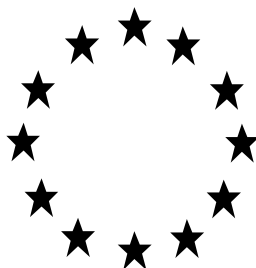


Draft Assessment Report



DIFENOCONAZOLE

Volume 3 **Annex B.6** **Toxicology and Metabolism**

Rapporteur Member State: Sweden

May 2006
Updated December 2006

Volume 1

Level 1: Statement of subject matter and purpose for which the monograph was prepared

Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

Appendix 3: List of endpoints

Level 3: Proposed decision with respect to the application for inclusion of the active substance in Annex I

Level 4: Further information to permit a decision to be made, or to support a review of the conditions and restrictions associated with the proposed inclusion in Annex 1

Volume 2

Annex A: List of the tests and studies submitted and of information available

Volume 3

Annex B: RMS summary, evaluation and assessment of the data and information

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Annex B.2: Phys/chem.

Annex B.3: Data application and further information.

Annex B.4: Proposal for classification and labelling

Annex B.5: Analytical method

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Appendix 1: Standard terms and abbreviations

Appendix 2: Specific terms and abbreviations

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Annex B.6: Toxicology and Metabolism

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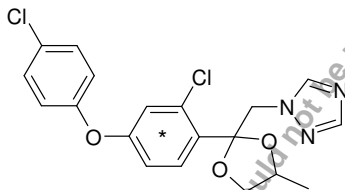
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B.6 Toxicology and metabolism

B.6.1 Absorption, distribution, excretion and metabolism (toxicokinetics) (Annex IIA 5.1)

B.6.1.1 Single dose (two dose levels) in rats (active substance)

Reference:	Esumi, Y (1992). Absorption, distribution and excretion of CGA 169374 in rats.
Guideline:	J-MAFF 59 NohSan No. 4200 (1985). (The test procedures followed the) OECD TG No. 417.
GLP:	No
Acceptability:	Yes
Test substance / purity:	¹⁴ C labelled Difenoconazole / Not specified. Specific radioactivity 1.14 MBq/mg (30.8 µCi/mg). Radiochemical purity > 97%.
	
	*) position of label
Species / Strain:	Rat / Sprague Dawley (SPF)
Doses / No. of animals:	0.5 and 300 mg/kg bw (corresponding radioactive doses approx. 15.4 and 30.8 µCi/kg bw) / <u>Experiment 1 (blood kinetics)</u> : 3 animals/sex/dose (p.o.), 1 male (i.v. 0.5 mg/kg bw), <u>Experiment 2 (bile excretion)</u> : 3 animals/sex/dose (p.o.), <u>Experiment 3 (entero-hepatic circulation)</u> : 3 males, <u>Experiment 4 (whole body autoradiography)</u> : 3 males, 0.5 mg/kg bw (p.o.), <u>Experiment 5 (tissue residues)</u> : 3 animals/sex/dose (p.o.)
Administration:	Orally, by gavage
Exposure time / Duration:	Single dose, 168 h observation period post dose
Expression of results:	Radioactivity concentrations expressed as equivalent of difenoconazole. AUC calculated by trapezoidal method. Detection limit of radioactivity was defined as two times the background value.

Materials and Methods:

Experiment 1 (blood kinetics): Blood samples (100 µl) were withdrawn for determination of radioactivity concentration at 0.5, 2, 4, 6, 8, 12, 24, 36, 48, 60, 72, 96, 120, 144 and 168 hr. Experiment 2 (bile excretion):

Bile ducts were cannulated for bile collection in anesthetized animals at 1, 2, 4, 6, 8, 24 and 48 h and urine were

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collected at 4, 8, 24 and 48 h and feces at 24 and 48 h after administration, for determination of radioactivity in samples. After the 48 h-samplings, the animals were sacrificed and the gastrointestinal content was collected for measurement of radioactivity. Finally, radioactivity in the carcass was determined. Experiment 3 (entero-hepatic circulation): Bile was collected from male rats (unspecified number) that had been gavaged with 0.5 mg ¹⁴C-phenyl difenoconazole/kg 24 hours before. The bile were injected into the duodenum of **3 other**, cannulated males at a dose level of 24.5 µg (difenoconazole equivalents)/0.76 µCi/8 ml bile/kg bw. Bile was then collected from the latter rats at 1, 2, 4, 6, 8, 24 and 48 h and urine at 4, 8, 24 and 48 h and feces at 24 and 48 h. After sampling, the rats were sacrificed and the gastro-intestinal content was collected and measured for radioactivity. Finally, radioactivity was measured in the carcasses. Experiment 4 (whole body autoradiography): Rats (1 per time point) were sacrificed at 2, 24 and 168 h post administration and the carcasses were cut in a cryomicrotome. The slices were applied on x-ray film for 35 days before developed for whole body autoradiograms. Experiment 5 (tissue residues): Rats were anesthetised and bled from the abdominal aorta at 2, 24 and 168 h (low-dose group) and at 4, 48 and 168 h (high-dose group) post administration. **More than 30 different tissues were sampled and weighed from each rat, including vital organs, fat, bone and reproductive organs.** The radioactivity concentration and the distribution ratio were determined.

Results:

Absorption at the 0.5 mg/kg-dose level

Experiment 1 (blood kinetics): Maximal concentration (T_{max}) and maximal blood concentration (C_{max}) were **2 h** and **0.327 ppm**, respectively, in the males. The area under curve (AUC) was **6.2** µg equivalent hr/ml up to 168 h after administration. In corresponding females, T_{max} and C_{max} were approximately **50%, respectively** of that of the **males**. The AUC was 2.8 µg equivalent hr/ml up to 168 h after administration (45% of the male value).

Absorption at the 300 mg/kg-dose level

Maximal concentration (T_{max}) and maximal blood concentration (C_{max}) were **4 h** and **47.89 ppm**, respectively in the males. The AUC was **2460** µg equivalent hr/ml up to 168 h after administration. In corresponding females, T_{max} and C_{max} were approximately **100 and 63%, respectively** of that of the **males**. The AUC was 1710 µg equivalent hr/ml up to 168 h after administration (70% of the male value).

The **disappearance of radioactivity in females** was slightly **faster** than that in males.

Table B.6.1.1-1: Key parameters of blood kinetics after single oral dose of difenoconazole in rat

	low dose (0.5 mg/kg bw)		high dose (300 mg/kg bw)	
	male	female	male	female
C_{max} (ppm)	0.327	0.169	47.89	30.02
T_{max} (hr)	2	0.5	4	4
$T_{1/2}$ 1st phase	6.2 hr	4.4 hr	22 hr	24 hr
$T_{1/2}$ 2nd phase	2.8 day	3.7 day	3.8 day	3.4 day
AUC (0-168 hr) (µg equiv.·hr/ml)	6.2	2.8	2460	1710

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Excretion

Experiment 2: The **major excretion route** was *via* the **bile**, which amounted to 40-80% of the dose by 48 h. There was no significant difference in the bile excretion between male and females in the low-dose group, whereas in the high-dose group, females had lower bile excretion and higher residual radioactivity in the gastrointestinal contents than males. The residual radioactivity in **carcass** at 48 h was **4 and 3%** of the dose for 0.5 and 300 mg/kg -dose **males**, respectively. The corresponding values for **females** were **3 and 2%**.

Table B.6.1.1-2: Cumulative excretion after single oral administration (0.5 mg/kg bw) of difenoconazole to bile duct cannulated rats

Time (hr)	Excreted radioactivity (% of administered dose)					
	Male			Female		
	Bile	Urine	Faeces	Bile	Urine	Faeces
48	73.3	13.9	3.9	76.4	8.9	1.8

Radioactivity in GI-tract contents at 48 hr: 1.9% (male) and 7.4% (female)

Table B.6.1.1-3: Cumulative excretion after single oral administration (300 mg/kg) of difenoconazole to bile duct cannulated rats

Time (hr)	Excreted radioactivity (% of administered dose)					
	Male			Female		
	Bile	Urine	Faeces	Bile	Urine	Faeces
48	55.6	1.0	17.1	38.6	1.2	22.0

Radioactivity in GI-tract contents at 48 hr: 20.0% (male) and 31.8% (female)

The excretion via bile was lower and the radioactivity in the gastrointestinal content was higher in the 300 mg/kg-dose group as compared with the 0.5 mg/kg-dose group.

Experiment 3 (entero-hepatic circulation): Following bile collection from male rats orally exposed to 0.5 mg/kg bw, and subsequent injection intraduodenally to other rats, 83.7% of the radioactivity injected was re-absorbed by 48 hr and 79.6% was re-excreted in the bile (4.1% in urine and 13.7% in feces). Thus, an **enterohepatic circulation is indicated**. No radioactivity was noted in gastrointestinal contents or carcass.

Distribution

Experiment 4 (whole body autoradiography): The highest levels of radioactivity were found in the **gastro-intestinal contents** and in **bile**, followed by the **liver and kidney** and the **adrenal gland** at 2 h. No radioactivity was observed in any tissues at 168 hr after administration.

Experiment 5 (tissue residues): In the **0.5 mg/kg-males**, radioactivity in tissues reached a maximum at 2 h and the highest radioactivity was found in the **liver, kidney** and **adrenal gland** followed by **plasma** and **fat**. The radioactivity concentration in any tissue was < 22% of the maximum at 168 hr after administration. In **corresponding females**, radioactivity concentrations were similar or higher than those in male rats at 2 hr and lower than those in male rats at 24 hr and thereafter. The disappearance of radioactivity after 24 hr in female rats was similar to that in male rats. The transfer of radioactivity into blood cells was <8% for both sexes.

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Table B.6.1.1-4: Tissue residues after single oral administration (0.5 mg/kg bw) of difenoconazole

Tissue	Radioactivity concentration (ppm equivalents of difenoconazole)					
	male			female		
	2 hr	24 hr	168 hr	2 hr	24 hr	168 hr
Plasma	0.414	0.098	0.029	0.344	0.036	0.012
Blood	0.246	0.060	0.019	0.201	0.021	0.007
Harderian gland	0.148	0.063	0.011	0.448	0.048	0.005
Liver	2.319	0.182	0.006	1.450	0.047	0.003
Kidney	0.832	0.133	0.006	0.657	0.041	0.003
Adrenal gland	0.550	0.018	< 0.011	1.160	0.011	< 0.010
Fat	0.113	0.021	0.025	0.389	0.012	0.011
Brown fat	0.114	0.032	0.016	0.297	0.023	0.009
Cerebrum	0.033	< 0.003	< 0.003	0.159	< 0.002	< 0.002
Cerebellum	0.050	< 0.003	< 0.003	0.162	< 0.003	< 0.003
Uterus	-	-	-	0.155	0.011	< 0.005
Ovary	-	-	-	0.216	0.010	< 0.005
Testis	0.074	0.014	0.004	-	-	-
Epididymis	0.116	0.021	0.009	-	-	-
Heart	0.107	0.011	0.003	0.167	0.006	< 0.004
Stomach	0.219	0.012	< 0.004	0.215	0.006	0.003

In the 300 mg/kg- males, radioactivity in tissues reached a maximum at 4h and in plasma and blood at 48 h.

