

Organ weight measurements at necropsy did not show any treatment-related inter-group differences. Gross examination at necropsy and microscopic examination of organs and tissues did not reveal any treatment-related changes at doses up to and including 7000 ppm. There were no treatment-related effects on the type or incidence of any of the neoplasms observed in this study. The non-neoplastic and neoplastic changes were observed by the study investigators to be typical of those that occur spontaneously in aged laboratory mice of this strain; and their type, incidence, and/or severity were not affected by treatment.

Conclusion: RH-7281 was not carcinogenic in the 18-month dietary study in mice. The NOAEL was 7000 ppm (1021 or 1286 mg/kg bw/day) the highest dose tested.

RMS comment: The study is acceptable. Only one case of follicular adenoma was noticed at middose in male, and one case of malignant lymphoma in females at 0; 350 and 7000 ppm dose levels. Zoxamide was not carcinogenic in the 18-month dietary study in mice.

Incidence of histomorphologic observations in thyroid

	Male				Female			
Dose level	*0*	*350*	*1750*	*7000*	*0*	*350*	*1750*	*7000*
Number examined	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**

Adenoma, follicular	0	1	0	0	0	0	0	0
Lymphoma, malignant	0	0	0	0	1	1	0	1

6.6 Reproductive Toxicity

6.6.1 Generational studies

In DAR May (2001)

Reference: CA 5.6.1/01, [redacted] (1998)

Title: RH-117,281 Technical: Two-generation reproductive toxicity study in rats.

Report number: No: 95R-272, ER Ref No: 26.1

Guidelines: OECD 416

GLP: Yes

In a study (1998, in life 1996-97) groups of 30 male and 30 female rats were administered RH-7281 (lot no: DSR-9510, purity 92.3 %) in the diet at concentrations of 0, 1000, 5000 or 20,000 ppm active ingredient over two generations. Parental animals of the first generation (P1) were exposed from approximately 6 weeks of age and of the second generation (P2) from weaning (21 days). P1 and P2 animals were mated initially (to produce F1a and F2a litters) after at least ten weeks of exposure to treated diets. Treatment continued throughout gestation, lactation, and until terminal necropsy. P1 and P2 animals were each mated a second time to produce a second set of litters (designated F1b and F2b, respectively). Animals were remated approximately one week after weaning of all litters from the first mating was complete. Care was taken to pair animals differently than in the first mating.

A second mating of the P1 animals was conducted to verify apparent findings among the F1a offspring. The P2 animals were re-mated to ascertain if the apparent findings observed in the F1a, F1b and F2a litters were related to decreased feed intake, direct toxicity of the test substance, and/or from maternally mediated effects. To accomplish this, litters resulting from the second mating of P2 animals (F2b pups) were randomly divided into two subgroups within each dose group on Day 0 postpartum (PP). From Day 0 PP through Day 14 PP, the treatment regimen and evaluation of the animals remained unchanged. On Day 14 PP through Day 21 PP, all dams and their litters were removed from their respective diets and placed on untreated feed. All dams were administered RH-7281 (suspended in corn oil and dosed at a constant volume of approximately 5 ml/kg) daily by oral gavage at dose 333levels of 0, 50, 250 or 1000 mg/kg bw/day (corresponding to 0, 1000, 5000, or 20,000 ppm in the diet, respectively). Pups in litters assigned to one subgroup (at each dose) remained untreated during this period. Pups in litters assigned to the second subgroup (at each dose) were dosed daily by oral gavage at the same dose level as their dams. All dams were placed back on treated feed on Day 21 PP and maintained on treated feed for 3-5 weeks until terminal necropsy.

Body weight, feed consumption, and clinical signs were monitored in parental animals throughout treatment. Oestrus cycling was evaluated in P1 and P2 females for three weeks prior to their initial mating. Male parental animals were killed and necropsied after the second mating. Female parental animals were killed and necropsied after weaning of their second litters. Sperm evaluation was performed for all P1 and P2 males at the time of necropsy. Selected tissues (including reproductive tissues) were weighed from all P1 and P2 animals at necropsy. Selected tissues were collected and preserved for histopathology. Histopathological evaluation was performed for all tissues in control and high dose animals, and animals found dead or sacrificed during the course of the study. Reproductive tissues were also examined in all animals suspected of reduced fertility.

Survival and growth of offspring were monitored throughout lactation. Litters were culled to 8 pups (4/sex/litter where possible) on day 4 of lactation. Stillborn pups, pups that died during lactation, pups culled

at day 4, or sacrificed at weaning were grossly examined (except for F1b pups not chosen for organ weight evaluation, which were discarded). Selected tissues were collected, weighed and preserved from F1a, F1b, F2a, and F2b litters at weaning (1 animal/sex/litter) for possible histopathology. Microscopic evaluation was performed on spleens in the F1a, F2a and F2b weanlings (both subgroups) and on stomachs in the F2a & F2b weanlings. Sexual maturation (age at preputial separation in males, vaginal patency in females) was evaluated in F1a offspring selected as P2 parental animals.

The achieved intakes of RH-7281 for the P1 and P2 parents during the pre-mating period were 71 - 100, 360- 489 and 1474 - 2091 mg/kg bw/day, respectively, in males and 82 - 108 , 409 - 534 , and 1624 - 2239 mg/kg bw/day, respectively in females corresponding to nominal doses of 1000, 5000 or 20,000 ppm.

Findings: There were no treatment-related deaths or clinical signs of systemic toxicity in P1 or P2 animals of either sex during pre-mating, in females during gestation, or in females and pups during lactation at any dose level.

At 20,000 ppm, a treatment-related decrease in cumulative body weight gain was noted in P1 animals during the first week of treatment in both sexes and the decrease was evident throughout the pre-mating period in P1 females (Table B.6.6.1-1). Female (P1) feed consumption was decreased at 20,000 ppm during the first week of treatment (Table B.6.6.1-1).

Table B.6.6.1-1Summary of the pre-mating body weights and feed consumption

Dose Level (ppm)	0	1000	5000	20,000
Parameter/Interval				
P1 - Males				
Body Weights (g)				
Week 10	523.6	528.8	525.4	521.1
Cumulative Body Weight Gain (g)				
Week 0-1	59.7	59.8	55.7	52.7*
Week 0-10	293.6	298.0	295.5	288.2
Feed Consumption (g/animal/day)				
Week 0	24.9	25.4	23.8	23.5
Week 9	27.3	27.1	27.5	28.6
P1 - Females kb				
Body Weights (g)				
Week 10	315.2	314.2	307.7	295.7
Cumulative Body Weight Gain (g)				
Week 0-1	25.2	25.0	25.3	21.9
Week 0-10	132.7	132.5	127.1	117.2*
Feed Consumption (g/animal/day)				
Week 0	19.9	18.8	18.6	16.8*

Dose Level (ppm)	0	1000	5000	20,000
Parameter/Interval				
Week 9	21.1	20.7	20.5	20.0

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

There were no treatment-related effects on oestrus cycling in P1 or P2 females at any dietary concentration, as determined by number of oestrus stages during the evaluation period or mean cycle length, at any dietary concentration. There were no treatment-related effects on sperm motility, morphology, epididymal sperm count or concentration, or testicular sperm count and concentration of P1 or P2 males at any dietary level.

Reproductive performance was not affected. There were no treatment-related effects on mating or fertility in either generation at any dietary concentration. There were no treatment-related effects on gestation index, gestation length, or number of pups per litter in either generation. There were no treatment-related deaths during delivery and there were no litters entirely stillborn. The viability of offspring or the ratio of male to female pups in either generation did not show any treatment-related differences.

Organ weight measurements showed increases in relative liver weights in both sexes at 5000 ppm and 20,000 ppm. Absolute liver weights (males) were also increased at 20,000 ppm (Table B.6.6.1-2). These liver weight changes were considered to be equivocal by the study investigators and not indicative of an adverse effect of RH-7281 since no histopathological changes were seen in livers of the high dose animals and liver weight changes were not seen in the P2 animals. No treatment related effects were seen in absolute or relative organ weights in the P2 animals at any dietary concentration.

Table B.6.6.1-2 Summary of the liver weight findings in the 2-generation reproductive toxicity with RH-7281

Dose Level (ppm)	0	1000	5000	20,000
P1 - Males				
Absolute Liver (g)	14.826	15.458 (↑4.2 %)	15.961 (↑7.7 %)	16.566* (↑11.7 %)
Relative Liver (%)	2.596	2.677(↑3.1 %)	2.795*(↑7.6 %)	2.925* (↑12.6%)
P1 - Females				
Absolute Liver (g)	10.552	10.658	11.053	10.565
Relative Liver (%)	2.943	3.001 (↑1.9 %)	3.163* (↑7.4 %)	3.173* (↑7.8 %)

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Gross examination at necropsy and histopathology did not reveal any treatment-related abnormalities.

There were no treatment-related gross findings in F1a, F1b, F2a or F2b pups. There were no treatment-related effects on sexual maturation in F1a males or females at any dose level. There was an apparent decrease in body weight gain in the F1a, F1b, and F2a pups at all dose levels, during the latter stages of lactation (Table B.6.6.1-3), which was accompanied by a decrease in spleen weights (Table B.6.39 and histopathological changes in the spleen (decreased extramedullary hematopoiesis) (Table B.6.40). It was noted by the investigators that decreased spleen weights and histopathological changes in the spleen have been

reported as secondary effects to decreased body weight gain in rats (Boorman, G.A., et.al., 1990. Pathology of the Fischer Rat. Academic Press, Inc., San Diego, CA, USA. page 371; Holsapple, M., et.al.,1998. Feed restrictions during in Utero and Neonatal Life: Effects on Immune Parameters in the Rat. The Toxicologist Vol. 42, No. 1-5, page 102). These effects were not observed in the F2b litters that were placed on untreated diets from Day 14 to 21 of lactation and dosed by gavage during that time with the equivalent dose of RH-7281. In these litters, no effect on body weight was evident and no changes in spleen weight or histopathology of the spleen were seen. The decrease in body weight seen in the F1a, F1b and F2a litters was judged to be a secondary effect related to the palatability of the treated diets and not a systemic toxic effect of RH-7281. The effects noted in the spleen (F1a, F1b, F2a) were considered to be secondary to the decreased body weight gain since these effects were not seen in the F2b litters where body weight gain was not affected.

Table B.6.6.1-3 Summary of the changes in pup body weight gain during lactation in 2-generation reproductive toxicity study with RH-7281.

Dose Level (ppm)	0	1000	5000	20,000
Parameter/Interval				
P1/F1a				
Pup Weight/Litter (g)				
Day 0	6.6	6.7	6.7	6.6
Day 7	18.3	17.8	18.5	17.4
Day 14	37.8	35.9	37.3	35.2*
Day 21	60.2	54.5*	54.8*	51.0*
P1/F1b				
Pup Weight/Litter (g)				
Day 0	6.4	6.4	6.5	6.3
Day 7	17.6	17.2	16.5	16.6
Day 14	36.0	34.6	34.6	33.8
Day 21	58.3	54.4	52.8*	51.2*
P2/F2a				
Pup Weight/Litter (g)				
Day 0	6.4	6.3	6.2	6.2
Day 7	17.3	16.2	15.8	15.6*
Day 14	35.4	34.1	33.7	32.4*
Day 21	55.9	52.1*	49.0*	47.8*
P2/F2b[1]				
Pup Weight/Litter (g)				
Day 0	6.6	6.3	6.5	6.2
Day 4 - preculling[2]	11.2	10.0*	9.6*	9.3*

Dose Level (ppm)	0	1000	5000	20,000
Parameter/Interval				
Day 4 - postculling[2]	11.2	10.0*	9.6*	9.3*
Day 7[2]	18.1	16.1*	15.7*	15.3*
Day 14	36.1	35.0	34.7	33.4*
Day 14 - gavaged pups only	35.0	34.1	34.3	33.1
Day 14 - non-gavaged pups only	37.2	36.0	35.1	33.7
Day 21 - gavaged pups	57.3	55.3	56.2	54.1
Day 21 - non-gavaged pups only	60.0	56.9	57.6	55.3

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

[1] Animals exposed to RH-7281 in the diet until day 14 lactation (postpartum). From day 14 to day 20 of lactation, all doses were administered by gavage at 0, 50, 250 or 1000 mg/kg bw/day (corresponding to 0, 1000, 5000, or 20,000 ppm in the diet, respectively). All dams received the appropriate gavage dose. Pups in half of the litters received the gavage dose; pups in the remaining litters were untreated during this period.

[2] Although significant decreases in pup weight (10-17%) were noted in all treatment groups on days 4 and 7 of lactation, these effects were not judged to be treatment related but most likely related to the increased litter size evident in all treatment groups. By day 14 of lactation, no difference in pup weight was evident in the 1000 and 5000 ppm groups relative to control pups indicating recovery once litter size had been equalised by culling.