The highest radioactivity was found in the **fat, liver, brown fat, Harderian gland, adrenal gland and stomach**. The radioactivity concentration in any tissue was less than 26% of the maximum at 168 hr after administration. **In corresponding females**, radioactivity concentrations were similar than those in male rats at 4 hr and lower (by about 50%) than those in male rats at 48 hr and thereafter. The transfer of radioactivity into blood cells at 4 h was 20 and 18% for males and females, respectively.

Table B.6.1.1-5: Tissue residues after single oral administration (300 mg/kg) of difenoconazole

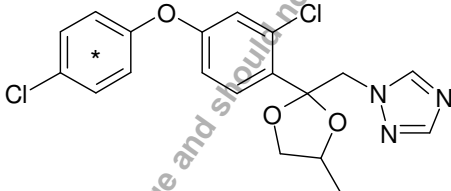
Tissue	Radioactivity concentration (ppm equivalents of difenoconazole)					
	male			female		
	4 hr	48 hr	168 hr	4 hr	48 hr	168 hr
Plasma	43.29	55.10	13.67	40.19	22.80	6.20
Blood	31.21	32.71	8.38	28.01	13.32	3.82
Harderian gland	169.61	110.48	5.75	188.61	61.91	3.18
Liver	194.79	47.87	2.51	214.53	20.01	1.50
Kidney	84.59	30.45	2.71	88.64	10.78	1.44
Adrenal gland	133.43	12.49	< 2.80	177.72	8.30	< 2.38
Fat	247.25	20.55	18.59	419.39	15.08	10.20
Brown fat	148.20	29.09	9.33	275.14	21.30	5.27
Cerebrum	69.45	1.40	< 0.76	78.03	1.15	< 0.78
Cerebellum	69.15	2.09	< 0.84	81.06	1.24	< 1.14
Uterus	-	-	-	34.94	6.81	1.87
Ovary	-	-	-	84.56	6.04	1.88
Testis	38.17	7.62	2.01	-	-	-
Epididymis	56.46	12.61	4.90	-	-	-
Heart	66.73	8.21	1.38	72.97	4.27	1.08
Stomach	157.62	2.59	1.00	143.34	3.17	< 1.08

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Conclusion:

Difenoconazole was almost **completely (80-90%)** absorbed after a single dose 0.5 mg/kg bw to rats. A dose of 300 mg/kg bw was absorbed by **40-60%**. Blood levels (AUC) were proportional to the dose. **Bile** was the **major elimination route**. There was evidence for **enterohepatic circulation**. **Tissue residues** were highest in liver, kidney, adrenals, fat, brown fat, Harderian gland and stomach. Maximum tissue residues were generally found at 2 or 4 hr after administration, with a rapid decline thereafter. Highest levels at 168 hr after administration were in fat and plasma.

B.6.1.2 Repeated dose (single dose level) in rats (active substance)

Reference:	Hassler S (2003a). Disposition of [4-chloro-phenoxy-U-¹⁴C] CGA 169374 in the rat after multiple oral administrations.
Guideline:	OECD TG 417
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	Radiolabelled Difenoconazole: [4-Chloro-phenoxy-U- ¹⁴ C] / 99.3%. Specific activity 2950 kBq/mg (80 µCi/mg). Radiochemical purity 98.5%
 <p style="text-align: right;">(* Position of [¹⁴C]-label)</p>	
Species / Strain:	Rat / HanBr: WIST (SPF) males
Doses / No. of animals:	0.5 mg/kg bw / 4 per subgroup (4 different sacrifice time points)
Administration:	Orally, by gavage
Exposure time / Duration:	Single daily dose for 1, 7 or 14 days / 20 days

Materials and Methods:

[4-chloro-phenoxy-U-¹⁴C] labelled difenoconazole was orally administered for up to 14 consecutive days. Four animals each were assigned to subgroups T1-T4 and sacrificed at different time points (day 1, 7, 14 and 20, respectively) after administration.

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Table B.6.1.2-1: Toxicokinetic studies in the rat - Dosing and sampling

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Dosing	X	X	X	X	X	X	X	X	X	X	X	X	X	X							
Sacrifice of Subgroup		T1						T2							T3						T4
Number of daily doses		1						7							14						14
Tissues		X						X							X						X
Serial Blood		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Excreta	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

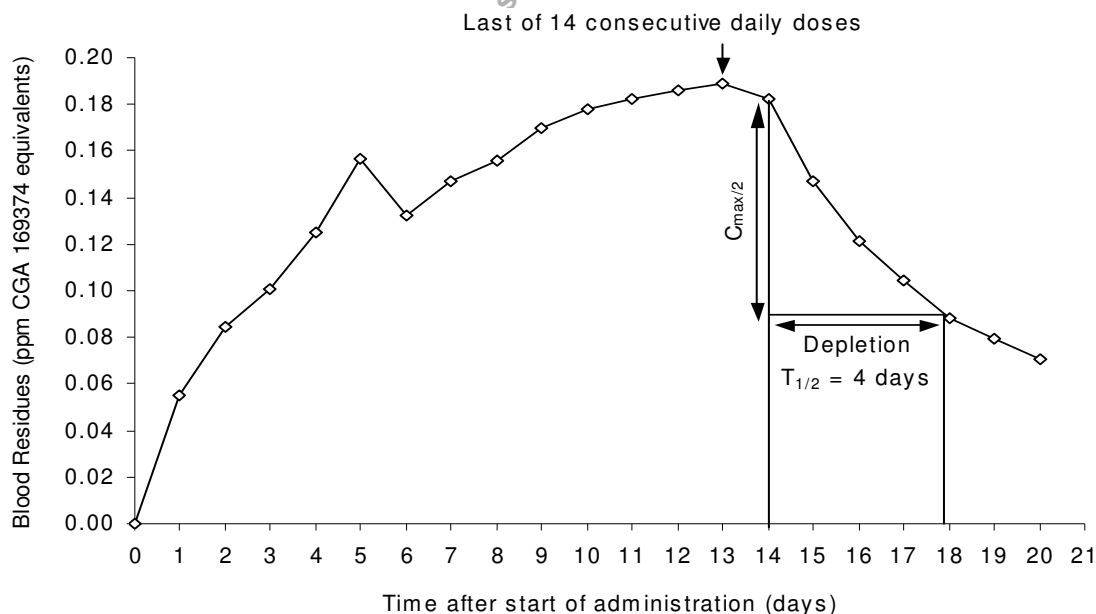
Excreted radioactivity was determined daily in urine and feces of subgroup T4 only. Male rats were chosen in this study because they had previously showed a slower depletion rate of blood residues and a higher AUC as compared with female rats.

Results:

Absorption, Excretion and Distribution

The **blood kinetics** showed increasing concentrations with ongoing administrations which appeared to have reached a **plateau after 11 days** of administration, at about 0.18 ppm difenoconazole equivalents. After cessation of dosing, the blood concentration was half the maximum concentration ($C_{\max/2}$) within 4 days – a moderately fast decline.

Figure B.6.1.2-1: Blood concentrations of radioactivity over a time course during and following 14 daily oral doses of 0.5 mg [^{14}C]-Difenoconazole/kg to male rats in subgroup T4



An **excretion steady state** was reached **3 days** after start of dosing. At steady state, **85%** of the daily dose was excreted *via faeces* and **12%** *via urine*. A previous study (Esumi, 1992) with bile cannulated rats showed a

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biliary excretion of 73% of given dose. Thus, **it is assumed that the majority of absorbed test substance re-enters the intestinal tract by biliary excretion and is finally excreted via feces.** More than 98% of the total administered radioactivity was excreted within 7 days after the last dose.

The **residues in most tissues** reached a **plateau 7 days after the start of dosing**. The **exceptions were liver, kidneys, fat and pancreas**, which did not reach a plateau during the dosing period. The **highest residue levels** were found in the **liver and kidneys** (0.8 ppm and 0.4 ppm difenoconazole equivalents, respectively). Less than 0.5% of the total administered dose remained in the tissues and carcass 7 days after the final dose. The **calculated half lives** ($t_{1/2}$) for the depletion of the residuals were, for most tissues and organs, **4-6 days**, assuming first order kinetics (absorption proportionate to concentration at site) and mono-phasic depletion kinetics for the depuration (cleansing xenobiotics from the body). Depletion was calculated to be more rapid in liver, kidneys and pancreas ($t_{1/2}$ **1-3 days**) and slower in fat ($t_{1/2}$ **9 days**).

Table B.6.1.2-2: Tissue concentrations of radioactivity during and following 14 daily oral doses of 0.5 mg [¹⁴C]-Difenoconazole/kg to male rats (Values are expressed as µg equivalents difenoconazole/g of tissue)

Subgroup	J1T1	J1T2	J1T3	J1T4	Half life (days)
Dose (mg/kg body weight)	0.56	0.52	0.49	0.48	
Days after start of dosing	1	7	14	20	
Days after last dose	1	1	1	7	
Adrenals	0.0170	0.0623	0.0588	0.0228	4
Blood	0.0414	0.2109	0.1834	0.0682	4
Bone	0.0133	0.0291	0.0235	0.0087	4
Brain	0.0017	0.0074	0.0072	0.0026	4
Fat	0.0077	0.0519	0.0671	0.0432	9
Heart	0.0132	0.0631	0.0526	0.0198	4
Kidneys	0.1574	0.3766	0.4030	0.0534	2
Liver	0.3309	0.6585	0.8148	0.0420	1
Lungs	0.0335	0.1778	0.1158	0.0501	5
Muscle	0.0055	0.0224	0.0189	0.0067	4
Pancreas	0.0229	0.0813	0.0932	0.0181	3
Plasma	0.0763	0.4186	0.3579	0.1379	4
Spleen	0.0068	0.0322	0.0284	0.0118	5
Testes	0.0070	0.0396	0.0315	0.0128	5
Thymus	0.0044	0.0237	0.0230	0.0121	6
Thyroid	0.0185	0.0894	0.0745	0.0312	5

The metabolite profiles in urine and faeces were not essentially influenced by multiple dosing. **Less than 2% of the daily administered dose was excreted as unchanged difenoconazole in faeces.**

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Table B.6.1.2-3: Distribution of metabolite fractions in urine and faeces after single and multiple oral dose administration of [¹⁴C]-Difenoconazole to male rats (Values are expressed as percentage of daily dose)

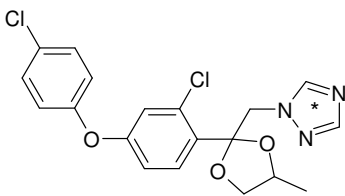
Urinary metabolite profiles				Faecal metabolite profiles			
Sample	Urine 0-1 d	Urine 6-7 d	Urine 13-14 d	Sample	Faeces 0-1 d	Faeces 6-7 d	Faeces 13-14 d
Number of doses	1	14	14	Number of doses	1	14	14
Metabolite fraction				Metabolite fraction			
Fr 1	0.3	0.6	0.6	Fr 1	4.4	8.7	10.7
Fr 2	0.9	1.9	2.2	Fr 2	32.0	57.6	61.5
Fr 3	0.8	2.6	2.5	Fr 3 (CGA 205375)	4.5	6.3	4.1
Fr 4	0.7	0.7	0.9	Fr 4	6.8	5.8	7.6
Fr 5	1.1	0.7	0.6	Fr 5 (CGA 169374)	1.6	1.6	1.6
Fr 6	2.2	2.5	2.8	Faeces extract	49.3	79.9	85.6
Fr 7	0.1	0.2	0.1	Non-extractable	6.3	6.8	7.3
Fr 8	0.2	1.5	1.5				
Fr 9	0.1	0.2	0.1				
Urine extract 2	6.5	10.9	11.4				
Urine extract 1	0.8	0.8	1.0				
Total	7.3	11.7	12.4	Total	55.6	86.7	92.9

Conclusion:

[¹⁴C]- difenoconazole was rapidly and almost **completely absorbed** into the systemic circulation in male rats following multiple oral doses of 0.5 mg/kg bw. More than 98% of the total administered radioactivity was excreted within 7 days after the last dose. Absorbed test substance was **excreted** predominantly **via faeces**. Less than 2% of the daily administered dose was excreted as unchanged difenoconazole in faeces. The **metabolite profiles** in urine and faeces were **qualitatively similar** at each time interval, **although quantitative differences** were observed following single and multiple oral dose administration. Residue levels in tissues plateaued after 7 days except in liver, kidneys, fat and pancreas which did not plateau during the dosing period. The depletion of test substance from tissues was moderately fast with typical half lives of 4-6 days.

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B.6.1.3 First metabolism report in rats: In-life phase triazole label

Reference:	Craine, EM (1987a). Metabolism of triazole-¹⁴C-CGA 169374 in the rat.			
Remark:	This report presents the biological part of a toxicokinetics and metabolism investigation on difenoconazole, the biochemical parts are reported by Capps, McFarland and Cassidy (1988) and Capps, Barr and Carlin (1990) (sections B.6.1.5- and 6, respectively).			
Guideline:	US-EPA FIFRA 85-1. The test procedures followed the OECD TG No. 417.			
GLP:	Yes; verified by the U.S. EPA			
Acceptability:	Yes			
Test substance / purity:	Difenoconazole / 94.5%.			
				
	*) position of label			
	Four batches of radiolabel were used. The low specific radioactive materials were used to dose animals at the high level, the high specific radioactive materials were used to dose rats at the low level.			
Specific activity	0.2 µCi/mg (0.0074 MBq)	0.2 µCi/mg (0.0074 MBq)	19.7 µCi/mg (0.73 MBq)	19.4 µCi/mg (0.72 MBq)
Radiochemical purity	98.1%	98.0%	98.1%	98.0%
Species / Strain:	Rat / Sprague Dawley Crl:CD®BR			
Doses / No. of animals:	0.5 and 300 mg/kg (corresponding to radioactive doses of approximately 9.8 µCi/kg bw and 60 µCi/kg bw) / 5/sex/treatment group, 3/sex as untreated controls.			
Administration:	Orally, by gavage			
Exposure time / Duration:	Single oral dose / 7 days observation period			

Materials and Methods:

The objective of this study was to determine the disposition of triazole-¹⁴C-CGA-169374 after oral administration to rats as a single dose of 300 mg/kg and as a single dose with or without prior treatment with unlabelled difenoconazole at a dose of 0.5 mg/kg. Animals were assigned to one of the following three study groups:

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- Group 1: single oral dose of 0.5 mg/kg bw ¹⁴C-difenoconazole
- Group 2: single oral dose of 300 mg/kg ¹⁴C-difenoconazole
- Group 3: repeated oral dose: daily doses (0.5 mg/kg) of unlabelled difenoconazole for 14 days, followed by a single dose (0.5 mg/kg) of ¹⁴C-difenoconazole

An additional pair of animals (one male and one female) was included in each group as untreated controls, receiving dosing solution without difenoconazole on the same schedule as the treated animals. Seven days after administration of radiolabelled dose, all animals were sacrificed. Urine and faeces were collected from each animal (including controls) for seven days after administration of the labelled dose. Collection periods were 0-4, 4-8, 8-12, 12-24, 24-48, 48-72, 72-96, 96-120, 120-144, and 144-168 hours after radioactive dosing. Cage wash was collected at the time of sacrifice. Blood was collected from each rat via aortic puncture.

Table B.6.1.3-1: After blood collection, tissues were collected as follows, weighed and stored:

Bone (femur)	Eyes	Heart	Lung	Uterus
Brain	Fat (perirenal)	Kidneys	Muscle (leg)	
Carcass	Gonads	Liver	Spleen	

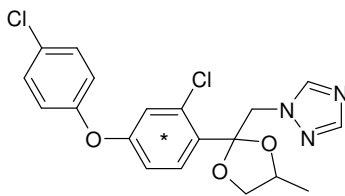
Results:

Body- and organ weights were comparable between the difenoconazole treated groups and the control groups. No remarkable differences in daily food intake were observed between the difenoconazole treated animals of group 3 and the respective control animals.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

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B.6.1.4 Second metabolism report in rats: In-life phase, phenyl label

Reference:	Craine, EM (1987b). Metabolism of phenyl-¹⁴C-CGA 169374 in the rat.	
Remark:	This report presents the biological part of a toxicokinetics and metabolism investigation on difenoconazole, the biochemical parts are reported by Capps, McFarland and Cassidy (1988) and Capps, Barr and Carlin (1990) (sections B.6.1.5- and 6, respectively).	
Guideline:	US-EPA FIFRA 85-1. The test procedures followed the OECD TG No. 417.	
GLP:	Yes; verified by the U.S. EPA	
Acceptability:	Yes	
Test substance / purity:	Difenoconazole / 94.5%.	
		
	*) position of label	
	Two batches of radiolabel were used. The low specific radioactive material was used to dose animals at the high level the high specific radioactive material was used to dose rats at the low level.	
Specific activity	0.2 µCi/mg (0.0074 MBq)	48.6 µCi/mg (1.80 MBq)
Radiochemical purity	98.6%	98.6%
Species / Strain:	Rat / Sprague Dawley Crl:CD®BR	
Doses / No. of animals:	0.5 and 300 mg/kg (corresponding to radioactive doses of approximately 24.3 µCi/kg bw and 60 µCi/kg bw) / 5/sex/treatment group, 3/sex as untreated controls	
Administration:	Orally, by gavage	
Exposure time / Duration:	Single oral dose / 7 days observation period	

Materials and Methods:

The objective of this study was to determine the disposition of phenyl-¹⁴C-CGA-169374 after oral administration to rats as a single dose of 300 mg/kg and as a single oral dose with or without prior treatment with unlabelled difenoconazole at a dose of 0.5 mg/kg. Animals were assigned to one of the following three study groups:

- Group 1: single oral dose of 0.5 mg/kg bw ¹⁴C-difenoconazole
 Group 2: single oral dose of 300 mg/kg ¹⁴C-difenoconazole

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Group 3: repeated oral dose: 14 daily doses (0.5 mg/kg) of unlabelled difenoconazole, followed by a single dose (0.5 mg/kg) of ^{14}C -difenoconazole

An additional pair of animals (one male and one female) was included in each group as untreated controls, receiving dosing solution without difenoconazole on the same schedule as the treated animals. Seven days after administration of radiolabelled dose, all animals were sacrificed. Urine and faeces were collected from each animal (including controls) for seven days after administration of the labelled dose. Collection periods were 0-4, 4-8, 8-12, 12-24, 24-48, 48-72, 72-96, 96-120, 120-144, and 144-168 hours after radioactive dosing. Cage wash was collected at the time of sacrifice. Blood was collected from each rat via aortic puncture. After blood collection, tissues were collected as follows, weighed and stored:

Table B.6.1.4-1:

Bone (femur)	Eyes	Heart	Lung	Uterus
Brain	Fat (perirenal)	Kidneys	Muscle (leg)	
Carcass	Gonads	Liver	Spleen	

Results:

One female in the 300 mg/kg bw- dose group became lethargic and emaciated with decreased urination and defecation. All clinical findings for this female were noted between 48 and 120 h following administration of the labelled dose. No remarkable observations were noted in this animal on the first two or last two days of exposure. Body- and organ weights were comparable between the difenoconazole treated groups and the vehicle control groups. No remarkable differences in daily food intake were observed between the difenoconazole treated animals of group 3 and the respective control animals.

B.6.1.5 Third metabolism report in rats: kinetics

Reference:	Capps, TM, McFarland JE and Cassidy JE (1988). Metabolism of triazole- ^{14}C and phenyl- ^{14}C -CGA 169374 in the rat – distribution of radioactivity.
Remark:	This report presents the kinetics part of toxicokinetics and metabolism investigations on difenoconazole, the biological parts are reported in sections B.6.1.3 and B.6.1.4 by <i>Crain (1987a and 1987b)</i> ; the metabolism part is reported in section B.6.1.6 by <i>Capps, Barr and Carlin (1990)</i> .
Guideline:	US-EPA FIFRA 85-1
GLP:	No. This study was performed according to sound scientific practices; deviations from GLP are detailed in the Tier I summary.
Acceptability:	Yes

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Materials and Methods:

The objectives of this report were to study:

- **Absorption, distribution and excretion** at a single low (0.5 mg/kg) and high (300 mg/kg) dose and at a single low dose after repeated (14 d) administration of non-labelled substance.
- Possible **tissue accumulation**
- The results for rats treated with phenyl- ¹⁴C- and triazole- ¹⁴C labelled difenoconazole to **determine whether the bridge between the phenyl and triazole rings is cleaved.**

Results:

Excretion

More than 98% of the radioactivity administered to all rats was recovered in faeces and urine. **Most of the elimination was via faeces (>78%)** (probably via the bile duct) and **10-20%** was eliminated **in urine**.