Table B.6.6.1-4 Summary of the changes in spleen weights in pups in 2-generation reproductive toxicity study in rats with RH-7281

Dose Level (ppm)	0	1000	5000	20,000
F1a - Males				
Absolute Spleen (g)	0.283	0.223*	0.236*	0.205*
Relative Spleen (%)	0.473	0.415*	0.420*	0.394*
F1a - Females				
Absolute Spleen (g)	0.286	0.248*	0.226*	0.208*
Relative Spleen (%)	0.489	0.461	0.424*	0.408*
F1b - Males				
Absolute Spleen (g)	0.263	0.242	0.220*	0.203*
Relative Spleen (%)	0.442	0.431	0.404	0.385*
F1b - Females				
Absolute Spleen (g)	0.267	0.235	0.216*	0.199*
Relative Spleen (%)	0.457	0.442	0.419	0.395*

Dose Level (ppm)	0	1000	5000	20,000
F2a – Males				
Absolute Spleen (g)	0.253	0.229	0.217*	0.172*
Relative Spleen (%)	0.439	0.425	0.425	0.354*
F2a – Females				
Absolute Spleen (g)	0.267	0.234	0.211*	0.184*
Relative Spleen (%)	0.479	0.460	0.429*	0.380*
F2b - Males (dams and pups dosed by oral gavage)				
Absolute Spleen (g)	0.269	0.280	0.270	0.252
Relative Spleen (%)	0.457	0.534	0.481	0.448
F2b - Females (dams and pups dosed by oral gavage)				
Absolute Spleen (g)	0.251	0.265	0.273	0.258
Relative Spleen (%)	0.453	0.455	0.483	0.481
F2b - Males (dams, only, dosed by oral gavage)				
Absolute Spleen (g)	0.290	0.295	0.282	0.285
Relative Spleen (%)	0.474	0.484	0.468	0.478
F2b - Females (dams, only, dosed by oral gavage)				
Absolute Spleen (g)	0.286	0.266	0.283	0.267
Relative Spleen (%)	0.483	0.485	0.510	0.476

* Significant difference from control ($p<0.05$); ANOVA, Dunnett's t-test

Table B.6.6.1-5 Summary of the incidence of extramedullary haematopoesis in the spleen of rats treated with RH-7281 in 2-generation reproductive toxicity study.

Dose Level (ppm)	0	1000	5000	20,000	0	1000	5000	20,000
Sex (Male/Female)	M	M	M	M	F	F	F	F
Decreased extramedullary hematopoiesis in spleen	# Affected / # Examined							
F1a Pups	0/24	6/26	7/24	9/26	1/24	5/25	12/25	15/27
F2a Pups	1/23	5/24	6/24	13/22	0/23	5/24	12/23	12/22
[1]F2b Pups * (Dams only)	0/10	-	-	1/11	0/9	-	-	0/11
[1]F2b Pups ** (Dams and pups)	0/10	-	-	0/11	0/9	-	-	0/10

[1]Animals exposed to RH-117,281 in the diet until day 14 postpartum. From day 14 to day 20 of lactation, all doses were administered by gavage.

* Only maternal animals were gavage dosed

** Maternal animals and the pups were gavage dosed

To conclude, there were no adverse reproductive effects in the rat following the administration of RH-7281 in rats. Some reductions in pup body weight gain were seen but this was shown to be related to palatability.

Conclusion: Since decreased BW gain during late lactation and spleen effects were not observed in gavaged pups (F2b) from PND14 to PND20, those effects observed in F1a, F1b and F2a were considered secondary to decreased palatability and not systemic effects. Decreased body weight gain observed at PND 14 in high dose groups F1a, F2a and F2b cannot be attributed to palatability since before PND15 diet intake is not significant. The offspring of NOAEL 5000 ppm (360 mg/kg bw/day) is proposed. The parental NOAEL is 5000 ppm (360 mg/kg bw/day) based on decreased body weight gain of P1 females during the pre-mating period and liver effects in males at 2000 ppm . An increase in relative liver weight was noted at dose levels of ≥ 5000 ppm in both sexes and in absolute liver weight in males only at 20000 ppm. No adverse effects were seen at doses up to and including 20000 (1474 - 2091 mg/kg/day in males; 1624 – 2239 mg/kg/day in females, reproductive NOAEL is 20000 ppm.

RMS comment: The study is acceptable.

6.6.2 Developmental toxicity studies

In DAR May (2001)

Reference: CA 5.6.2/01, [redacted] (1995b)

Title: RH-117,281 Technical: oral (gavage) developmental toxicity study in rats.

Report number: Ref No: 94R-079, ER Ref No: 6.1

Guidelines: OECD 414

GLP: Yes

In a study (1995, inlife 1994) groups of 25 mated female rats were administered by gavage from days 6 – 15 of presumed gestation RH-7281 (lot no: DK2011, 94.2 % purity) in corn oil at dose levels of 0 (control), 100, 300, and 1000 mg/kg bw/day. All animals were examined daily for signs of ill health or reaction to treatment. Body weight and feed consumption were monitored throughout gestation. Dams were killed on Day 20 of presumed gestation, and subjected to a gross necropsy. Uteruses were weighed, and the numbers of corpora lutea, implantation sites, live and dead foetuses, and resorptions were recorded. Each live fetus was removed, weighed individually, and examined for external abnormalities. One half of each litter was examined for soft tissue and head alterations; skeletal examinations were performed on all foetuses.

Findings: There were no treatment-related mortalities or clinical signs of toxicity at any dose. Maternal body weight, body weight gain, feed consumption or gravid uterine weights showed no treatment-related differences (Table 6.2.2-1).

Table 6.2.2-1 Mean maternal body weight and feed consumption during gestation

Dose Level (mg/kg bw/day)	Dosage	0	100	300	1000

Dose Level (mg/kg bw/day)	Dosage	0	100	300	1000
Mean maternal body weight during gestation					
Day 0	**Mean**	**234.3**	**237.4**	**234.2**	**234.0**
	S.E.	2.41	2.22	2.22	2.43
	N	23	22	24	24
Day 20	Mean	**430.0**	**429.8**	**430.1**	**428.9**
	S.E.	7.19	4.73	5.34	6.12
	N	23	22	24	24
Mean maternal feed consumption during gestation					
N		23	22	24	24
Days 0 to 6	Mean	**25.0**	**24.2**	**24.6**	**24.5**
	S.E	0.47	0.45	0.49	0.52
Days 6 to 10	Mean	**22.0**	**21.2**	**22.3**	**21.9**
	S.E	0.69	0.50	0.67	0.66
Days 10 - 16	Mean	**23.1**	**23.0**	**22.9**	**23.1**
	S.E	0.58	0.43	0.60	0.53
Days 16 - 20	Mean	**31.0**	**31.0**	**30.4**	**29.9**
	S.E	0.60	0.54	0.81	0.61

There were no treatment-related effects on the numbers of early or late resorptions, live fetuses per litter, fetal body weight or sex ratio. There were no treatment- related external, soft-tissue, head or skeletal malformations, variations, or developmental retardations observed at any dose level.

Conclusion: The NOAEL was 1000 mg/kg bw/day based on the absence of maternal foetal toxicity at dose levels of ≤ 1000 mg/kg bw/day. RH-7281 was not teratogenic in the developmental toxicity study in rats under the conditions of this assay.

RMS: Comment: The study is acceptable. The agreed NOAEL for maternal and developmental toxicity is 1000 mg/kg bw/day.

In DAR May (2001)

Reference: CA 5.6.2/02, [redacted] (1997)

Title: RH-117,281 Technical: oral (gavage) developmental study in rabbits.

Report number: No: : 95R-267, ER Ref No: 8.2

Guidelines: OECD 414

GLP: Yes

In a study (1997,in-life 1996), groups of 16 artificially-inseminated New Zealand White rabbits were administered by gavage triallate (lot no: DSR9510, purity 92.3 %) suspended in 0.5% aqueous methylcellulose solution at dose levels of 0 (control), 100, 300 or 1000 mg of a.i./kg/day on Days 7-19 of presumed gestation. All doses were administered at a constant volume of 20 ml/kg. Clinical signs were recorded once daily on Days 2-6 and 20-29 of presumed gestation, and twice daily from Days 7-19. The does were weighed on Days 0, 7, 9, 11, 14, 17, 20 and 29. Feed consumption was recorded daily on Days 2-29. On Day 29, the does were killed and the thoracic and abdominal cavities were examined for gross changes. Each intact gravid and non-gravid uterus was weighed, and corpora lutea, implantation sites and resorptions were counted. The number of fetuses per litter was counted and their locations within the uterus recorded. All live fetuses were weighed individually and examined for external and visceral alterations (Staples' technique). Sex was determined for each fetus during the visceral examination. Following the visceral examinations, fetuses were skinned and eviscerated, then macerated, stained with Alizarin Red S, and examined for skeletal alterations.

Findings: There were no treatment-related deaths or clinical signs of toxicity in does. No treatment-related effects were noted for maternal body weight, body weight change, feed consumption or gravid uterine weights were noted at any dose level. No treatment-related gross lesions were observed in does during postmortem examinations at any dose level.

The number of viable litters, mean numbers of resorptions, live or dead fetuses, sex ratio per litter did not reveal any treatment-related intergroup differences. No treatment-related differences were observed in foetal body weights. External, visceral and skeletal examinations of foetuses did not reveal any treatment-related abnormalities.

Conclusion: The NOAEL for RH-7281 in the developmental study for developmental and maternal toxicity in rabbits was 1000 mg/kg bw/day based on the absence of treatment-related toxicity in dams or foetuses at the highest test dose. RH-7281 was not teratogenic to rabbits

RMS comment: The study is acceptable.

B.6.7 Neurotoxicity

B.6.7.1 Neurotoxicity studies in rodents

n DAR May (2001)

Reference: CA 5.7.1/01, [redacted] (1997)

Title: RH-117,281 Technical: acute oral (gavage) neurotoxicity study in rats.

Report number: 95R-182, ER Ref No: 10.1

Guidelines: OECD 417 ≅ OPPTS 870.7485 ≅ JMAFF 4200

GLP: Yes

In an acute neurotoxicity study (1997, inlife 1995), groups of 10 male and 10 female Crl:CD®BR rats were administered by gavage RH-7281 (lot no: LG3517; 92.9% active ingredient) suspended in corn oil at dose levels of 0 (control), 125, 500 and 2000 mg /kg bw (10 ml/kg). All rats were observed daily for signs of ill health or reaction to treatment. Each rat received a pretest Functional Observational Battery (FOB) and motor activity assessment 7 days prior to dosing. FOB and motor activity testing was repeated approximately 5 hours

post-dosing (day 0) and on post-dose days 7 and 14. On the day after the final FOB/motor activity assessment, rats were anaesthetized, perfused with neutral buffered formalin and given a limited gross necropsy. Twelve randomly selected control animals (6 males and 6 females) and 12 randomly selected high dose animals (6 males and 6 females) received a special neuropathology evaluation that included microscopic examination of the brain, spinal cord, peripheral nerves of the hindlimb and selected ganglia.

Findings: There were no mortalities, treatment-related clinical signs of toxicity or body weight effects observed during the study period. There were no treatment-related effects on motor activity or any of the FOB parameters. No treatment-related morphologic alterations occurred in any of the examined areas of the central or peripheral nervous systems.

Conclusion: The NOAEL in the acute neuroxicity study in the rat was 2000 mg/kg bw the highest test dose level.

RMS comment: The study is acceptable.

In DAR May (2001)

Reference:	CA 5.7.1/02 (5.3.2/02), [redacted] (1996a)
Title:	RH-117,281: three-month dietary toxicity/neurotoxicity study in rats.
Report number:	No: 94R-233, ER Ref No: 3.1
Guidelines:	Not stated, but satisfied the essential criteria of OECD guideline # 408
GLP:	Yes

Groups of 15 male and 15 female Crl:CD®BR rats were administered RH-117,281 in the diet for three months at concentrations of 0 (control), 1000, 5000, and 20,000 ppm. The NOAEL for RH-117,281 in the 3-month neurotoxicity dietary study in rats was 20000 ppm, the top dose tested (equivalent to 1509 or 1622 mg/kg bw/day in males or females respectively) based on the absence of any treatment related effects.

RMS: The study is acceptable.

B.6.7.2. Delayed polyneuropathy studies

Since the chemical structure of zoxamide is not similar or related structures to those capable of inducing delayed polyneuropathy, a delayed polyneuropathy study is not considered required.

B.6.8. Other toxicological studies

B.6.8.1. Toxicity studies on metabolites and relevant impurities

For the first inclusion of zoxamide in Annex I to Directive 91/414/EEC, data on some metabolites was evaluated and is summarised below. .

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R141,452 and RH-141,455 were the major metabolites of RH-7281 found in the potato metabolism study. They were also isolated as minor metabolites of RH-7281 in the rat metabolism study. Although essentially no human exposure is anticipated because actual residues in field trials never exceeded trace levers approximating

the limits of quantification, metabolism and limited toxicity studies were conducted to confirm the absence of any hazard.