Regardless of gender or label, **50% of the radioactivity was excreted in 20h** in the 0.5 mg/kg bw dose group. In the 300 mg/kg bw-dose group, 50% of the given dose was excreted within **48h**. Preconditioning the rats for 14 days at the low dose rate resulted in an elimination half-life of **22 h**. Also, preconditioned rats given the **triazole label excreted** the radioactivity **faster than** rats given the **phenyl label** ($t_{1/2}$ = 21 vs. 24h for males and 21 vs. 22h for females).

At the 300 mg/kg-dose level, the 50% **elimination times for triazole labelled rats were lower than** those for **phenyl labelled rats** - 33 vs. 37 h for males and 33 vs. 48 h for females. It is noted that the **female metabolism** of the phenyl label is **slower** ($t_{1/2}$ = 48 h) **than** the corresponding metabolism **in males** ($t_{1/2}$ = 37 h).

Distribution

Tissue residues at sacrifice (7 days after dosing) were **<1%** of the administered dose. Highest levels were found in **plasma** and **fat** in animals given phenyl-labelled substance and in the **livers** of animals given the **high** dose of triazole-labelled substance (0.9 and 0.7 ppm or 0.02 and 0.01% of the dose in males and females, respectively).

Tissue residues in **females** were generally **below** those in **males**. Multiple pre-treatment with non-labelled difenoconazole had no effect on the tissue distribution.

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Table B.6.1.5-1: Tissue residues in males 7 days after dosing with ¹⁴C-phenyl labelled difenoconazole

	low dose		high dose		multiple low dose	
	ppm	% dose	ppm	% dose	ppm	% dose
Heart	0.005	0.00	3.84	0.01	0.006	0.01
Lung	0.007	0.01	4.99	0.01	0.010	0.01
Spleen	LQ	0.00	1.65	0.00	0.002	0.00
Kidney	0.005	0.01	3.64	0.01	0.007	0.02
Liver	0.005	0.07	3.71	0.07	0.007	0.10
Fat	0.011	0.26	11.10	0.42	0.012	0.36
Gonads	LQ	0.00	1.86	0.01	0.005	0.01
Muscle	LD	0.00	1.18	0.19	LQ	0.10
Brain	LD	0.00	LQ	0.00	LQ	0.00
Bone	LQ	0.00	1.50	0.00	0.003	0.00
Carcass	0.004	0.67	2.71	0.84	0.004	0.67
Plasma	0.027	0.23	15.64	0.23	0.030	0.37
RBC	0.005	0.03	3.68	0.03	0.008	0.06
Eyeballs	LQ	0.00	0.94	0.00	LQ	0.00

LQ ≤ limit of quantification (0.003 ppm for low dose; ~ 0.8 ppm for high dose)

LD ≤ limit of detection (0.001 ppm)

Table B.6.1.5-2: Tissue residues in females 7 days after dosing with ¹⁴C-phenyl labelled difenoconazole

	low dose		high dose		multiple low dose	
	ppm	% dose	ppm	% dose	ppm	% dose
Heart	LQ	0.00	2.52	0.00	0.004	0.00
Lung	0.005	0.01	3.57	0.01	0.008	0.01
Spleen	LD	0.00	1.17	0.00	LQ	0.00
Kidney	0.003	0.00	2.36	0.01	0.005	0.01
Liver	0.004	0.01	2.58	0.04	0.005	0.05
Fat	0.009	0.18	8.57	0.29	0.008	0.19
Gonads	LD	0.00	7.82	0.00	LQ	0.00
Uterus	0.005	0.00	4.38	0.00	0.008	0.00
Muscle	LD	0.00	0.85	0.12	LQ	0.00
Brain	LD	0.00	LD	0.00	LQ	0.00
Bone	LD	0.00	1.07	0.00	LQ	0.00
Carcass	LQ	0.12	2.28	0.63	0.004	0.22
Plasma	0.019	0.14	9.02	0.12	0.021	0.19
RBC	0.004	0.02	2.04	0.01	0.005	0.03
Eyeballs	LQ	0.00	0.73	0.00	LQ	0.00

LQ ≤ limit of quantification (0.003 ppm for low dose; ~ 0.8 ppm for high dose)

LD ≤ limit of detection (0.001 ppm)

Metabolic cleavage

More radioactivity was found in tissues of rats dosed with the phenyl label (0.4-1.0%) than in rats dosed with the triazole label (0.00-0.02%) indicating that **the bridge between the phenyl and triazole rings must be susceptible to metabolic cleavage.**

Conclusion:

More than 98% of the radioactivity administered to all rats was recovered in faeces and urine. Most of the elimination was via faeces (>78%). T_½ for the excretion of applied radioactivity was between 20 and 48 h in all dose groups. Tissue residues at sacrifice were <1% with the highest levels in fat and plasma (phenyl label) and in liver (triazole label). Residues from the triazole labelled compound were below those from the phenyl labelled compound.

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B.6.1.6 Fourth metabolism report in rats: dual label

Reference:	Capps, TM, Barr, HP and Carlin HJ (1990). Characterization and identification of major triazole-¹⁴C and phenyl-¹⁴C-CGA 169374 metabolites in rats.
Amendment:	Capps, T and Anderson, W (1993). Supplemental Report on the metabolism of ¹⁴C-phenyl-CGA-169374 in rats – Identification of the major urinary metabolites.
Remark:	This report presents the metabolism part of toxicokinetics and metabolism investigations on difenoconazole, the biological parts are reported in sections B.6.1.3 and B.6.1.4 by <i>Crain (1987a and 1987b)</i> ; the kinetics part is reported in section B.6.1.5 by <i>Capps, McFarland and Cassidy (1988)</i> .
Guideline:	US-EPA FIFRA 85-1
GLP:	Yes.
Acceptability:	Yes

Materials and Methods:

The objectives of this report were to:

- **identify** the significant **metabolites** of difenoconazole in urine and feces from rats
- **determine** common **metabolites between dose levels and gender** and their respective abundance

The objective of the amendment was additional isolation and identification of the major urine metabolites of phenyl-¹⁴C-CGA-169374. Focus was held on faecal metabolites since the majority of the radioactivity was excreted via feces. In the amendment, day-1-urine samples from high dose ¹⁴C-phenyl female rats were pooled for further identification work of metabolites.

Results:

Identification of metabolites

The **major faecal metabolites** were referred to as **A, B and C** and accounted together for (on average) **69% of the dose**. Metabolite **C** was **only observed at high dose** (300 mg/kg bw) level. Metabolites A and B did not match any available standard, whereas **metabolite C** was readily **identified as CGA-205375**. However, metabolites A and B could be identified as **hydroxyl-CGA-205375** and **hydroxyl-CGA-169374**, respectively after further investigations, and **two isomers** each were identified for the two. Metabolite profiles for **urine** were more **complex** and no individual urine metabolite accounted for $\geq 10\%$ of the total radioactivity. In urine of **triazole** dosed rats, a single urinary metabolite was isolated and identified as **CGA-71019** (free 1,2,4-triazole). This metabolite **represents** the only **significant example of cleavage of the alkyl bridge between the ring systems**. The **phenyl** labelled urine samples tended to contain less polar metabolites and showed **greater complexity** in distribution than the triazole labelled samples. One tissue metabolite was isolated and characterised from phenyl labelled **liver** samples as the acetic acid metabolite, **CGA 189138**.

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Abundance of metabolites

Some **gender differences** were observed in the relative abundance of the faecal metabolites:

Table B.6.1.6-1: Quantification of major metabolites of difenoconazole in rat faeces (% of applied dose)

	low dose (0.5 mg/kg bw)		high dose (300 mg/kg bw)		multiple low dose (0.5 mg/kg bw)	
	male	female	male	female	male	female
phenyl label						
metabolite A	74.7	66.2	78.5	30.8	51.2	40.5
metabolite B	1.6	14.5	--	20.3	7.9	18.7
metabolite C	--	--	10.2	24.2	--	--
triazole label						
metabolite A	62.9	18.4	71.8	48.3	51.6	46.2
metabolite B	12.9	18.2	2.1	16.0	5.5	17.6
metabolite C	--	--	6.7	9.6	--	--

Individual urinary metabolites were found in low amounts, no one exceeding 10% of elimination. The metabolite distribution was more complex in urine than in faeces, with greater **variability resulting from label difference**.

Table B.6.1.6-2: Quantification of major metabolites of difenoconazole in rat urine (% of applied dose)

	high dose (300 mg/kg bw)	
	male	female
phenyl label		
CGA 205375	n.d.	0.2
hydroxy-CGA 205375	n.d.	1.7
sulfate conj. CGA 205375	n.d.	2.8
sulfate conj. hydroxy-CGA 205375	n.d.	2.0
hydroxy acetic acid	n.d.	1.8
triazole label		
CGA 71019	<10%	n.d.

n.d. not determined

Metabolic Pathway

The proposed metabolic pathway involves **hydroxylation of the outer phenyl ring**. The observations of the metabolite CGA-71019 in triazole labelled urine and CGA-189138 in liver provide evidence that **bridge cleavage is occurring**.

Conclusion:

The major steps of the metabolism of difenoconazole in the rat involve **hydrolysis** of the ketal **resulting in CGA 205375 (metabolite C)** with the ketone CGA 205374 as a postulated but not identified intermediate **and hydroxylation** on the outer phenyl ring of the parent and in CGA 205375 (metabolite A). The resulting metabolites accounted together for an average of 68% of the applied dose. As **a minor process cleavage of the alkyl chain between the triazole and the inner phenyl ring** occur, resulting in a hydroxy acetic acid or an acetic acid moiety and free triazole. Sulfate conjugates were identified for CGA 205375 and for hydroxy-CGA 205375. It appears likely, that the **triazole and the hydroxyacetic acid** metabolites are **excreted in urine** because of their **polar nature** while the free **phenyl acid** is absorbed by the **tissues** due to its **lipophilic character**.

Comments:

All urine metabolites identified in the amendment were metabolites previously identified or conjugates of these known metabolites.

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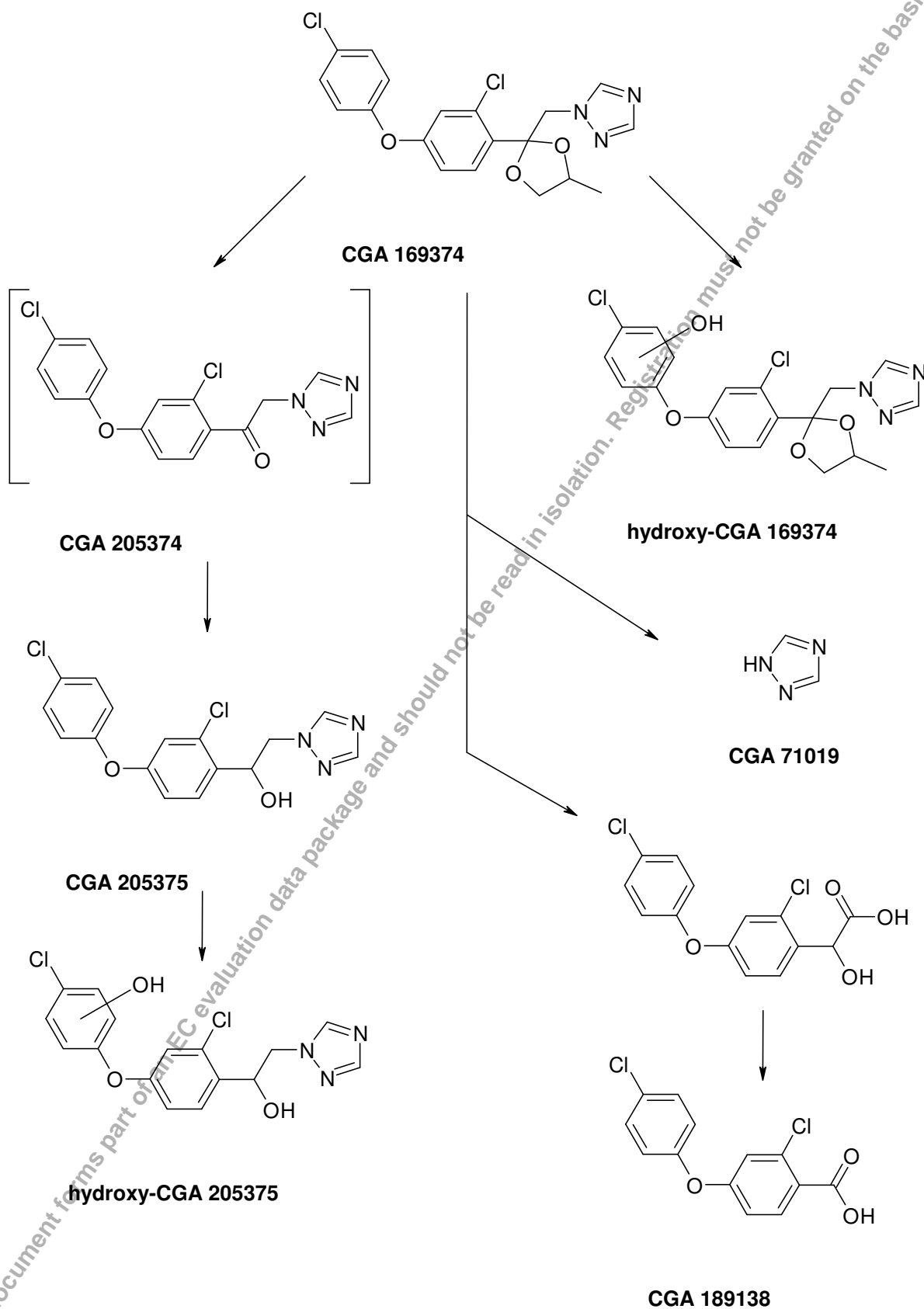


Figure B.6.1.6-1: Metabolic Pathway of Difenconazole in Animals

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B.6.1.7 Summary and conclusions on absorption, distribution, excretion and metabolism studies in mammals

The **oral absorption** of difenoconazole is **80 - 90%** at lower (0.5 mg/kg bw) dose levels and **40-60%** at higher (300 mg/kg bw) dose levels.

A single oral dose of **0.5 mg** [¹⁴C-phenyl]-difenoconazole/kg was **almost completely absorbed** by male and female rats and eliminated predominantly *via bile* (accounting for **73 and 76%** of a 0.5 mg/kg dose in males and females, respectively). **Urinary** excretion in bile duct cannulated rats accounted for **14 and 9%** in males and females, respectively and **less than 4% faecal** excretion which confirm the high absorption. Corresponding results in non-cannulated rats were 13 - 22% in urine and 81 - 87% in faeces. An **entero-hepatic recirculation** was demonstrated experimentally. Biliary metabolites were largely excreted in faeces. There was **no difference in excretion profiles** between the sexes or between two radiolabelled forms (¹⁴C-phenyl- or ¹⁴C-triazole-difenoconazole). Furthermore, there was neither a sex difference nor differences in excretion profiles in rats given the same radiolabelled dose (0.5 mg/kg) following pre-treatment with multiple oral doses of radiolabelled difenoconazole (0.5 mg/kg), from the rats with no pre-treatment. At the 0.5 mg/kg bw- dose level, the **half life** of excretion was approximately **20 hours**.

At 2 and 24 hours, most of the radioactivity was present in the **gastrointestinal tract contents** and in **bile** (as shown by whole body autoradiography sections) and only **liver and kidney** had higher tissue concentrations than plasma, after a dose of 0.5 mg [¹⁴C-phenyl]-difenoconazole/kg bw. After 7 days, only **fat** had comparable concentrations with those present in plasma. Residues in female tissues tended to be lower than in males. Pre-treatment with unlabelled test substance had no effect on tissue distribution.

A single oral dose of **300 mg** [¹⁴C-phenyl]-difenoconazole/kg was **less extensively absorbed**, however still eliminated predominantly *via bile* (accounting for **56 and 39%** of the dose in males and females, respectively). **Urinary** excretion in bile duct cannulated rats accounted for **1%** (both sexes) and **faecal** excretion for **17 and 22%** in males and females, respectively which confirm the **lower absorption**. Corresponding results in non-cannulated rats were 8 - 15% in urine and 85 - 95% in faeces. As seen at the low dose level, there was **no difference in excretion profiles** between the sexes or between two radiolabelled forms. Non-cannulated rats excreted more of the dose in urine than bile duct cannulated rats, apparently following reabsorption and further metabolism of some biliary metabolites. However, the predominant route of excretion of biliary radioactivity was in faeces, as observed at the low dose level. At the 300 mg/kg bw- dose level, the **half life** of excretion was **33 - 48 hours**.

At 4 hours, the highest tissue concentrations were present in **fat** (both sexes) with progressively lower levels in **liver, Harderian glands, adrenal glands, kidney and pancreas**, after a dose of 300 mg [¹⁴C-phenyl]-difenoconazole/kg bw. After 7 days, only **fat** had higher concentrations with those present in plasma. Residues in female tissues tended to be lower than in males.

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Tissue depletion results showed that at 4 hours after a 300 mg/kg [^{14}C -phenyl]-dose, most tissue concentrations were similar to or higher than in plasma in both sexes. All other tissues that initially showed concentrations higher than in plasma declined rapidly by 48 hours after dosing and by 168 hours all [^{14}C -phenyl] tissue levels had declined markedly with only fat showing residues higher than in plasma. [^{14}C -triazole]-tissue residues were significantly lower than [^{14}C -phenyl]-residues and by 168 hours were measurable only in the liver. Measurements in the gastrointestinal tract contents were consistent with the observed absorption and elimination profiles.

Metabolites were isolated from urine and faeces of male and female rats administered a single oral dose of 0.5 or 300 mg [^{14}C -phenyl] and [^{14}C -triazole]-difenoconazole/kg, or a 0.5 mg/kg dose after 14 daily oral doses of 0.5 mg unlabelled difenoconazole/kg. **Three main metabolites, A, B and C** were isolated from faeces and together **accounted for** an average of **68% of the dose**. The urinary metabolite profile was more complex and showed more variability between the two radiolabelled forms (^{14}C -phenyl and ^{14}C -triazole). Other urinary metabolites included metabolite C. One metabolite - CGA 189138 (chlorophenoxy-chlorobenzoic acid) - was also isolated from liver.

Hence, at both dose levels, elimination kinetics were independent of sex and radiolabel position. The difenoconazole molecule was **extensively metabolised**, although with limited cleavage of the triazole and dioxolane rings. The extensive **biliary elimination** was **consistent with** the relatively **high molecular weights** of the major **metabolites**.

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B.6.2 Acute toxicity including irritancy and skin sensitization (Annex IIA 5.2)

B.6.2.1 Oral

B.6.2.1.1 Acute Oral Toxicity Study in Rats

Reference:	Argus MA, Ricci JM, Huber KR, Schiavo DM, Hazelette JR and Green JD (1987). CGA 169374 technical: Acute oral toxicity study in rats.
Guideline:	OECD TG 401, 87/302/EEC B.1
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole Technical / Not specified, see under comments
Vehicle:	cornstarch / polysorbate 80
Species / Strain:	Rat / Sprague Dawley
Doses / No. of animals:	1000, 2000 and 3000 mg/kg bw, 5/sex/dose
Administration:	Orally, by gavage
Exposure time / Duration:	Single dose / Observation period of 14 days post dose

Materials and Methods:

The test material was suspended in vehicle and administered in a dosing volume of 50 ml/kg to approximately 8-week-old rats. Control animals received vehicle only. Body weights were recorded pre-test and on test days 1, 8 and 15. Necropsy was performed on all animals. The LD₅₀ was calculated by the method of Bliss, modified by Carmines et al., (1980).

Results:

Mortality rates were 40, 40 and 100% in the 1000, 2000 and 3000 mg/kg bw-dose groups, respectively. Clinical signs were observed at all dose levels and included hypoactivity, gastrointestinal symptoms, chromodacryorrhea, ataxia and spasm, with an onset between 0.5 h and 7 days post administration. All deaths occurred at day 3, 4 or 5 post dosing. In animals found dead after receiving 2000 mg/kg bw (2 males and 2 females), pronounced stomach lesions were observed at necropsy. Necropsy findings of all other animals sacrificed at day 15 were negative.