In DAR May (2001)

Reference: CA 5.8.1/01, [redacted] (1998a)

Title: 14C-RH-141,452: Rat metabolism study, Tier I testing

Report number: No: 97RC-154, ER Ref No: 27.1

Guidelines: OECD 417 ≅ OPPTS 870.7485 ≅ JMAFF 4200

GLP: Yes

In a study (1998), the absorption, distribution, metabolism and elimination of [^{14}C]- RH-141,452 (Lot No. 955.0005, Specific activity 75.38 mCi/g) in male rats were studied. Four male rats were each administered a single oral dose of [^{14}C]- RH-141,452 in pH adjusted water at a nominal dose of 1000 mg/kg body weight. After 78 hours post dose, the rats were sacrificed and the total radiolabelled residues were determined.

Metabolite analyses were conducted on pooled day-1 and day-2 and day-3 urine and faeces samples. These samples accounted for >99% of the dosed radiochemical. Urine samples were filtered and directly analysed by reversed-phase high-performance liquid chromatography (RP-HPLC). Day-1, day-2, and day-3 faeces homogenates were extracted three times with methanol, which was concentrated and analysed by RP-HPLC. The total radioactive residue in the post extraction solids (PES) fraction was determined by combustion analysis using a biological sample oxidiser followed by liquid scintillation counting (LSC).

Metabolites were isolated and purified using preparative HPLC followed by preparative TLC. Each purified metabolite was then characterised and identified using normal phase thin layer chromatography (NP-TLC) and/or liquid chromatography/mass spectrometry (LC/MS).

Findings: The total radioactivity levels excreted from urine, cage rinse, faeces and expired air are summarised in the following table (Table B.6.8.1-1)

Table B.6.8.1-1 Mass balance of the distribution of [^{14}C]-RH-141,452 in rats at 78 hours post-dose

Animal	% Dose in Urine	% Dose in Cage Rinse	% Dose in Feces	% Dose in Expired Air	Total % Recovered
G1-001-M	87.68	2.99	3.32	0.01	94.00
G1-002-M	108.29	2.77	0.63	0.01	111.70
G1-003-M	94.98	1.77	1.03	0.01	97.79
G1-004-M	91.02	2.55	1.80	<0.01	95.37
Mean	95.49	2.52	1.70	0.01	99.72

The majority of the radioactivity was eliminated in the urine (approximately 98% in urine and cage rinse), with only a small amount excreted from faeces (<2%). Approximately 0.01% of dosed radioactivity was found in the expired air. Tissues were not analysed since more than 95% of radioactivity was found in the excreta. The excretion of the ^{14}C radioactivity was rapid, with more than 97% excreted within 24 hours. The average recovery of the administered dose was >99%.

Metabolism studies showed the majority of RH-141,452 was eliminated unchanged through urine, accounting for >94 % of the administered dose. Three minor conjugates, M-2, M-3 (glucuronide conjugates), and M-4 (glycine conjugate) were also found in the urine samples, accounting for approximately 3 % of the administered dose. An additional 1.6 % of the administered radioactivity was excreted in the faeces as the parent chemical (Table B.6.8.1-2). The metabolite pathways are illustrated in Figure 6.8.1-1.

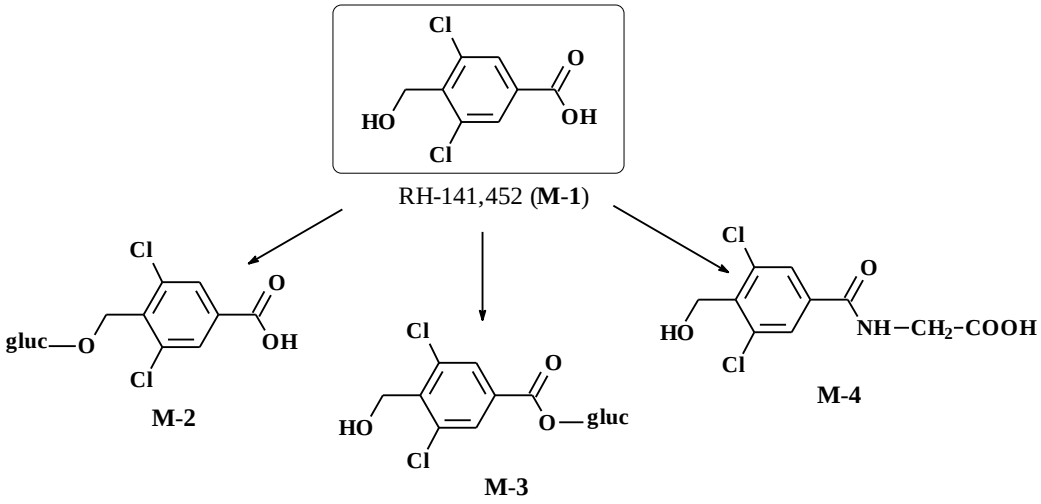
Table B.6.8.1.-2 Distribution of metabolites in rat urine and faeces after oral administration of [^{14}C]-RH-141,452 (% administered dose)

	% of Dose		Total
Metabolite ID	in Urine	in faeces	% of Dose
M-1	94.41	1.60	96.01
M-2 M-3 M-4	3.25	ND	3.25
Unknown	ND	0.03	0.03

ND: Not detected

NB. There is no correlation in the nomenclature of the metabolites (M-1 to M-4) with those found in other studies

Figure B.6.8.1-1 Metabolite pathways for RH-141,452



Conclusion: The absorption, distribution, metabolism and elimination of [14C]-RH-141,452 was investigated in male rats. The majority of RH-141,452 was eliminated unchanged through urine, accounting for >94% of the administered dose. Three minor conjugates, M-2, M-3 (glucuronide conjugates), and M-4 (glycine conjugate) were also found in the urine samples, accounting for approximately 3% of the administered dose. An additional 1.6% of the administered radioactivity was excreted in the faeces as the parent chemical.

RMS: The study is acceptable.

In DAR May (2001)

Reference:	CA 5.8.1/02 [redacted] (1998d)
Title:	RH-141,452: Acute oral toxicity study in male and female mice.
Report number:	No: 98R-049, ER Ref No: 25.2
Guidelines:	OECD 401
GLP:	Yes

In an acute oral toxicity study (1998) six male and six female Crl:CD-1®(ICR)BR mice were administered by gavage a limit dose of 5000 mg/kg bw (20 ml/kg) of the metabolite RH-141,452 (lot no: BM-3915, containing 97.7 % active ingredient) in a corn oil suspension was assessed in mice.

Findings: There were no treatment-related mortalities, clinical signs of toxicity or effects on body weight when compared to historical control data. Necropsy revealed no gross changes. The acute oral LD_{50} for RH-141,452 in male and female mice was > 5000 mg/kg bw.

Conclusion: The acute oral LD50 of RH-141,452 in male and female mice was > 5000 mg/kg bw.

RMS comment: The study is acceptable.

In DAR May (2001)

Reference:	CA 5.8.1/03, Sames, J.L., Ciaccio, P.J.(1998a)
Title:	RH-141,452: Salmonella typhimurium gene mutation assay (Ames test)
Report number:	No: 98R-050, ER Ref No: 25.3

Guidelines: OECD 471

GLP: Yes

In a bacterial gene mutation assay(1998), histidine-dependent TA98, TA100, TA1535, TA1537, and TA102 strains of *Salmonella typhimurium* were exposed to the metabolite, RH-141,452 (lot no: BM 3915, 97.7 % active ingredient) dissolved in dimethyl sulfoxide at concentrations of 0 (solvent control) to 5000 μg/plate in the presence and absence of an Arochlor 1245-treated rat S-9 liver fraction. In the tests with metabolic activation, 2-anthramine was used as the positive control for all strains. In the tests without metabolic activation, the positive controls used were: 2- nitrofluorene (TA98), sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), and mitomycin-c (TA102). The number of revertants was determined. The results were confirmed in an independent assay.

Findings: RH-141,452 did not induce an increase in revertants when compared to solvent controls. This was true for all tester strains both with and without metabolic activation.

Conclusion: RH-141,452 was not mutagenic in the *Salmonella* gene mutation assay under the conditions of this study.

RMS comment: The study is acceptable. It can be concluded that metabolite RH-141,452 **is not** of greater toxicity than parent.

In DAR May (2001)

Reference: CA 5.8.1/04, [redacted] (1998b)

Title: ^{14}C-RH-141,455: Rat metabolism study, Tier I testing

Report number: No: 98RC-017, ER Ref No: 27.2

Guidelines: OECD 417 ≅ OPPTS 870.7485 ≅ JMAFF 4200

GLP: Yes

In a study (1998), the absorption, distribution, metabolism and elimination of [^{14}C]- RH-141,455 (Lot No. 958.0005, Specific activity 74.46 mCi/g) in male rats were studied. Four male rats were administered a single oral dose of [^{14}C]- RH-141,455 in pH adjusted water at a nominal dose of 1000 mg/kg bw. At 168 hours post dose, the rats were sacrificed and the total radiolabelled residues were determined.

Metabolite analyses were conducted on pooled day-1 and day-2 feces and day-1 urine. These samples accounted for >74 % of the dosed radiochemical. Urine sample was filtered and directly analysed by reversed-phase high-performance liquid chromatography (RP-HPLC). Day-1 and day-2 faeces homogenates were extracted three times with methanol (CH_3OH), which was concentrated and analysed by RP-HPLC. The total radioactivity residue in the post extraction solids (PES) fraction was determined by combustion analysis using a biological sample oxidiser followed by liquid scintillation counting (LSC).

The total radioactivity levels excreted from urine, cage rinse, faeces and expired air are summarised in Table B.6.8.1-3)

Table B.6.8.1-3 Distribution of radiolabel in rats 168 hours after oral administration of [^{14}C]-RH-141,455:

Animal	% Dose in Urine	% Dose in Cage Rinse	% Dose in Faeces	% Dose in Expired Air	Total % Recovered
G1-001-M	12.24	17.98	62.86	0.01	93.09
G1-002-M	16.07	8.51	68.53	0.01	93.12
G1-003-M	7.01	2.42	84.68	0.01	94.12
G1-004-M	8.88	8.27	73.99	0.01	91.15
Mean	11.05	9.30	72.52	0.01	92.87

RH-141,455 was excreted from the rat in a moderate rate following oral administration. Approximately 73% of the administered dose was recovered in the faeces, and 20% was recovered in the urine and cage rinse, which may have contained faeces owing to the animals having diarrhoea.

The data indicate that the majority of the radioactivity was eliminated in the faeces (73%), and 11% of the radioactivity was eliminated in the urine (with 9% in the cage rinse). Only 0.01% of dosed radioactivity was found in the expired air. Tissues were not analysed since more than 92% of radioactivity was found in the excreta. The excretion rate of the ^{14}C radioactivity was moderate with approximately 47% excreted within 24 hours, and an additional 32% excreted during the 24-48 hour interval. The average recovery of the administered dose was >92%.

Metabolites were isolated and purified using preparative HPLC followed by preparative TLC. Each purified metabolite was then characterised and identified using normal phase thin layer chromatography (NP-TLC) and/or liquid chromatography/mass spectrometry (LC/MS).

Conclusion: Greater than 96% of radioactivity excreted from faeces and urine was identified to be unchanged RH-141,455. Some very minor metabolites were also observed in urine samples but were not identified due to their extremely low percentage of dose.

RMS: The study is acceptable.

In DAR May (2001)

Reference: CA 5.8.1/05, [redacted] (1998b)

Title: RH-141,455: Acute oral toxicity study in male and female mice.

Report number: No: 98R-047, ER Ref No: 27.3

Guidelines: OECD 401

GLP: Yes

In an acute oral toxicity study (1998), six male and six female Crl:CD-1®(ICR)BR mice were administered by gavage a limit dose of 5000 mg/kg bw (10 ml/kg) of the metabolite RH-141,455 (Lot No. WJZ 4091B, 98.74% a.i.)

Findings: There were no mortalities or effects on body weight when compared with historical control data. Scant faeces was noted in males and/or females on days 1 and 2. Necropsy revealed no gross changes.

Conclusion: The acute oral LD_{50} for RH-141,455 in male and female mice was greater than 5000 mg/kg bw.

RMS: The study is acceptable.

In DAR May (2001)

Reference:	CA 5.8.1/06 Sames, J.L., Ciaccio, P.J. (1998b)
Title:	RH-141,455: Salmonella typhimurium gene mutation assay (Ames test).
Report number:	No: 98R-048, ER Ref No: 27.4
Guidelines:	OECD 471
GLP:	Yes

In a bacterial gene mutation assay(1998), histidine-dependent TA98, TA100, TA1535, TA1537, and TA102 strains of *Salmonella typhimurium* were exposed to the metabolite, RH-141,455 (Lot number WJZ 4091B, 98.74% active ingredient) dissolved in dimethyl sulfoxide at concentrations of 0 (solvent control) to 5000 μg/plate in the presence and absence of an Arochlor 1245-treated rat S-9 liver fraction. In the tests with metabolic activation, 2-anthramine was used as the positive control for all strains. In the tests without metabolic activation, the positive controls used were: 2- nitrofluorene (TA98), sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), and mitomycin-c (TA102). The number of revertants was determined. The results were confirmed in an independent assay.