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Conclusion:

The oral LD₅₀ was calculated to be 1453 mg/kg for both males and females. Thus, the acute oral toxicity in rats is moderate and according to the Council Directive 67/548/EEC, Difenoconazole Technical should be **classified** as harmful with the risk phrase **R22 Harmful if swallowed**.

Comments:

According to the original report (page 2), records pertaining to the purity of the test substance are on file with the sponsor.

B.6.2.1.2 Acute Oral Toxicity Study in Mice

Reference:	Hartmann HR (1990). CGA 169374 tech.: Acute oral toxicity in the mouse.
Guideline:	OECD TG 401, 87/302/EEC B.1
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / Not specified
Vehicle:	arachis oil
Species / Strain:	Mouse / Tif: MAG f (SPF)
Doses / No. of animals:	1000 and 2000 mg/kg bw, 5/sex/dose
Administration:	Orally, by gavage
Exposure time / Duration:	Single dose / 14 days observation period post dosing

Materials and Methods:

The test material was suspended in vehicle and administered at a volume of 10 ml/kg. Body weights were recorded before dosing and on days 7 and 14. All animals were necropsied at death or termination.

Results:

Mortality rates were, **20 and 30%** in the 1000 and 2000 mg/kg dose groups, respectively.

Table B.6.2.1.2-1: Mortality in mice after oral administration of difenoconazole

Dose(mg/kg)	Males		Females	
	dead/treated	time to death (days)	dead/treated	time to death (day)
1000	0/5	-	1/5	2
2000	1/5	2	2/5	3, 5

Clinical signs were seen in all dose groups and included decreased locomotion, piloerection, ataxia and dyspnoea. Furthermore, spasms were observed in animals of the 2000 mg/kg dose group. Surviving animals recovered within 8 days. Necropsy was negative.

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Conclusion:

LD₅₀ was calculated to be >1000 mg/kg bw for females and **>2000 mg/kg bw** for males. Thus, the acute oral toxicity in female mice is moderate and according to the Council Directive 67/548/EEC, difenoconazole should be classified as harmful with the risk phrase **R22 Harmful if swallowed**.

Comment from notifier:

"The acute oral LD₅₀ was calculated to be greater than 2000 mg/kg for male and female mice. Difenoconazole is therefore of low toxicity to Tif: MAG f (SPF) mice after a single oral application."

B.6.2.2 Percutaneous**B.6.2.2.1 Acute dermal toxicity study in rabbits**

Reference:	Mastrocco F, Ricci J, Huber K, Schiavo DM, Hazelette JR and Green JD (1987a). CGA 169374 technical: Acute dermal toxicity study in rabbits.
Guideline:	OECD TG 402, US-EPA FIFRA 81-2
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole tech. / not specified, see under comments
Species / Strain:	Rabbit / New Zealand White
Doses / No. of animals:	Limit test, 2010 mg/kg, 5/sex
Administration:	Dermally
Exposure time / Duration:	24 h / 14 days observation period post exposure

Materials and Methods:

The test material was prepared as a 50% solution in ethanol and applied to area of 240 cm² of 12-13 week-old rabbits. The test article was held in place for 24 hours by a semi-occlusive wrapping. After exposure the skin was washed with ethanol. Body weights were recorded prior to treatment on day 1 and on days 8 and 15. Necropsy was performed on all animals.

Results:

No **mortality** occurred. Erythema (Draize grade 1) at the treatment site of 3 animals was observed at the end of the dosing period. Fissuring was noted in 1 male at 72 h and desquamation of the skin was noted in all animals at day 7 and in most rabbits at day 14, **see comments**. All animals gained weight during the study and gross visceral evaluations performed on all animals at the termination of the study were unremarkable.

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Conclusion:

The dermal **LD₅₀** was estimated to be **>2010 mg/kg** in male and female rabbits. According to the Council Directive 67/548/EEC, **no classification is required**.

Comments:

According to the original report (page 2), records pertaining to the **purity** of the test substance are on file with the sponsor. The original report refers the observed erythema, fissuring and desquamation at treatment sites to be a result of exposure to the ethanol vehicle. However, according to the OECD TG 402 (1987) the influence of the vehicle should be taken into account and test substance should be moistened with water if possible.

Difenoconazole is soluble in water.

B.6.2.3 Inhalation**B.6.2.3.1 Acute Inhalation Toxicity in the Rat**

Reference:	Hartmann HR (1991). CGA 169374 tech.: Acute inhalation toxicity in the rat.
Amendment:	Hartmann HR (1992). The correct value for water solubility of the test substance used in the study is 3.3 ppm . It is erroneously indicated to be 20 ppm in the original (page ten).
Guideline:	OECD TG 403
GLP:	Yes, consistent with OECD principles of GLP
Acceptability:	Yes
Test substance / purity:	Difenoconazole tech. / 96.2%.
Vapour pressure:	3.7 mPa (DAR Section Volume 3 Annex B2.1.1.2)
Species / Strain:	Rat / Tif RAI f (SPF)
Doses / No. of animals:	3285 mg/m³ = 3.3 ppm, Limit test (technically highest achievable concentration), (3967/3458 nominal/gravimetric mg/m ³), see comments, 5/sex /dose
Administration:	Inhalation, nose-only
Exposure time / Duration:	4 h / 14 days observation period post exposure

Materials and Methods:

Difenoconazole was mixed with an inert silica (5% Sipernat 50S) in order to maintain a constant **aerosol** concentration with acceptable particle size distribution. The aerosol was generated from the solid test material by means of a brush-feed micronizing jet mill. The aerosol from the dust generator was diluted with filtered humidified air. Care was taken to avoid re-breathing of the exhaled air. The exposure conditions were as follows:

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Table B.6.2.3.1-1

Parameter	Value determined
Mean exposure concentration corrected for 5% admixed Sipernat	3285 mg/m ³
Mass median aerodynamic diameter (MMAD) (geometric standard deviation)	1.1 – 1.5 µm (2.8 – 3.8)
Particles < 7 µm	89 - 95%
Particles < 3 µm	73 – 81%
Air flow through chamber	48 L/min

Controls were exposed to purified air under the same conditions. Body weights were recorded prior to exposure and on days 7 and 14. Body weight of exposed and control animals were compared by Analysis of Variance. Necropsy was performed on all animals with particular attention given to the respiratory tract.

Results:

No animals **died** during the study. Both sexes showed signs of discomfort with piloerection, hunched posture, dyspnoea, and reduced locomotor activity but recovered within 7 days. The mean **body weight** of exposed males was significantly lower (2%) than controls at day 7 post exposure. Exposed females however, had significantly higher (12%) mean body weight than controls at day 14. No treatment-related **macroscopic findings** were observed.

Conclusion:

The inhalation **LC₅₀** of Difenconazole suspended in air was determined to be > **3300 mg/m³** (3.3 mg/l) for male and female rats. No effects were detected in this study at 3.3 mg/l after 7 days of recovery. **Thus, no classification should be required according to the Council Directive 67/548/EEC.**

Comments:

Due to the properties of the test material, it was not possible to generate higher aerosol concentrations than **3458 mg/m³**. Furthermore, in order to maintain a constant aerosol concentration with acceptable particle size distribution it was necessary to blend difenoconazole with 5% Sipernat 50S, an inert silica. After correction for the 5%-Sipernat admix, the **mean exposure concentration was 3285 mg/m³**. Particle size analysis was performed by means of a Marple personal cascade impactor instead of an APS-33 aerodynamic particle sizer, which deviates from protocol.

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B.6.2.4 Skin irritation

B.6.2.4.1 Primary Dermal Irritation Study in Rabbits

Reference:	Glaza SM (1991a). Primary dermal irritation study of CGA 169374 technical in rabbits.
Report supplement:	Tisdell M (1992). Supplemental information for primary dermal irritation study of CGA 169374, see comments.
Guideline:	US-EPA FIFRA 81-5
GLP:	Yes; verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole / purity 91.5%.
Species / Strain:	Rabbit / Hra:(New Zealand White) SPF
Doses / No. of animals:	0.5 g/animal, 3/sex
Administration:	Dermally, semi-occluded conditions
Exposure time / Duration:	4 h/ 72 h observation period post treatment

Materials and Methods:

The test material was moistened with 0.9% saline and applied to a 6.25 cm² gauze pad which was placed on the shaved dorsal area of rabbits weighing 2-3 kg. The patch was covered with Saran Wrap[®] and Elastoplast[®] tape. At the end of exposure, patches were removed and the test sites were washed with tap water. Adjacent areas of untreated skin of each animal served as controls for the test. Signs of skin erythema or oedema were scored after 30 minutes, 24, 48, and 72 h post dosing, **see comments**. Dermal irritation was scored and recorded according to the Draize technique.

Results:

One female was found with a **grade 1 erythema** at the 30-min reading. After 24 h this reaction was clear. No other skin reactions were noted with any animal. Average of the 30-minute, 24-, 48, and 72-h scores was **0.1, see comments**.

Conclusion:

Based on the degree of the skin reaction (mean skin irritation score 24 to 72 hours after removal of the test material; 0.1) **no classification is required** according to the Council directive 67/548/EEC.

Comments:

The purpose of the **supplemental document** by Tisdell was to provide requested information by the EPA reviewer on the size of the test site and the purity and stability of the test material. The test site was approximately 6.25 cm² and the purity and stability of test substance 91.5% and 24 months in room temperature, respectively according to CIBA-GEIGY response.

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Deviations from the OECD TG No 404 (2002).

Animals should be scored at 60 minutes, 24, 48 and 72 h after patch removal according to the TG. In the present study the first and second scorings were made at 30 minutes and 24 h respectively. Also the temperature (21-25°C) and the humidity (66-77%) of the animal room deviated from the TG recommendations of 20±3°C and 50-60%, respectively.

B.6.2.5 Eye irritation

B.6.2.5.1 Primary Eye Irritation Study in Rabbits

Reference:	Glaza SM (1991b). Primary eye irritation study of CGA 169374 technical in rabbits.
Guideline:	US-EPA FIFRA 81-4
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole / 91.5%.
Species / Strain:	Rabbit / New Zealand White (Hra: (NZW) SPF
Doses / No. of animals:	0.05g / animal (0.1 ml equivalent), <u>Group 1 (unwashed)</u> : 3 males and 3 females, <u>Group 2 satellite (flushed)</u> : 2 males and 1 female
Administration:	Instillation into the everted lower lid of the right eye
Exposure time / Duration:	96 h (sufficient to determine reversibility of the effects)

Materials and Methods:

Animals were acclimatized for an unknown period before treatment. The test material was administered as received (powder form) into the conjunctival sac and the lids were held together for a second. The eyes of the satellite group were flushed with water for 60 seconds starting 30 seconds after instillation. Eyes were examined at 1, 24, 48 72 and 96 h post dosing. Eye irritation was scored and recorded according to the Draize technique. Body weights were recorded prior to dosing only.

Results:

Conjunctival irritation with involvement of the cornea and iris were observed in of all rabbits with unwashed eyes, beginning 1 hour after application and persisting through 48 or 72 hours. Reactions in the washed eyes (satellite group) were generally weaker and of shorter duration. All reactions were clear by day 4 (96 h) after treatment.

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Table B.6.2.5.1-1: Eye irritation in rabbits after instillation of difenoconazole

	Draize scores at indicated time after removal*					
	1 hour	24 hours	48 hours	72 hours	96 hours	Mean score (24-72 hours)
Group 1 (unwashed eye)						
Cornea opacity	0/0/0/0/0/0	1/1/1/2/0/0	1/0/0/1/0/0	0/0/0/1/0/0	0/0/0/0/0/0	
Mean score	0	0.83	0.33	0.17	0	0.44
Iris lesions	0/1/0/0/1/1	1/1/1/1/1/0	1/1/0/1/0/0	0/0/0/0/0/0	0/0/0/0/0/0	
Mean score	0.5	0.83	0.5	0	0	0.44
Conjunctivae - redness	2/2/2/2/2/2	2/3/3/3/2/2	2/3/2/3/1/2	2/2/1/2/0/1	0/0/0/0/0/0	
Mean score	2.0	2.5	2.16	1.33	0	2.0
Conjunctivae - chemosis	1/1/1/1/1/1	2/1/1/2/1/1	1/1/1/2/0/1	0/0/0/1/0/0	0/0/0/0/0/0	
Mean score	1.0	1.33	1.0	0.17	0	0.83
Group 2 (washed eye)						
Cornea opacity	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	
Mean score	0	0	0	0	0	0
Iris lesions	1/0/1	1/0/0	0/0/0	0/0/0	0/0/0	
Mean score	0.67	0.33	0	0	0	0.11
Conjunctivae - redness	2/2/2	2/2/2	1/2/2	1/1/1	0/0/0	
Mean score	2.0	2.0	1.67	1.0	0	1.56
Conjunctivae - chemosis	1/1/1	1/1/0	1/0/0	0/0/0	0/0/0	
Mean score	1.0	0.67	0.33	0	0	0.33

*) The numbers are the individual scores from each animal tested.

Conclusion:

Based on the degree of the eye reactions in the un-washed eyes (mean scores 24 to 72 hours after instillation of the test material; 0.44, 0.44, 2.0 and 0.83) **no classification is required** according to the Council Directive 67/548/EEC.

Comments:

Deviations from the OECD TG No 405 (2002).

The temperature (19-25°C) and the humidity (54-77%) of the animal room deviated from the TG recommendations of 20±3°C and 50-60%, respectively.

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B.6.2.6 Skin sensitization

B.6.2.6.1 Skin Sensitization Test in Guinea Pigs – Buehler Test

Reference:	Mastrocco F, Ricci JM, Huber KR, Schiavo DM, Hazelette JR and Green JD (1987b). CGA 169374 technical – Dermal sensitization study in female Guinea pigs.
Guideline:	OECD 406, US-EPA FIFRA 81-6
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole tech. / not specified <u>Positive control:</u> 1-chloro-2,4-dinitrobenzene (DNCB), 0.05%
Species / Strain:	Guinea pigs / Hartley (females)
Doses / No. of animals:	0.5 g per animal (undiluted test substance), 0.5 ml per animal (positive control) / 10 per treatment group , see comments
Administration:	Topical induction, topical challenge
Exposure time / Duration:	<u>Induction:</u> 10 x 6-h periods, <u>Challenge:</u> 1 x 6-h period / 48-h observation period post challenge

Materials and Methods:

Animals were acclimatized for 2 weeks prior to dosing according to the scheme below:

Table B.6.2.6.1-1: Dosing schedule was a modification of the Buehler (1965) method.

group	No. of animals	Topical induction	Topical challenge
1. non-sensitized	10	not performed	difenoconazole
2. sensitized	10	difenoconazole	difenoconazole
3. non-sensitized	10	not performed	positive control substance
4. sensitized	10	positive control substance	positive control substance

Body weights were recorded weekly on days 1, 8, 15, 22, 29 and 36.

Epidermal Irritation Screening Pre-test:

An additional four animals were used to determine the irritative potential of the test material.

Epidermal Induction – days: 1-22:

An un-known area on one flank was shaved prior to dosing. Test substance or positive control solution was applied to a gauze patch of un-known size (**see comments**) and placed on the flanks of group-2 and 4 animals (the sensitized groups), with an occlusive tape and left in place for 6 h. The induction process was repeated on days 1, 3, 6, 8, 10, 13, 15, 17, 20 and 22 giving a total of ten 6-hour exposures. At exposure termination, patches

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were removed and the test site was wiped with 95% ethanol and water. Dermal observations were made 24 h after each induction dose. Animals of groups 1 and 3 were left un-treated during the induction phase.

Epidermal Challenge – day 36:

The test substance was applied, in a manner identical to the induction treatments, to a previously untreated skin site of all animals, including non-sensitized group-1-and 3-animals. Skin sites were examined 24 and 48 hours after the challenge dose.

Results:

Epidermal Induction:

Epidermal **induction** caused **no skin reactions** in any of the animals dosed with undiluted difenoconazole (group 2), whereas **erythema** grade 1 was variously observed in the majority of animals of the **positive control** group (group 4).

Epidermal Challenge:

No positive reactions were evident at the application site in any of the non-sensitized animals (groups 1 and 3) or in the difenoconazole-sensitized animals (group 2) 24 and 48 hours **after challenge** application. The positive control-sensitized animals (group 4) revealed positive skin reactions in 6 out of 9 animals after 24 hours and in 4 out of 9 animals after 48 hours.

One animal in the **positive control** group (group 4) was **found dead** on test day 12, without showing clinical signs prior to death. Spontaneous deaths in guinea pigs are reported to happen in the study laboratory and the present death was not considered related to treatment. There were no other clinical signs in any animal during the test period. **Body weights** were not affected by treatment.

Conclusion:

The negative result of this study is based on fewer animals than recommended. However, the conclusion is that difenoconazole not induces sensitisation in the guinea pig. Based on the degree of the skin reactions (sensitization rate <15%) in 10 animals/test group, **no classification is required**, according to the Council Directive 67/548/EEC.

Comments:

Deviations from the OECD TG No. 406 (1992)

The size of the pads used in the induction and challenge phases is not given in the original report but should, according to the TG be approximately 4-6 cm². Furthermore, only 10 animals per treatment are used in the study, which is fewer than the recommended 20 per treatment group. The TG states that **“When fewer than 20 test and 10 control guinea pigs have been used - and it is not possible to conclude that a the test substance is a sensitizer - testing in additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.”**

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B.6.2.7 Summary and conclusions on acute toxicity studies

A classification as "Harmful if swallowed" (R22) is considered warranted according to the **Council Directive 67/548/EEC** based on the acute oral LD₅₀ of 1453 mg/kg. No signs of dermal irritation were noted. Signs of ocular irritation were observed in the rabbit. However, as the mean values of the readings were below the thresholds defined in Directive 2001/59/EC, no classification is required. In a modified Buehler test no sensitisation effects were detected and therefore no classification is considered necessary.

Table B.6.2.7:

Study author	parameter	species	Dose levels	Results (incl ev classification)
Argus et al., 1987	Acute oral LD ₅₀	rat	1000, 2000 and 3000 mg/kg	male and female 1453 mg/kg Xn, R22
Hartmann, 1990	Acute oral LD ₅₀	mouse	1000 and 2000 mg/kg	>2000 mg/kg
Mastrocco et al., 1987	Acute dermal LD ₅₀	rabbit	Limit test, 2010 mg/kg	>2010 mg/kg bw
Hartmann H.R., 1991	Acute inhalation LC ₅₀	rat	Limit test, 3285 mg/m ³	>3300 mg/m ³
Glaza, S.M., 1991	Skin irritation	rabbit	0.5g	non-irritating
Glaza, S.M., 1991	Eye irritation	rabbit	0.05g	non-irritating
Mastrocco et al., 1987	Skin sensitisation (modified Buehler)	female Guinea pigs	0.5g	non-sensitising

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B.6.3 Short-term toxicity (Annex IIA 5.3)

B.6.3.1 Oral

B.6.3.1.1 Oral 28-day study: rat

Reference:	Suter, P. (1986a). 28-Day cumulative oral toxicity (feeding) study with CGA 169374 in the rat.
Guideline:	OECD TG 407
GLP:	No
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / $\geq 95\%$
Species / Strain:	Rat / SPF Wistar
Doses / No. of animals:	0, 250, 1 500 or 10 000 ppm corresponding to 0, 27, 156 and 914 mg/kg bw/day for males and 0, 27, 166 and 841 mg/kg bw/day for females, respectively / 10/sex/dose
Administration:	Orally, via the diet
Exposure time:	33 days
Statistics:	One-way Analysis of Variance (ANOVA), Dunnett's test (for normally distributed data), Steel-test (for non-normally distributed data) on body weight, organ weights and clinical laboratory data.

Materials and Methods:

The experimental procedures followed the guideline in principle. However, eye examinations (not mandatory) were conducted in all animals during the acclimatization period and at day 28. Blood and urine samples were collected on study day 28. Animals were sacrificed on study day 33. The required organs and tissues were weighed (except epididymidis) and fixed for histopathological examination. However, full histopathology according to guideline was not performed, **see comments**.

Results:

General observations

There were no spontaneous **deaths** or signs of ill health during the experiment. **Food consumption** was reduced in the 10 000-ppm group during the whole experimental period (reductions of 75% and 71% for males and females, respectively during the first study week) and mean **body weights** of both males and females in this group were consistently lower than controls from day five and throughout the study.

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Table B.6.3.1.1-1: Mean body weights (g) and food consumption (g/animal/day), 28-day feeding study with difenoconazole in rats.

Dose level (ppm)	males				Females			
	0	250	1500	10000	0	250	1500	10000
Body weight								
Day 26	283	267	286	164** ↓ 42%	182	179	175	116** ↓ 36%
Food consumption								
Days 22-28	25	23	24	16	18	18	18	10

No statistical tests were performed on food consumption due to the low number of observations (n=2 cages per dose group). **Ophthalmoscopic** examination of the eyes revealed no treatment-related findings.