Findings: The metabolite, RH-141,455 did not induce an increase in revertants compared to solvent controls. This was true for all tester strains both with and without metabolic activation. RH-141,455 was not mutagenic in the *Salmonella* gene mutation assay under the conditions of this assay.

Conclusion: RH-141,455 was not mutagenic in this Ames test.

RMS: The study is acceptable.

Submitted for the purpose of renewal.

Reference:	CA 5.8.1/07 Woods, I. (2014b)
Title:	RH-141455: In Vitro Micronucleus Test In Human Lymphocytes
Report number:	FRK0050
Guidelines:	OECD 487.
GLP:	Yes

Executive Summary:

This study was designed to assess the potential of RH-141,455 to cause an increase in the induction of micronuclei in cultured human lymphocytes (stimulated by phytohaemagglutinin (PHA)) *in vitro*. Following a preliminary toxicity test, cells were exposed to RH-141455 for 3 hours in both the absence and presence of exogenous metabolic activation (S9 mix), and for 20 hours in the absence of S9 mix. The maximum final concentration to which the cells were exposed was 587.5 µg/mL, in order to test up to the maximum concentration that was seen not to alter the pH by more than 1 pH unit. Vehicle (DMSO) and positive control cultures were included in all test conditions. Following 3-hour treatment in the absence or presence of S9 mix, no significant reductions in cytokinesis block proliferative index (CBPI) were observed. In the absence of S9 mix following 20 hour treatment, no significant reductions in CBPI were observed. Concentrations of RH-141,455 selected for micronucleus analysis were 146.88, 293.75 and 587.5 µg/mL, in all exposure conditions. In both the absence and presence of S9 mix following 3-hour treatment, and in the absence of S9 mix following 20 hour treatment, RH-141,455 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei compared to the vehicle controls. The positive control compounds caused significant increases in the number of binucleate cells containing micronuclei under appropriate conditions, demonstrating the efficacy of the S9 mix and the sensitivity of the test system. Based on these findings, it is concluded that RH-141,455 administered for 3 hours in both the absence and presence of S9 mix and for 20 hours in the absence of S9 mix only, at concentrations of up to 587.5 µg/mL, did not show any evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes in this *in vitro* test system under the experimental conditions described.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test material RH-141,455

Description: White powder

Lot/Batch: Batch No.: 76791-1-31

Purity: 100%

Stability: Stable for duration of the study.

Solvent: Dimethyl sulphoxide (DMSO)

2. Control materials:

Negative: DMSO (1% v/v)

Positive:

Nonactivation (-S9): Mitomycin C, Colchicine

Activation (+S9) Cyclophosphamide

3. Activation: S9 fraction was prepared from phenobarbital and 5,6-benzoflavone-induced male rat liver according to standard protocols. Aliquots were frozen and stored at *ca.* -80ºC, then thawed for preparation of the S9 mix. The co-factors were filter-sterilised with a 0.2 µm non-pyrogenic sterile filter prior to use.

S9 Mix composition:	Component	Concentration
	S9 fraction	10% v/v
	$MgCl_2$	8 mM
	Glucose-6-phosphate	5 mM
	NADP	4 mM
	KCl	33 mM
	Sodium phosphate buffer (pH 7.4)	100 mM

4. Test cells: Human blood was collected aseptically from two healthy, non-smoking, adult donors, pooled (in equal volumes from each donor) and diluted with RPMI 1640 tissue culture medium supplemented with 10% foetal calf serum, 0.2 IU/mL sodium heparin, 20 IU/mL penicillin / 20 µg/mL streptomycin and 2.0 mM L-glutamine. As lymphocytes do not normally undergo cell division, they were stimulated to do so by the addition of phytohaemagglutinin (PHA). Cultures were established from the prepared (pooled) sample and dispensed as 5 mL aliquots (in sterile universal containers) so that each contained blood (0.4 mL), culture medium (4.5 mL) and PHA solution (0.1 mL). All cultures were then incubated at 37°C, and the cells re-suspended (at least once daily) by gentle inversion

5. Culture medium: RPMI 1640 tissue culture medium.

6. Test concentrations

(a) Preliminary cytotoxicity assays

Without S9 mix*: 0, 1.15, 2.29, 4.59, 9.18, 18.36, 36.72, 73.44, 146.88, 293.75, 587.50 µg/mL

With S9 mix*: 0, 1.15, 2.29, 4.59, 9.18, 18.36, 36.72, 73.44, 146.88, 293.75, 587.50 µg/mL

Without S9 mix**: 0, 1.15, 2.29, 4.59, 9.18, 18.36, 36.72, 73.44, 146.88, 293.75, 587.50 µg/mL

* 3-hour treatment; **20-hour treatment

(b) Chromosomal aberration assay:

Without S9 mix*: 36.72, 73.44, 146.88, 293.75, 587.50 µg/mL

With S9 mix*: 36.72, 73.44, 146.88, 293.75, 587.50 µg/mL

Without S9 mix**: 36.72, 73.44, 146.88, 293.75, 587.50 µg/mL

* 3-hour treatment; **20-hour treatment

Underlined treatments: concentrations selected for micronucleus analysis

B. TEST PERFORMANCE

This study was conducted between 29 April 2014 and 12 June 2014.

1. Test procedure

In the preliminary test, after stimulation with PHA, lymphocyte cultures were incubated for approximately 48 hours, before addition of the test material. The test material was prepared in the vehicle and dilutions made for both sets of cultures. Single cultures were prepared for each treatment level and duplicate cultures were prepared for vehicle controls. S9 homogenate was

present in appropriate cultures at a final concentration of 2% v/v.

Before treatment all cultures were centrifuged and resuspended in fresh medium. Test material preparations were added to cultures at 1% v/v, and incubated at 37°C for 3 hours. The cells were centrifuged again, and the medium replaced. Cytochalasin B, at a final concentration of 6 µg/mL, was then added to all cultures. The cultures were incubated for a further 17 hours until the scheduled harvest time.

For the 20-hour treatment in the absence of S9 mix, the human lymphocyte cultures were set up as previously described. A 20-hour continuous treatment (1.5 to 2 normal cell cycles) at 37°C was used in the absence of S9 mix. Test material preparations were added to cultures at 1% v/v in the presence of Cytochalasin B (6 µg/mL).

The procedure for the three main tests was the same as that for the preliminary tests, with the following exceptions: positive control cultures were included for all tests; duplicate cultures were prepared for each treatment level and positive control cultures; quadruplicate cultures were prepared for vehicle controls.

Following treatment in all preliminary and main test assays, the cells were harvested and slides prepared, so that binucleate cells could be examined for micronucleus induction by fluorescence microscopy.

2. Statistics

The analysis assumed that the replicate was the experimental unit. An arcsine transformation was used to transform the data. RH-141455 treated groups were then compared to control using Williams' tests, unless there was evidence against a monotonic dose-response relationship, in which case Dunnett's test was used instead. Positive controls were compared to control using *t*-tests. Trend tests have also been carried out using linear contrasts by group number. These were repeated, removing the top dose group, until there were only 3 groups. Statistical significance was declared at the 5% level for all tests. Data were analysed using SAS 9.1.3 (SAS Institute 2002) and Quasar 1.4 (Quasar 1.4 2012).

3. Evaluation criteria

Criteria for assessment of assay acceptability were as follows:

Positive controls must show clear unequivocal positive responses.

The negative control (solvent, vehicle control or untreated cultures) must show reproducible low and consistent micronucleus frequencies.

The test material was considered to be positive if the following conditions are met:

Statistically significant increases in the frequency of micronucleated cells are observed at one or more test concentrations compared with the solvent control.

The increases are reproducible between replicate cultures.

The increases are not associated with large changes in pH, osmolality of the treatment medium or extreme toxicity.

There is evidence of a concentration-response relationship.

A negative response is claimed if no statistically significant increases in the number of micronucleated cells above concurrent solvent control frequencies are observed at any concentration, and there is no evidence of a concentration-response relationship.

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

The pH of the test substance in medium was tested. Concentrations of 1175 µg/mL, and 2350 µg/mL caused a change of greater than 1 pH unit. On the basis of these fluctuations in pH, the highest concentration was set at 587.50 µg/mL.

In all of the assays in the preliminary test, under all treatment regimens, both in the presence and absence of S9 mix, no significant reduction in cytokinesis block proliferative index (CBPI) was seen compared to vehicle control values, following exposure to RH-141455 at up to 587.50 µg/mL. Concentrations of RH-141455 selected for the main micronucleus test were 36.72, 73.44, 146.88, 293.75 and 587.5 µg/mL.

B. CYTOGENICITY AND CHROMOSOME ANALYSIS

In all of the assays in the main test, under all treatment regimens, both in the presence and absence of S9 mix, no significant reduction in CBPI was seen compared to vehicle control values, following exposure to RH-141455 at up to 587.50 µg/mL. Concentrations of RH-141455 selected for micronucleus analysis were 146.88, 293.75 and 587.5 µg/mL.

RH-141455 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls.

Mean micronucleus induction in the vehicle control was within the historical control range. The positive control compounds caused significant increases in the number of binucleate cells containing micronuclei, demonstrating the sensitivity of the test system.

Table 6.8.1-4: Summary of Results

Main test: 3-hour treatment in the absence of S9 mix								
Treatment	**Conc. (µg/mL)**	**CBPI**	**Mean CBPI**	**Mean cytotoxicity (%)**	**Binucleated cells containing micronuclei**			
					per 1000 cells	**Mean**	**Pairwise *p*-value**	**Trend test *p*-value**
Vehicle#		1.83	1.81	0	5	7.0		
		1.75			8			
		1.82			6			
		1.82			9			
Test material	146.88	1.74	1.75	6.5	5	5.0	0.301	
		1.76			5			
	293.75	1.80	1.80	0.9	5	4.5	0.301	0.058
		1.80			4			
	587.5	1.79	1.78	3.4	7	7.0	0.301	0.931
		1.76			7			
MMC	0.3	1.46	1.45	44.0	30	27.0	<0.001***	
		1.45			24			
COL	0.07	1.69	1.67	16.4	18	17.5	<0.001***	
		1.66			17			
Main test: 3-hour treatment in the presence of S9 mix								
Treatment	**Conc. (µg/mL)**	**CBPI**	**Mean CBPI**	**Mean cytotoxicity (%)**	**Binucleated cells containing micronuclei**			
					per 1000 cells	**Mean**	**Pairwise *p*-value**	**Trend test *p*-value**
Vehicle#		1.70	1.70	0	6	6.8		
		1.72			10			
		1.67			6			

		1.71			5			
Test material	146.88	1.71	1.69	1.6	7	6.5	1.000	
		1.67			6			
	293.75	1.74	1.69	1.8	8	8.0	0.443	0.343
		1.64			8			
	587.5	1.74	1.72	-2.4	7	8.0	0.443	0.241
		1.70			9			
CPA	10	1.45	1.45	36.0	18	17.5	<0.001***	
		1.45			17			
Main test: 20-hour treatment in the absence of S9 mix								
Treatment	**Conc. (µg/mL)**	**CBPI**	**Mean CBPI**	**Mean cytotoxicity (%)**	**Binucleated cells containing micronuclei**			
					per 1000 cells	**Mean**	**Pairwise *p*-value**	**Trend test *p*-value**
Vehicle#		1.78	1.74	0	9	8.5		
		1.72			7			
		1.70			10			
		1.75			8			
Test material	146.88	1.72	1.69	6.9	10	9.0	1.000	
		1.65			8			
	293.75	1.76	1.76	-2.6	10	7.5	1.000	0.47
		1.75			5			
	587.5	1.66	1.66	11.0	10	9.0	0.963	0.968
		1.65			8			
MMC	0.10	1.46	1.50	31.8	23	21.0	<0.001***	
		1.54			19			
COL	0.020	1.50	1.52	29.9	16	17.0	<0.001***	
		1.53			18			

CBPI: cytokinesis block proliferative index
MMC: mitomycin C
COL: colchicine
CPA: cyclophosphamide
vehicle control = DMSO (1% v/v)
*** $p < 0.001$

III. CONCLUSIONS

It is concluded that RH-141,455 administered for 3 hours in both the absence and presence of S9 mix and for 20 hours in the absence of S9 mix only, at concentrations of up to 587.5 µg/mL, did not show any evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes in this *in vitro* test system under the experimental conditions described.

RMS: The study is acceptable.

Submitted for the purpose of renewal.

Reference: CA 5.8.1/08 Woods, I. (2014a)

Title: RH-141455: In Vitro Mutation Test Using Mouse Lymphoma L5178Y Cells

Report number: FRK0049

Guidelines: US EPA OPPTS 870.5300; OECD 476; EU Council Regulation No.440/2008, Part B, Method B17.

GLP: Yes

Executive Summary:

An *in vitro* mammalian cell mutation assay was performed in mouse lymphoma L5178Y TK+/- 3.7.2c cells to test the potential of RH-141,455 to cause gene mutation at the TK locus. The study consisted of a preliminary toxicity test and three independent mutagenicity assays. The cells were exposed for either 3 or 24 hours in the absence of exogenous metabolic activation (S9 mix) or 3 hours in the presence of S9 mix. A final concentration of 1175 μg/mL (0.005 M) was used as the maximum concentration in the preliminary toxicity test, and main tests, due to a pH change greater than 1 unit observed at 2350 μg/mL. Limited toxicity was observed in the preliminary toxicity test. Following a 3-hour exposure to RH-141,455 at concentrations from 2.29 to 1175 μg/mL , relative suspension growth (RSG) ranged between 152 to 82% and from 91 to 44% in the absence and presence of S9 mix, respectively. Following a 24-hour exposure in the absence of S9 mix RSG ranged between 110 to 42%. In the main mutation test, following 3-hour treatment in the absence and presence of S9 mix, there were no increases in the mean mutation frequencies that exceeded the sum of the mean concurrent vehicle control mutant frequency and the Global Evaluation Factor (GEF: 126 x 10^{-6}) for microwell assays. The maximum concentration assessed for mutant frequency in both the 3-hour treatment in the absence and presence of S9 mix was 1175 μg/mL. In the absence and presence of S9 mix relative total growth (RTG) was reduced to 64 and 69%, respectively, which were within acceptable levels of toxicity. Following 24-hour treatment in the absence of S9 mix, the maximum concentration assessed for mutant frequency was 1175 μg/mL. No increase in mutant frequency exceeded the sum of the mean concurrent vehicle control mutant frequency and the GEF of 126 x 10^{-6} for microwell assays. There was no reduction in RTG compared to the concurrent vehicle controls. In all tests, the concurrent vehicle and positive controls were within acceptable ranges. RH-141455 did not demonstrate mutagenic potential in this *in vitro* cell mutation assay, under the experimental conditions described.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: RH-141,455

Description: White powder

Lot/Batch: Batch No.: 76791-1-31

Purity: 100%

Stability: Stable for duration of the study.

2. Control Materials:

Negative: Dimethyl sulphoxide (DMSO) (1% v/v)

Positive:

Nonactivation (-S9): Methyl methanesulphonate (MMS)

Activation (+S9): Benzo[a]pyrene (BaP)

3. Activation:	S9 fraction was prepared from phenobarbital and 5,6-benzoflavone-induced male rat liver according to standard protocols. Aliquots were frozen and stored at *ca.* -80ºC, then thawed for preparation of the S9 mix. The S9 mix contained: S9 fraction (5% v/v), glucose-6-phosphate (6.9 mM), NADP (1.4 mM) in supplemented RPMI 1640 medium. The co-factors were prepared, neutralised with 1N NaOH and filter sterilised before adding to S9 fraction and the medium.
4. Test cells:	L5178Y mouse lymphoma (3.7.2c) cells were obtained from American Type Culture Collection (ATCC), Virginia. Spontaneous thymidine kinase deficient mutants, TK $^{-/-}$, were eliminated from the cultures by a 24-hour incubation in the presence of methotrexate, thymidine, hypoxanthine and glycine two days prior to storage at -196°C, in heat-inactivated donor horse serum (HiDHS) containing 10% DMSO. Cultures were used within ten days of recovery from frozen stock. Cell stocks are periodically checked for freedom from mycoplasma contamination.
5. Culture medium:	RPMI 1640, buffered with 2 mg/mL sodium bicarbonate, supplemented with L-glutamine (to 2.0 mM) and gentamicin (to 50 μg/mL), Synperonic F68 (to 0.1% v/v), sodium pyruvate (to 1.0 mM) and heat-inactivated donor horse serum (to 10% v/v)
6. Locus examined:	TK locus
7. Test concentrations:	
(a) Preliminary cytotoxicity (range finding) assay:	Without S9 mix*: 2.29, 4.59, 9.18, 18.36, 36.72, 73.44, 146.88, 293.75, 587.5, 1175 μg/mL With S9 mix*: 2.29, 4.59, 9.18, 18.36, 36.72, 73.44, 146.88, 293.75, 587.5, 1175 μg/mL Repeat without S9 mix**: 2.29, 4.59, 9.18, 18.36, 36.72, 73.44, 146.88, 293.75, 587.5, 1175 μg/mL * 3 hour treatment ** 24 hour treatment
(b) Mutation assay:	Without S9 mix*: 385, 481.3, 601.6, 752, 940, 1175 μg/mL With S9 mix*: 385, 481.3, 601.6, 752, 940, 1175 μg/mL Repeat without S9 mix**: 385, 481.3, 601.6, 752, 940, 1175 μg/mL * 3 hour treatment ** 24 hour treatment

B. TEST PERFORMANCE

This study was conducted between 29 April 2014 and 7 June 2014.

1. Test procedure

Doses were selected on the basis of a cytotoxicity pre-test. There was limited evidence of toxicity, however the maximum concentration assessed for mutant frequency in the main test was 1175 μg/mL in the absence and presence of S9 mix, based on pH.

For 3-hour exposures, cultures contained a total of 1.2×10^7 cells. The final volume of the cultures was 10 mL and the final concentration of the S9 fraction was 2% v/v, if present. For 24-hour exposures, cultures contained a total of 3×10^6 cells in a total volume of 10 mL. Duplicate cultures were prepared for each concentration of test material and positive control. Quadruplicate cultures were prepared for vehicle

controls. Aliquots of 100 µL of test material dilution (at 100 times the desired final concentration), vehicle or positive control were added, then all cultures were incubated with continuous shaking for 3 hours at 37°C. At least four serial dilutions of the test material were tested.

Following the 3-hour exposure, the cells were washed once, re-suspended in media to nominally 2×10^5 cells/mL (assuming no cell loss) and incubated for a further 48 hours to allow for expression of mutant phenotype. The cultures were sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to 2×10^5 cells/mL where necessary. After 48 hours cultures with a density of more than 1×10^5 cells/mL were assessed for cloning efficiency (viability) and mutant potential by plating in 96-well plates. Cloning efficiency was assessed by plating 1.6 cells/well , two plates being prepared per culture. Mutant potential was assessed by plating 2×10^3 cells/well in selective medium, two plates being prepared per culture. The plates were placed in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air.

After the plates had been incubated for at least 7 days for viability plates, and approximately 10 to 14 days for mutant plates, the number of empty wells was assessed for each 96-well plate (P0). P0 was used to calculate the cloning efficiency (CE) and mutant frequency (MF). The colony size distribution in the vehicle and positive controls was examined to ensure that there was an adequate recovery of small colony mutants.

Following the 24-hour exposure, the cells were washed once, re-suspended, and counted to ascertain treatment growth. The cultures were then diluted to 2×10^5 cells/mL, incubated and sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to 2×10^5 cells/mL, the intention being to retain at least 1×10^7 cells. Following this, the procedure was the same as in the 3-hour treatment.

2. Statistics

The data were analysed using Fluctuation application SAFEStat (SAS statistical applications for end users) version 1.1, which follows the methods described by Robinson et al. (1989).

3. Evaluation criteria

The assay was considered valid if all of the following criteria were met:

The highest concentration of the test material tested was one that allowed the maximum exposure up to 5000 µg/mL or 10 mM for freely soluble compounds, or the limit of toxicity (i.e. relative total growth reduced to approximately 10 to 20% of the concurrent vehicle control) or the limit of solubility. For a toxic substance, at least 4 analysable concentrations should have been achieved which ideally spanned the toxicity range of 100 to 10% relative total growth (RTG).

The mean vehicle control value for mutant frequency was between 50 to 170×10^{-6}; the mean cloning efficiency was between 65 to 120%; and, the mean suspension growth was between 8 to 32 on Day 2 following 3-hour treatments and between 32 to 180 on Day 2 following a 24-hour treatment. Obvious outliers were excluded. However, there were at least 2 vehicle control cultures remaining.

Positive controls showed an absolute increase in mean total MF above the mean concurrent vehicle control MF of at least 300×10^{-6} (at least 40% of this was due to the number of small mutant colonies); mean RTG's for the positive controls were greater than 10%; there was an absence of confounding technical problems such as contamination, excessive numbers of outliers and excessive toxicity; and, there was not excessive heterogeneity between replicate cultures.

The test material was assessed for mutagenic potential in accordance with the following criteria which were applied for assessment of individual assay results using data for MF where the RTG normally exceeded 10%:

The assay was considered valid in accordance with the assay acceptance criteria.

The test material was regarded as negative if the mean mutant frequency of all test concentrations was less

than the sum of the mean concurrent vehicle control mutant frequency and the GEF (Global Evaluation Factor; for microwell assays this is 126 x 10^{-6}).

If the mutant frequency of any test concentrations exceeded the sum of the mean concurrent solvent control mutant frequency and the GEF, a linear trend test was applied: if the linear trend test was negative, the result was regarded as negative; if the linear trend test was positive, this indicated a positive, biologically relevant response.

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

No precipitate was observed at any concentration tested in the absence or presence of S9 mix, following a 3-hour exposure. Exposure to RH-141455 at concentrations from 2.29 to 1175 µg/mL in the absence and presence of S9 mix (3-hour exposure) resulted in relative suspension growth (RSG) values from 152 to 82% and from 91 to 44%, respectively.

Following a continuous exposure for 24 hours in the absence of S9, no precipitation was observed. Exposure to concentrations from 2.29 to 1175 µg/mL resulted in RSG values from 110 to 42%.

B. MUTATION ASSAYS

No precipitate was observed at any concentration tested at the end of treatment either in the absence of presence of S9 for both exposure conditions. Following the 3-hour exposure period in the absence of S9 mix, relative total growth (RTG) values of 91 to 64% were obtained relative to the vehicle control. Following the 3 hour exposure period in the presence of S9 mix, RTG values of 102 to 69% were obtained relative to the vehicle control. In both the absence and in the presence of S9 mix there were no increases in the mean mutant frequencies of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control mutant frequency and the Global Evaluation Factor (GEF), within acceptable levels of toxicity.

In the 24 hour assay in the absence of S9 mix, no reduction in RTG values were obtained relative to the vehicle control. There were no increases in the mean mutant frequencies of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control mutant frequency and the GEF, within acceptable levels of toxicity.

The positive controls induced an acceptable increase in mutation frequency and an acceptable increase in the number of small colony mutants.

Table 6.8.1-5: Main Mutation Test Results

Main mutation test - 3 hour treatment in the absence of S9 mix								
Treatment (µg/mL)		**Rep.**	**Cell conc. (x 10^5/mL)**		**Viability plate count***	**Mutant plate count***	**Mean RTG (%)**	**Mean MF (x 10^{-6})**
			24 h	**48 h**	**Day 2**	**Day 2**		
Vehicle control		A	4.29	8.17	38	150	100	98
		B	4.19	8.58	40	156		
		C	4.04	8.82	31	157		
		D	3.71	9.44	35	162		
Test material	385	A	3.69	8.83	41	162	91	95
		B	4.04	8.71	38	156		
	481.3	A	3.55	8.19	35	154	91	102
		B	3.93	9.00	37	156		

	601.6	A	3.40	8.20	31	152	83	109
		B	3.67	8.47	41	154		
	752	A	4.16	7.17	34	145	72	116
		B	2.50	8.50	39	157		
	940	A	1.83	4.91	NP	NP		
		B	2.69	8.22	NP	NP		
	1175	A	2.33	7.75	37	152	64	109
		B	2.56	9.96	32	152		
MMS	10	A	2.57	7.09	92	43	26	1578
		B	3.29	7.09	92	47		

Main mutation test - 3 hour treatment in the presence of S9 mix

Treatment (µg/mL)		**Rep.**	**Cell conc. (x 10^5/mL)**		**Viability plate count***	**Mutant plate count***	**Mean RTG (%)**	**Mean MF (x 10^{-6})**
			24 h	**48 h**	**Day 2**	**Day 2**		
Vehicle control		A	6.68	10.33	42	161	100	89
		B	5.90	11.18	37	162		
		C	6.53	10.43	48	164		
		D	5.79	11.37	44	163		
Test material	385	A	6.75	10.55	45	163	102	102
		B	6.13	11.03	42	155		
	481.3	A	5.77	10.58	NP	NP		
		B	4.92	11.37	NP	NP		
	601.6	A	6.14	9.05	36	156	99	88
		B	5.51	11.74	37	164		
	752	A	4.51	10.35	31	161	91	84
		B	6.16	9.63	36	159		
	940	A	5.44	11.49	35	163	91	94
		B	4.93	11.39	46	157		
	1175	A	3.99	12.60	39	164	69	83
		B	3.38	10.45	35	160		
BaP	1	A	4.90	12.27	47	41	77	897
		B	4.49	12.16	53	44		

Main mutation test - 24 hour treatment in the absence of S9 mix

Treatment (µg/mL)		**Rep.**	**Cell conc. (x $10^{5/}$mL)**			**Viability plate count***	**Mutant plate count***	**Mean RTG (%)**	**Mean MF (x 10^{-6})**
			0 h	**24 h**	**48 h**	**Day 2**	**Day 2**		
Vehicle control		A	7.37	4.56	12.44	41	156	100	99
		B	7.32	4.30	13.04	45	163		
		C	7.54	5.18	11.04	47	161		
		D	7.50	4.71	12.72	47	162		
Test material	385	A	7.09	4.69	13.56	NP	NP		
		B	8.05	4.16	13.13	NP	NP		
	481.3	A	7.29	4.58	13.13	40	158	124	92
		B	7.14	6.51	11.81	41	163		
	601.6	A	7.18	5.22	12.64	50	160	114	100
		B	7.44	4.73	13.76	39	160		
	752	A	6.96	5.12	14.14	27	156	141	87
		B	7.26	5.01	13.99	43	163		
	940	A	7.54	5.10	13.48	41	166	147	78
		B	7.12	5.24	14.47	29	159		
	1175	A	6.86	5.00	13.58	38	161	118	99
		B	7.12	5.01	13.52	44	156		

MMS	5	A	5.17	3.27	12.54	113	45	16	2521
		B	4.17	4.55	10.94	124**	41		

*Number of non-colony bearing wells (out of a total number of 192 wells, with the exception of ** which had a total well number of 189)
NP – Culture not plated for mutant frequency
MMS – Methyl methanesulphonate
BaP – Benzo[a]pyrene
RTF Relative Total Growth
MF – Mutant Frequency

III. CONCLUSIONS

It was concluded that RH-141455 did not demonstrate mutagenic potential in this *in vitro* cell mutation assay, under the experimental conditions described.

RMS: The study is acceptable. RH-141455 did not demonstrate mutagenic potential in this in vitro cell mutation assay, under the experimental conditions described.

Submitted for the purpose of renewal.

Reference: CA 5.8.1/09, Sokolowski, A. (2013)

Title:

Report number: Harlan study number 1549300

Guidelines: OECD 471

GLP: Yes

Executive Summary:

RH-150721 was evaluated for its mutagenic potential by a reverse mutation test with five strains of Salmonella typhimurium (TA1535, TA1537, TA98, TA100, and TA102). The test was conducted according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) in the presence and absence of metabolic activation (rat liver S9 mix).

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations:

Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II without S9 mix: 6.5; 12.5; 25; 50; 100; 200; 400; and 800 µg/plate
Experiment II with S9 mix: 3; 10; 33; 100; 333; 750; 1500; and 3000 µg/plate

The test item precipitated in the overlay agar in the test tubes from 1000 to 5000 µg/plate with and without S9 mix in experiment I, and at 3000 µg/plate with S9 mix in experiment II. Precipitation of the test item in the overlay agar on the incubated agar plates was observed at 5000 µg/plate in experiment I. No precipitation (visible to the unaided eye) was observed in experiment II. The undissolved particles had no influence on the data recording. Reduced background growth was observed in both experiments in all strains used. Toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), were observed in all strains used.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with RH-150721 at any dose level, neither in the presence nor in the absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

RH-150721 is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test material: RH-150721

Description: Not stated

Lot/Batch: Lot No.: F1132-136

Purity: 98.8%.

CAS#: Not available

Stability: Expiry date: 24 April 2015

2. Control materials:

Negative: Solvent, DMSO

Positive:

Non-activation (-S9): Sodium azide – TA1535, TA100 (10 µg/plate)

4-nitro-o-phenylene-diamine, 4-NOPD – TA1537, TA98 (10 µg/plate in strain TA98, 50 µg/plate in strain TA1537)

methyl methane sulfonate, MMS – TA102 (2 µg/plate)

Activation (+S9): 2-aminoanthracene – TA1535, TA1537, TA98, TA100, TA102 (2.5 µg/plate, 10.0 µg/plate for TA 102)

3. Activation: Phenobarbital/β-naphthoflavone induced rat liver S9 were used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the Harlan CCR SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

An appropriate quantity of S9 supernatant is thawed and mixed with S9 cofactor solution, to result in a final concentration of approx. 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the concentrations below in the S9 mix in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. During the experiment, the S9 mix is stored in an ice bath.

S9 Mix composition:

Component	Parts per ml
$MgCl_2$	8 mM
KCl	33 mM
Glucose-6-phosphate	5 mM
NADP	4 mM

4. Test organism:	*Salmonella typhimurium* (TA1535, TA1537, TA98, TA100, TA102). Regular checking of the properties of the Salmonella typhimurium strains regarding the membrane permeability, ampicillin resistance, UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates is performed in Harlan CCR.
5. Test concentrations:	
(a) Pre-Experiment/ Experiment I:	With and without S9 mix: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
(b) Experiment II:	without S9 mix: 6.5; 12.5; 25; 50; 100; 200; 400; and 800 µg/plate
	with S9 mix: 3; 10; 33; 100; 333; 750; 1500; and 3000 µg/plate

B. TEST PERFORMANCE

1. Test procedure

For each strain and dose level, including the controls, three plates were used.
The following materials were mixed in a test tube and poured onto the selective agar plates:

Experiment I (Plate Incorporation)
100 µL Test solution at each dose level (solvent or reference mutagen solution (positive control)),
500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
100 µL Bacteria suspension,
2000 µL Overlay agar

Experiment II (Pre-Incubation)
In the pre-incubation assay 100 µL test solution (solvent or reference mutagen solution (positive control)), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates. After solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark.

Colonies were counted using the Petri Viewer Mk2 (Perceptive Instruments Ltd, Suffolk, UK) with the software program Ames Study Manager (v.1.21). The counter was connected to a PC with printer to print out the individual values and mean values of the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates. Due to precipitation and reduced background growth, the colonies were partly counted manually.

2. Statistics

According to OECD 471, a statistical analysis of the data is not mandatory.

3. Evaluation criteria

The assay is considered acceptable if it meets the following criteria:
- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of our historical data
- the positive control substances should produce a significant increase in mutant colony frequencies
- a minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA98, TA100, and TA102) or three times (strains TA1535 and TA1537) the colony count of the corresponding solvent control is observed.
A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.
An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.
A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Not determined.

B. CYTOTOXICITY (RANGE-FINDING ASSAY)

The test item precipitated in the overlay agar in the test tubes from 1000 to 5000 µg/plate with and without S9 mix in experiment I, and at 3000 µg/plate with S9 mix in experiment II. Precipitation of the test item in the overlay agar on the incubated agar plates was observed at 5000 µg/plate in experiment I. No precipitation (visible to the unaided eye) was observed in experiment II. The undissolved particles had no influence on the data recording.

C. MUTATION ASSAY

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with RH-150721 at any concentration level, neither in the presence nor in the absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

6.8.1-6: Results – Experiment I (also the pre-experiment for toxicity)

WITHOUT S9											Positive controls			
Strain	Controls		RH-150721 concentration / plate								NaN_3	4-NOPD	4-NOPD	MMS
	Solvent Control	Untreated	3 µg	10 µg	33 µg	100 µg	333 µg	1000 µg	2500 µg	5000 µg	10 µg	10 µg	50 µg	2.0 µg
TA1535	17 ± 4	12 ± 2	16 ± 4	17 ± 4	16 ± 3	18 ± 4	9 ± 3 M R	2 ± 1 M R	0 ± 0 M R	0 ± 0 P M R	2343 ± 147			
TA1537	10 ± 4	14 ± 4	12 ± 3	12 ± 4	13 ± 3	9 ± 1	2 ± 1	1 ± 1	0 ± 0 M R	0 ± 0 P M R			75 ± 9	
TA98	28 ± 7	29 ± 2	30 ± 8	22 ± 2	28 ± 5	25 ± 3	21 ± 6 R	2 ± 1 M R	0 ± 0 M R	0 ± 0 P M R		296 ± 35		
TA100	98 ± 1	106 ± 10	90 ± 18	104 ±17	95 ± 12	100 ±19	23 ± 6 M R	0 ± 0 M R	0 ± 0 M R	0 ± 0 P M R				
TA102	370 ± 27	345 ± 16	355 ± 7	369 ± 26	384 ± 13	406 ± 18	39 ± 7 M R	0 ± 0 M R	0 ± 0 M R	0 ± 0 P M R				4503 ± 748

WITH S9											Positive controls	
Strain	Controls		RH-150721 concentration / plate								2-AA	2-AA
	Solvent Control	Untreated	3 µg	10 µg	33 µg	100 µg	333 µg	1000 µg	2500 µg	5000 µg		
TA1535	21 ± 4	16 ± 3	20 ± 5	16 ± 2	18 ± 1	13 ± 1	16 ± 2	17 ± 6	11 ± 3 M R	0 ± 0 P M R	475 ± 56	
TA1537	22 ± 6	20 ± 3	21 ± 7	19 ± 3	21 ± 1	19 ± 5	20 ± 5	17 ± 5	6 ± 3 M R	0 ± 0 P M R	446 ± 4	
TA98	26 ± 2	39 ± 4	24 ± 8	30 ± 5	30 ± 3	30 ± 1	38 ± 5	42 ± 3	33 ± 2 M R	0 ± 0 P M R	2844 ± 285	
TA100	146 ± 21	144 ± 1	138 ± 8	129 ± 6	144 ± 0	145 ± 12	149 ± 6	52 ± 7 M R	30 ± 5 M R	0 ± 0 P M R	3696 ± 426	
TA102	452 ± 27	483 ± 1	479 ± 38	492 ± 16	545 ± 48	543 ± 78	464 ± 49	542 ± 50	75 ± 8 M R	0 ± 0 P M R		2688 ± 42

NaN_3: Sodium Azide, 2-AA: 2-aminoanthracene, 4-NOPD: 4-nitro-o-phenylene-diamine, MMS: methyl methane sulfonate, P:precipitate, M: manual count R:reduced background growth

6.8.1-7: Results – Experiment II

WITHOUT S9											Positive controls			
Strain	Controls		RH-150721 concentration / plate								NaN_3	4-NOPD	4-NOPD	MMS
	Solvent Control	Untreated	6.5 µg	12.5 µg	25 µg	50 µg	100 µg	200 µg	400 µg	800 µg	10 µg	10 µg	50 µg	3.0 µg
TA1535	15 ± 1	9 ± 2	11 ± 4	16 ± 2	16 ± 3	19 ± 4	21 ± 5	15 ± 1 M R	18 ± 10 M R	1 ± 2 M R	2448 ± 212			
TA1537	10 ± 3	10 ± 2	12 ± 3	14 ± 2	14 ± 2	13 ± 3	13 ± 1	8 ± 4 M R	9 ± 5 M R	0 ± 0 M R			61 ± 9	
TA98	22 ± 12	28 ± 5	28 ± 4	20 ± 5	21 ± 0	28 ± 8	23 ± 5	25 ± 3 M R	24 ± 3 M R	0 ± 0 M R		266 ± 18		
TA100	90 ± 13	131 ± 8	100 ± 13	93 ± 14	89 ± 7	113 ± 21	97 ± 14	56 ± 7 M R	0 ± 0 M R	0 ± 0 M R	1657 ± 151			
TA102	**359** ± 16	**393** ± 22	453 ± 75	379 ± 16	387 ± 11	408 ± 29	470 ± 32	471 ± 48 M	196 ± 12 R	114 ± 15 M R				4302 ± 436

WITH S9											Positive controls	
Strain	Controls		RH-150721 concentration / plate								2-AA	2-AA
	Solvent Control	Untreated	3 µg	10 µg	33 µg	100 µg	333 µg	750 µg	1500 µg	3000 µg	2.5 µg	10.0 µg
TA1535	12 ± 7	11 ± 3	9 ± 3	8 ± 1	11 ± 5	11 ± 6	10 ± 5	9 ± 1	10 ± 3	23 ± 8 M R	397 ± 31	
TA1537	10 ± 6	20 ± 5	19 ± 3	18 ± 3	11 ± 2	17 ± 5	18 ± 4	14 ± 4	14 ± 5	16 ± 7 M R	304 ± 64	
TA98	34 ± 4	46 ± 5	36 ± 13	44 ± 16	30 ± 6	35 ± 5	43 ± 7	45 ± 9	30 ± 3	3 ± 3 M R	1312 ± 61	
TA100	123 ± 17	130 ± 18	119 ± 5	103 ± 9	91 ± 9	97 ± 11	160 ± 23	82 ± 17	28 ± 8	0 ± 0 M R	2743 ± 153	
TA102	350 ± 9	383 ± 28	414 ± 55	347 ± 10	360 ± 3	359 ± 37	495 ± 44	553 ± 69	478 ± 43	248 ± 34 M R		3667 ± 80

2-AA: 2-aminoanthracene, P:precipitate, M: manual count, R reduced background growth

III. Conclusions

RH150721 was not mutagenic under the conditions of this bacterial reverse mutation assay.

RMS: The study is acceptable. RH150721 was not mutagenic under the conditions of this bacterial reverse mutation assay.

Submitted for the purpose of renewal.

Reference:	CA 5.8.1/10, Thandi, A. (2013)
Title:	DEREK evaluation of the toxicities of zoxamide and metabolite RH-150,721
Report number:	LSR Associates Ltd study number FRK0046
Guidelines:	QSAR: DEREK NEXUS VERSION 2.0.2 (Lhasa Ltd, Leeds, UK)
GLP:	No

A comparative structural activity-relationship analysis was carried out for RH-150,721 and Zoximide using the proprietary software DEREK NEXUS VERSION 2.0.2 (Lhasa Ltd, Leeds, UK). No new toxicological hazards were identified for RH-150,721 over those identified zoxamide. Those that were highlighted as being common to both RH-150,721 and zoxamide ("***116 Polyhalogenated aromatic****: Carcinogenicity in mammal is PLAUSIBLE (Extrapolation from alpha-2mu-Globulin nephropathy); and* ***264 Polyhalogenated benzene****: alpha-2-mu-Globulin nephropathy in mammal is EQUIVOCAL*"), did not manifest for zoxamide during *in vivo* testing. In conclusion, a structural activity relationship analysis did not highlight any new areas of toxicological concern for RH-150,721 over those identified for zoxamide.

RMS: Concern was raised regarding the possible presence of metabolite RH-150-721 in high amounts in vine. RH-150-721 has not been isolated in the rat or in the comparative in- vitro metabolism study with human, dog, rat and mouse hepatocytes.

According to the applicant in wine, parent zoxamide is generally not present (<0.01 mg/kg), however the metabolite/degradate RH-150721 is found at significant levels (>10% TRR in wine in the radiolabelled vinification study, and up to 0.49 mg/kg in processing studies). RH-150721 is produced by hydrolysis, which is a major route of metabolism in mammals. RH-150721 is believed to be an intermediate in mammalian metabolism, although it has not been isolated in the rat or in the comparative in- vitro metabolism study with human, dog, rat and mouse hepatocytes. RH-150721 has been shown to be non -mutagenic in the AMES test and comparative structural - activity relationship analysis using DEREK did not highlight any new areas of concern for RH-150721 over those identified for parent zoxamide.
On the basis that RH-150721 is likely to be an intermediate in the metabolism of zoxamide in mammals, and that the toxicity data and DEREK analysis indicates that RH-150721 is no more toxic than parent zoxamide, RH-150721is considered not to be of toxicological concern. It is therefore proposed that the residue definition for wine remains as parent zoxamide only for both risk assessment a nd monitoring.
RMS opinion: Although it's stated that this is an intermediate metabolite, in the result it could not be concluded whether this metabolite is less toxic than parent zoxamide. It can be concluded that metabolite

RH-150721 is not mutagenic in AMES test. For further surveyed for toxicological hazard DEREK NEXUS software analysis was submitted where no new areas of toxicological concern were found. RMS is on the opinion that because of high amount of metabolite RH-150721 in wine, genotoxic and carcinogenic potential could not be excluded. RMS believes that DEREK analysis is reliable in the results regarding mutagenicity rather than for genotoxicity or carcinogenicity. **Overall conclusion is that it could not be concluded whether metabolite RH-150721 is less toxic than parent zoxamide**

B.6.8.2. Supplementary studies on the active substance

Reference:	CA 5.4.2/02 (5.8.2.2/01), Young, D.H. (1998)
Title:	Mechanism of action of the oomycete fungicides RH-54032 and RH-117,281 on *Phytophthora capsici*, tobacco, mouse lymphoma cells and isolated bovine tubulin
Report number:	No: 98R-1098, ER Ref No: 23.5
Guidelines:	Not applicable. Mechanistic study
GLP:	Not applicable. Mechanistic study

In a series of experiments, the antitubulin benzamide (ATB) compounds RH-117,281 and RH-54032 inhibited nuclear division in the Oomycete fungus *Phytophthora capsici* by disruption of cellular microtubules as the result of a highly specific covalent binding to the ß-subunit of tubulin. In plants, RH-54032 reportedly caused morphological effects of leaf cupping and club-shaped roots which are considered typical of antimicrotubule agents. More detailed studies in tobacco suspension-cultured cells demonstrated covalent binding of RH-54032 to ß-subunit of tubulin, and an accumulation of cells in arrested metaphase. Mouse lymphoma cells were shown to be less sensitive than P. capsici and tobacco to RH-117,281 and RH-54032, however an accumulation of metaphase cells and covalent binding to ß - tubulin was also demonstrated. *In vitro* microtubule assembly assays demonstrated an inhibition of assembly by RH-117,281 and RH-54032, which was noted to be unusual in requiring a prolonged incubation with tubulin. RH-117,281 was comparable in potency to carbendazim in inhibiting microtubule assembly and the growth of mouse lymphoma cells, and was considerably less active than colchicine and vinblastine. Consistent with whole cell labelling experiments, the binding of radiolabeled RH-54032 to isolated bovine tubulin was shown to involve the ß-subunit. Since binding of radiolabeled RH-54032 to isolated tubulin was strongly inhibited by colchicine, podophyllotoxin and nocodazole, but not by vinblastine, it was considered likely that the ATBs bind at or near the colchicine binding site of tubulin.

RMS: The study is acceptable.

B.6.8.3. Studies on endocrine disruption

The fungicidal mode of action does not directly act on an endocrine system or receptor. It acts by inhibiting nuclear division, binding specifically to the β subunit of tubulin, causing disruption of cellular microtubules.

The existing studies have been evaluated with respect to evidence for effects on endocrine systems. There were no effects e.g. effects on endocrine organs weights or pathology, reproduction, time to reach developmental miles stones, oestrus cycling, or gamete production or function that would indicate zoxamide possessed any endocrine activity. Zoxamide is not an Endocrine Disruptor.

B.6.9 Medical data and information

B. 6.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

No adverse events have been associated with exposure of manufacturing plant personnel or workers exposure to zoxamide.

B.6.9.2 Data collected on humans

There are no reported incidents or case studies of accidental or deliberate exposure to zoxamide.

B.6.9.3 Direct observations

There are no reported incidents or case studies of accidental or deliberate exposure to zoxamide.

B.6.9.4 Epidemiological studies

There are no epidemiological studies associating zoxamide exposure with adverse outcomes on the population.

B.6.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

No clinical signs or symptoms of poisonings have been observed in any of the acute exposure studies of zoxamide, and no clinical signs or symptoms of poisoning are anticipated to result from accidental human exposure.

B.6.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

Inhalation: Move subject to fresh air. In case of unconsciousness place patient stably in side position for transportation.

Eye Contact: Flush eyes with a large amount of water for at least 15 minutes. Consult a physician if irritation persists.

Skin Contact: Remove contaminated clothing. Wash affected skin areas thoroughly with soap and water. Consult a physician if irritation persists. Wash contaminated clothing thoroughly before reuse. Do not take clothing home to be laundered.

Ingestion: If swallowed, give 2 glasses of water to drink. Never give anything by mouth to an unconscious person. Consult a physician.

Note to Physician: If swallowed, careful evacuation of the stomach is advisable.

Medical Treatment

No therapeutic treatment is expected to be required for exposures to zoxamide.

B.6.9.7 Expected effects of poisoning

No effects are anticipated

LITERATURE REVIEW

A literature review was carried out by the applicant for zoxamide and its metabolites according to regulation (EC) no 1107/2009.

Report summarises the search for published information on zoxamide, its metabolites and the plant protection product 'Zoxium 240 SC'. The search strategy was based on a single-concept search (in both STN and Dialog databases).

The selection process resulted in only one category of publication:

- Publications that do not meet the relevance criteria.

The reliability assessment for relevant studies was carried out according to Klimisch et al. (1997)

The following search terms were identified and used as a basis for the literature search:

Table 1 Overview and justification of search terms used

Description/justification of search terms	**Search terms**	
Search period	**1 January 2004 to 31 january 2014**	
ISO common names of active substance	Zoxamide	
Company developmental name / code:	RH-117,281, RH-7281	
IUPAC names:	(RS)-3,5-dichloro-N-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-p-toluamide	
Chemical names (CA):	3,5-dichloro-N-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-4-methylbenzamide	
CAS numbers:	156052-68-5	
Primary metabolites of concern, plus synonyms:	RH-141,452	3,5-dichloro-4-hydroxymethylbenzoic acid (CAS 89894-53-1)
	RH-141,455	3,5-dichloro-1,4-benzenedicarboxylic acid
	RH-150,721	(3-amino-3-methyl-2-oxo)pentyl-(3,5-dichloro-4-methyl)benzoate
	RH-24549	3,5-dichloro-4-methylbenzoic acid

		(CAS 39652-34-1)
	RH-139432	3,5-dichloro-4-methylbenzamide
	RH-127450	3,5-dichloro-4-methyl-N-(3-methyl-2-oxopentan-3-yl)benzamide
	RH-163353	3,5-dichloro-4-methyl-N-(1-carboxy-3-methyl-2-oxopentan-3-yl)benzamide
Plant protection product	Zoxium 240 SC	

Articles of potential relevance to the regulatory data package for the active substance were investigated in further detail by examining the abstract and/or the full article text. The reliability of articles considered to meet the criteria for relevance was assessed using the approach described in Klimisch *et al*., (1997).

Only **one** study was considered as being potentially relevant and reliable for toxicology.

No publications were found that showed new/unknown effects or information potentially contradictory to the regulatory data package for the active substance, relevant metabolites and/or plant protection products with respect to human health, animal health and/or the environment, which could impact the regulatory endpoints or the risk assessment parameters. Therefore, it was unnecessary to assess the reliability of any of the publications reviewed.

This review of the published literature for zoxamide and its metabolites did not reveal any studies considered to significantly affect the regulatory assessment of human health, animal health or the environment.

RMS: The literature review is adequate and considered in accordance with EFSA guidance on Submission of Scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) N°1107/2009 (EFSA Journal 2011; 9(2):2092).
For zoxamide and its metabolites no information was identified which was considered to have an impact on an EU-agreed endpoint, or would require to adapt any of the risk assessments in the zoxamide supplementary (renewal) dossier.

B.6.10 References relied on

Data point	**Annex point (Old)**	**Author(s)**	**Year**	**Title, Source (where different from company), Company, Report No, GLP or GEP status (where relevant), Published or not**	**Verteb rate study Y/N**	**Data Protecti on Claimed Y/N**	**Justificat ion if data protectio n claimed**	**Owner**
CA, 5.1.1/0 1	IIA, 5.1/01	[redacted]	1998a	14C-RH-117,281: Pharmacokinetic and metabolism study in rats. [redacted] Report No: 94R-235 ER Ref No: 24.1 US Ref No: 94R-235, GLP, Unpublished	Y	N	NA	Gowan
CA, 5.1.1/0 2 (5.4.2/0 3)	IIA, 5.1/02 (5.4.2/03)	[redacted]	1998b	Distribution of 14C-RH-117,281 to the bone marrow of mice. [redacted] Report No: 97R-173 ER Ref No: 24.2 US Ref No: 97R-173, GLP, Unpublished	Y	N	NA	Gowan
CA, 5.1.1/0 3 Add. study	IIA, 5.1/03 Add. study	Reibach P.H., Detweiler B.S.	2001	Identification of RH-139432 from zoxamide (RH-117281) Rat Pharmacokinetic Study Samples Rohm and Haas Co. Report No: 34-00-105 ER Ref No: 45.3, GLP, Unpublished	N	N	NA	Gowan
CA, 5.2.1/0 1	IIA, 5.2.1/01	[redacted]	1996a	RH-117,281 Technical: acute oral toxicity study in male and female rats. [redacted] Report No: 95R-268 ER Ref No: 1.3, US Ref No: 95R-268 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.2.1/0 2	IIA, 5.2.1/02	[redacted]	1998a	RH-117,281 Technical: Acute oral toxicity study in male and female mice. [redacted] Report No. 98R-165 ER Ref No: 24.3, US Ref No: 98R-165 GLP, Unpublished	Y	N	NA	Gowan

Data point	Annex point (Old)	Author(s)	Year	Title, Source (where different from company), Company, Report No, GLP or GEP status (where relevant), Published or not	Vertebrate study Y/N	Data Protection Claimed Y/N	Justification if data protection claimed	Owner
CA, 5.2.2/01	IIA, 5.2.2/01	[redacted]	1996b	RH-117,281 Technical: acute dermal toxicity study in male and female rats. [redacted] Report No: 95R-269 ER Ref No: 1.4, US Ref No: 95R-269 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.2.3/01	IIA, 5.2.3/01	[redacted]	1996	RH-117,281 Technical: acute inhalation toxicity study in rats. [redacted] Report No: 95R-266 ER Ref No: 2.2, US Ref No: 95R-266 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.2.4/01	IIA, 5.2.4/01	[redacted]	1996c	RH-117,281 Technical: skin irritation study in rabbits. [redacted] Report No: 95R-270 ER Ref No: 1.5, US Ref No: 95R-270 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.2.5/01	IIA, 5.2.5/01	[redacted]	1996d	RH-117,281 Technical: eye irritation study in rabbits. [redacted] Report No: 95R-271 ER Ref No: 1.6 US Ref No: 95R-271 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.2.6/01	IIA, 5.2.6/01	[redacted]	1997	Dermal sensitization study of RH-117,281 Technical in guinea pigs - maximization test. [redacted] Project No. 6228-112 [redacted] Report No: 95RC-170 ER Ref No: 4.2 US Ref No: 95RC-170 GLP, Unpublished	Y	N	NA	Gowan

Data point	**Annex point (Old)**	**Author(s)**	**Year**	**Title, Source (where different from company), Company, Report No, GLP or GEP status (where relevant), Published or not**	**Vertebrate study Y/N**	**Data Protection Claimed Y/N**	**Justification if data protection claimed**	**Owner**
CA, 5.2.6/02	IIA, 5.2.6/02	[redacted]	1998a	RH-117,281 Technical: Delayed contact hypersensitivity study in guinea pigs. [redacted] Report No: 97R-074 ER Ref No: 23.2 US Ref No: 97R-074 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.2.6/03	IIA, 5.2.6/03	[redacted]	1998b	RH-117,281 Technical: Delayed contact hypersensitivity (dilution) study in guinea pigs. [redacted] Report No: 98R-154 ER Ref No: 24.4 US Ref No: 98R-154 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.3.1/01	IIA, 5.3.1/01	[redacted]	1996	RH-117,281 Technical: four-week range-finding toxicity study in dogs. [redacted] Report No: 94R-234 ER Ref No: 2.3 US Ref No: 94R-234 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.3.2/01	IIA, 5.3.2/01	[redacted]	1996	RH-117,281: three-month dietary toxicity study in mice. [redacted] Report No: 94R-075 ER Ref No:5.3 US Ref No: 94R-075 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.3.2/02 (5.8.2.1/02)	IIA, 5.3.2/02 (5.8.2.1/02)	[redacted]	1996a	RH-117,281: three-month dietary toxicity/neurotoxicity study in rats. [redacted] Report No: 94R-233 ER Ref No: 3.1 US Ref No: 94R-233 GLP, Unpublished	Y	N	NA	Gowan

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CA, 5.3.2/03	IIA, 5.3.2/03	[redacted]	1997	RH-117,281 Technical: three-month dietary toxicity study in dogs. [redacted] Report No: 96R-030 ER Ref No: 9.1 US Ref No: 96R-030 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.3.2/04	IIA, 5.3.2/04	[redacted]	1998c	RH-117,281 Technical: one-year chronic dietary toxicity study in dogs. [redacted] Report No: 95R-277 ER Ref No: 25.1 US Ref No: 95R-277 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.3.3/01	IIA, 5.3.3/01	[redacted]	1998d	RH-117,281 Technical: twenty-eight day dermal toxicity study in rats. [redacted] Report No: 97R-075 ER Ref No: 23.3 US Ref No: 97R-075 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.4.1/01	IIA, 5.4.1/01	Sames, J.S., Ciaccio, P.C.	1996a	RH-117,281 Technical: Salmonella Typhimurium gene mutation assay (Ames test). Rohm and Haas Co. Report No: 95R-262 ER Ref No: 2.7 US Ref No: 95R-262 GLP, Unpublished	N	N	NA	Gowan
CA, 5.4.1/02	IIA, 5.4.1/02	Riley, S.	1998	RH-117,281: Test for chemical induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells. Covance Laboratories, UK Project No. 616/20-D5140 ER Ref No: 23.6 US Ref No: 96RC-125 GLP, Unpublished	N	N	NA	Gowan

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CA, 5.4.1/03	IIA, 5.4.1/03	Pant, K.	1994	RH-117,281: Test for chemical induction of gene mutation at the HGPT locus in cultured Chinese hamster ovary cells with and without metabolic activation. Sitek Study No: 0282-2510 ER Ref No: 23.4 US Ref No: 94RC-077 GLP, Unpublished	N	N	NA	Gowan
CA, 5.4.2/01	IIA, 5.4.2/01	Sames, J.S., Vandenberghe, Y.L.	1996b	RH-117,281 Technical: micronucleus assay in CD-1 mouse bone marrow cells. Rohm and Haas Co. Report No: 95R-264 ER Ref No: 1.9 US Ref No: 95R-264 GLP, Unpublished	N	N	NA	Gowan
CA, 5.4.2/02 (5.1/02)	IIA, 5.4.2/03 (5.1/02)	[redacted]	1998b	Distribution of 14C-RH-117,281 to the bone marrow of mice. [redacted] Report No: 97R-173 ER Ref No: 24.2 US Ref No: 97R-173 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.4.2/02 (5.8.2.2/01)	IIA, 5.4.2/02 (5.8.2.2/01)	Young, D.H.	1998	Mechanism of action of the oomycete fungicides RH-54032 and RH-117281 on *Phytophthora capsici*, tobacco, mouse lymphoma cells and isolated bovine tubulin. Rohm and Haas Co. Report No: 98R-1098 ER Ref No: 23.5 US Ref No: 98R-1098 GLP, Unpublished	N	N	NA	Gowan

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CA, 5.5/01	IIA, 5.5/01	[redacted]	1998a	RH-117,281 Technical: 24-month dietary chronic/oncogenicity study in rats. Covance project No. 417-505 [redacted] Report No: 94RC-236 ER Ref No: 21.1 US Ref No: 94RC-236 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.5/02	IIA, 5.5/03	[redacted]	1998c	RH-117,281 Technical: Eighteen-month dietary oncogenicity study in mice. [redacted] Report No: 96R-094 ER Ref No: 20.1 US Ref No: 96R-094 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.6.1/01	IIA, 5.6.1/01	[redacted]	1998	RH-117,281 Technical: Two-generation reproductive toxicity study in rats. [redacted] Report No: 95R-272 ER Ref No: 26.1 US Ref No: 95R-272 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.6.2/01	IIA, 5.6.2/01	[redacted]	1995b	RH-7281 Technical: oral (gavage) developmental toxicity study in rats. [redacted] Report No: 94R-079 ER Ref No: 6.1 US Ref No: 94R-079 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.6.2/02	IIA, 5.6.2/02	[redacted]	1997	RH-117,281 Technical: oral (gavage) developmental study in rabbits. [redacted] Report No: 95R-267 ER Ref No: 8.2 US Ref No: 95R-267 GLP, Unpublished	Y	N	NA	Gowan

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CA, 5.7.1/01	IIA, 5.8.2.1/01	[redacted]	1997	RH-117,281 Technical: acute oral (gavage) neurotoxicity study in rats. [redacted] Report No: 95R-182 ER Ref No: 10.1 US Ref No: 95R-182 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.7.1/02 (5.3.2/02)	IIA, 5.8.2.1/02 (5.3.2/02)	[redacted]	1996a	RH-117,281: three-month dietary toxicity/neurotoxicity study in rats. [redacted] Report No: 94R-233 ER Ref No: 3.1 US Ref No: 94R-233 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.7.1/03	IIA, 5.8.2.1/03	[redacted]	1995	Carbaryl and DDT: Neurotoxicity Evaluation of Positive Control Substances in Rats. [redacted] Report No: 94R-224 ER Ref No: 27.5 US Ref No: 94R-224 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.7.1/04	IIA, 5.8.2.1/04	[redacted]	1995a	d-Amphetamine and Chlorpomazine: Motor Activity Assessment of Positive Control Substances in Rats. [redacted] Report No: 94R-225 ER Ref No: 27.6 US Ref No: 94R-225 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.7.1/04	IIA, 5.8.2.1/05	[redacted]	1995	Triethyltin and Acrylamide: Neurotoxicity Evaluation of Positive Control Substances in Rats. [redacted] Report No: 94R-211 ER Ref No: 28.1 US Ref No: 94R-211 GLP, Unpublished	Y	N	NA	Gowan

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CA, 5.8.1/01	IIA, 5.8.1.1/01	[redacted]	1998a	14C-RH-141,452: Rat metabolism study, Tier I testing [redacted] Report No. RPT00410 ER Ref No: 27.1 US Ref No: 97RC-154 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.8.1/02	IIA, 5.8.1.1/02	[redacted]	1998d	RH-141,452: Acute oral toxicity study in male and female mice. [redacted] Report No: 98R-049 ER Ref No: 25.2 US Ref No: 98R-049 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.8.1/03	IIA, 5.8.1.1/03	Sames, J.L., Ciaccio, P.J.	1998a	RH-141,452: *Salmonella typhimurium* gene mutation assay (Ames test). Rohm and Haas Co. Report No: 98R-050 ER Ref No: 25.3 US Ref No: 98R-050 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.8.1/04	IIA, 5.8.1.2/01	[redacted]	1998b	14C-RH-141,455: Rat metabolism study, Tier I testing [redacted] Report No. RPT00411 ER Ref No: 27.2 US Ref No: 98RC-017 GLP, Unpublished	Y	N	NA	Gowan
CA, 5.8.1/05	IIA, 5.8.1.2/02	[redacted]	1998b	RH-141,455: Acute oral toxicity study in male and female mice. [redacted] Report No: 98R-047 ER Ref No: 27.3 US Ref No: 98R-047 GLP, Unpublished	Y	N	NA	Gowan

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CA, 5.8.1/06	IIA, 5.8.1.2/03	Sames, J.L., Ciaccio, P.J.	1998b	RH-141,455: Salmonella typhimurium gene mutation assay (Ames test). Rohm and Haas Co. Report No: 98R-048 ER Ref No: 27.4 US Ref No: 98R-048 GLP, Unpublished	N	N	NA	Gowan

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KCA, 5.1.1/04	Scullion, P.	2013	A study to compare the metabolite profile of 14C-zoxamide in cryopressed human, dog, rat and mouse hepatocytes. CXR Biosciences James Lindsay Place, Dundee Technopole, Dubdee DD1 5JJ, UK Report Number: CXR1237 Not GLP, Not pubplished	N	Y	New data requirement.	Gowan	

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KCA, 5.1.1/05	Powrie, R.H.	2014	A study to investigate the presence of a specific metabolite of zoxamide, RH-150721, in Analytical Data From Previous Study CXR1237 CXR Biosciences James Lindsay Place, Dundee Technopole, Dubdee DD1 5JJ, UK Report Number: CXR1416 Not GLP, Not pubplished	N	Y	New data requirement.	Gowan	

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KCA, 5.2.7/01	Heppenheimer, A.	2014	Zoxamide: Cytotoxicity assay *in vitro* with Balb/c 3T3 cells: Natural red test at simultaneous irradiation with artificial sunlight. Harlan Cytotest Cell Research GmbH In den Leppsteinswiesen 19, 64380 Rossdorf, Germany GLP, Not Published	N	Y	New data requirement.	Gowan	

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KCA, 5.8.1/07	Woods, I	2014	RH-141455: *In vitro* mutation test using mouse lymphoma L5178Y Huntingdon Life Sciences Eye Research Centre, Suffolk, IP23 7PX, UK Study No. FRK0049 GLP, Not published	N	Y	To demonstrate that the metabolite is not of toxicological relevance.	Gowan	

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CA, 5.8.1/08	Woods, I	2014	RH-141455: *In Vitro* Micronucleus Test in Human Lymphocytes Huntingdon Life Sciences Eye Research Centre, Suffolk, IP23 7PX, UK Study No. FRK0050 GLP, Not published	N	Y	To demonstrate that the metabolite is not of toxicological relevance.	Gowan	

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KCA, 5.8.1/09	Sokolowski, A.	2013	RH-150,721: Salmonella typhimurium reverse mutation assay Harlan CCR, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany Report Number 1549300 GLP, Not Published	N	Y	To address potential toxicity of metabolite found in wine.	Gowan	

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KCA, 5.8.1/10	Thandi, K	2013	DEREK evaluation of the toxicities of zoxamide and metabolite RH-150,721. LSR Associates Ltd , Woolley Road, Alconbury, Cambridgeshire, PE28 4HS, UK Study No: FRK0046 Not GLP, Not Published	N	Y	To address potential toxicity of metabolite found in wine.	Gowan	