Haematology, clinical chemistry and urinalysis

The haematology data showed significant reductions in **Hb, haematocrit, MCV, MCH** and **thromboplastin time (PT)**, primarily in animals of the high dose group (10 000 ppm). Also, high-dose females had significantly reduced **platelet count** and increased **reticulocyte count**. The haematological effects were of **dose-response** nature.

Table B.6.3.1.1-2: Haematology, 28-day feeding study with difenoconazole in rats (day 28, means).

Dose level (ppm)	Males				Females			
	0	250	1500	10 000	0	250	1500	10 000
haemoglobin (mmol/l)	10.1	9.9	9.7	9.5*	10.0	9.6*	9.8	9.1*
Haematocrit (%)	45	44	44	43*	45	43*	44	41*
mean corpuscular volume (fl)	51.9	50.9	51.0	48.6*	51.1	51.2	50.7	47.2*
mean corpuscular haemoglobin (fmol)	1.16	1.13	1.12	1.08*	1.14	1.14	1.12	1.04*
reticulocyte count (%)	3.8	4.1	4.0	4.2	2.5	2.5	2.5	4.2*
platelet count (G/l)	1047	1036	1076	923	1051	1153	1032	928* ↓ 12%
thromboplastin time PT (sec)	12.5	12.2	11.8* ↓ 6%	11.5* ↓ 8%	12.6	12.1*	12.1*	11.5*

* = p < 0.05, Dunnett test or Steel test

It should be noted however, that although several haematological parameters were significantly altered (primarily in the 10 000-ppm-dose group) the majority of the recorded values are still **within the normal ranges** in literature and within laboratory reference values of Wistar rats of similar age (7-9 weeks). **Thus, after scrutinizing the results, the only parameter deviating from normal range is the thromboplastin time (PT) for males in the two highest dose groups, which is shorter than the laboratory reference values for males of similar age (12.5-14.7 s).** Since the partial thromboplastin time (ptt) was unaffected in all animals this finding is not considered to be adverse, but may indicate an impact on liver function.

The **clinical biochemistry** data showed significant results, primarily in the 10 000-ppm dose group, with respect to **cholesterol, liver enzymes** and **sodium**. Also, **protein electrophoresis** revealed treatment-related effects at all dose levels.

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Table B.6.3.1.1-3: Clinical chemistry, 28-day feeding study in rats with difenoconazole (day 28, means)

Dose level (ppm)	Males				Females			
	0	250	1500	10000	0	250	1500	10000
cholesterol (mmol/l)	2.15	2.26	2.46	7.10* ↑ 230%	2.16	2.52	2.72	7.44* ↑ 428%
ALAT, alanine aminotransferase, (ukat/l)	0.78	0.86	0.78	0.92	0.80	0.71*	0.74	0.82
ASAT, aspartate aminotransferase (ukat/l)	1.59	1.59	1.53	1.76*	1.69	1.58*	1.59	1.77
ALP, alkaline phosphatase (ukat/l)	5.39	5.37	5.87	7.36*	4.00	2.94	3.58	6.71* ↑ 68%
GT, γ-glutamyl transferase (nkat/l)	77.18	77.02	67.35	135.4*	88.52	90.35	92.52	153.4*
absolute albumin (g/l)	35.0	36.8	37.8*	40.4*	40.4	40.9	40.7	43.9*
relative^a albumin	0.544	0.585*	0.600*	0.655*	0.632	0.638	0.626	0.662*
relative sum of β-globulins	0.243	0.215*	0.192*	0.178*	20.8	20.7	20.4	19.3
relative γ-globulin	0.017	0.019	0.021	0.015	2.4	1.9	2.3	1.7*
A/G ratio	1.20	1.41*	1.51*	1.90*	1.73	1.78	1.67	1.96*

* = p < 0.05, Dunnett test or Steel test, ^arelative values based on 1

The **Cholesterol increase** in high-dose animals of both sexes was above laboratory reference values and indicates an effect on fat metabolism in hepatocytes. However, this finding was not accompanied by any significant histological findings. (The **ALAT** values in females were higher than the given reference values from the laboratory (0.21-0.63 µkat/l) in all groups, including controls.) All values recorded for **ASAT** are within laboratory tolerance limits. Increase in **ALP** and **GT** indicates affection on liver and cholestasis. However, reference values for **GT** were not given by the laboratory, and only in 10 000-ppm females was the **ALP** increase above the laboratory reference value. Again, these findings were not supported by any significant liver findings histologically.

The significance of the **dysproteinemia revealed by electrophoresis is uncertain**. There is no clear dose-response and results from total protein analyses (g/l) reveal no significant change in treated animals as compared to controls. **Relative albumin** levels are elevated as compared to controls in treated males and in high-dose females. The levels are above laboratory reference values for males of dose groups 1 500 and 10 000 ppm and for females of dose groups 250 and 10 000 ppm. **Absolute albumin** levels (g/l) are above laboratory reference for 10 000-ppm males and females. **Hyperalbuminemia** in general could be due to dehydration and there is no indication that albumin synthesis in liver is impaired.

Urinalysis displayed increased ketone body formation in treated males and in high-dose females, probably explained by reduced food consumption (starvation) in these animals.

Pathology, organ weights and histopathology

Macroscopic examinations at the end of the treatment period (day 33) did not reveal any treatment-related findings. Absolute **organ weights** were reduced for all tissues but the adrenals in the 10 000 ppm-males, with the highest reduction found in thymus (49%). In the 10 000-ppm-females, absolute organ weights were reduced in all tissues but the liver and thyroid, with the largest reduction for the ovaries (56%). **Liver weight** was

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unaffected in treated females whereas treated males showed increased (at 1 500 ppm) and decreased (at 250 and 10 000 ppm) absolute weight.

Table B.6.3.1.1-4: Organ weights, 28-day feeding study in rats with difenoconazole (day 33, means)

dose level (ppm)		Males				Females			
		0	250	1500	10000	0	250	1500	10000
body (carcass)	absolute ^a	302.9	261.7** ↓ 14%	291.1	169.0** ↓ 44%	192.6	187.8	181.2* ↓ 6%	115.6** ↓ 40%
adrenals	absolute	0.06	0.06	0.07	0.06	0.07	0.08	0.07	0.05** ↓ 29%
	relative/body	0.2	0.2	0.2	0.3**	0.4	0.4	0.4	0.4*
brain	absolute	2.02	1.93	1.95	1.74** ↓ 14%	1.84	1.79	1.79	1.63** ↓ 11%
	relative/body	6.7	7.4	6.7	10.4**	9.6	9.6	9.9	14.2**
heart	absolute	0.89	0.81	0.93	0.54** ↓ 39%	0.66	0.65	0.62	0.43** ↓ 35%
	relative/body	2.9	3.1	3.2	3.2	3.4	3.4	3.4	3.7
kidneys	absolute	2.04	1.83	2.15	1.28** ↓ 37%	1.33	1.26	1.22* ↓ 8%	0.96** ↓ 35%
	relative/body	6.7	7.0	7.4*	7.6**	6.9	6.7	6.7	8.3**
liver	absolute	12.5	10.0** ↓ 20%	14.6** ↑ 17%	10.7* ↓ 14%	7.9	8.4	8.6	8.3
	relative/body	41.3	38.4	50.2**	63.4**	41.1	44.5	47.3**	71.6**
spleen	absolute	0.89	0.67** ↓ 25%	0.81	0.55** ↓ 38%	0.67	0.66	0.68	0.39** ↓ 42%
	relative/body	2.9	2.6	2.8	3.3	3.5	3.5	3.7	3.3
testes / ovaries	absolute	3.18	3.02	3.37	2.62** ↓ 18%	0.09	0.09	0.08	0.04** ↓ 56%
	relative/body	10.5	11.6	11.6	15.6**	0.5	0.5	0.5	0.3**
thymus	absolute	0.53	0.50	0.61	0.27** ↓ 49%	0.49	0.50	0.47	0.26** ↓ 47%
	relative/body	1.7	1.9	2.1	1.6	2.5	2.7	2.6	2.3
thyroids	absolute	0.021	0.02	0.02	0.016** ↓ 24%	0.02	0.02	0.02	0.02
	relative/body	0.1	0.1	0.1	0.1*	0.1	0.1	0.1	0.1*

^a All absolute weights in grams

^b Relative organ weights = % of body weight (g/g); * = $p \leq 0.05$, ** = $p \leq 0.01$, Dunnett test or Steel test

It should be noted that despite the marked weight reduction in several organs, histopathology did not reveal any changes that were considered treatment-related by the reporting laboratory or the notifier.

Table B.6.3.1.1-5: Histopathology findings, 28-day feeding study in rats with difenoconazole (incidence at day 33)

dose level (ppm)		Males				Females			
		0	250	1500	10000	0	250	1500	10000
<u>kidneys</u>	No. exam.	1	0	1	0	0	0	0	0
pelvic dilatation		1	-	0	-	-	-	-	-
<u>liver</u>	No. exam.	10	10	10	10	10	10	10	10
Kupffer cell proliferation		9	7	10	6	10	10	9	4
single cell necrosis		4	2	2	4	1	0	2	5
multiple cell necrosis		0	0	1	0	0	0	0	0
hepatocellular vacuolation		0	3	1	2	0	0	0	0
<u>testes</u>	No. exam.	1	1	0	0	-	-	-	-
tubular atrophy		1	1	-	-	-	-	-	-
aspermia		1	1	-	-	-	-	-	-

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It should be noted that **histopathological examination was not performed on testes or ovaries** from animals of the highest (10 000 ppm) dose group where weight reductions were 18 and 56%, respectively.

Conclusion:

The **NOAEL** was considered to be **1 500 ppm** (corresponding to 156 and 166 mg/kg bw for males and females, respectively) based on reduction in body-, carcass and organ weights in the highest (10 000 ppm) dose group. A **LOEL** was set to **250 ppm** due to effects on carcass- and organ weights, changes in RBC parameters, thromboplastin time (PT) and findings of dysproteinemia revealed by electrophoresis. The **NOEL** was **<250 ppm**.

Comments:

Deviations from the OECD TG No 407 (1995).

The date of necropsy was changed to day 33 from day 28 for technical reasons, and the study was performed without surveillance of a Quality Assurance Unit (comments from author of original report).

The dose intervals in the present study are somewhat larger (6 to 7 fold) than recommended (2 to 4 fold) in the TG, and the histopathological examination is not complete as the TG requires full histopathology carried out on preserved tissues in all control animals and in all animals of the highest dose group. The required tissues were preserved but only the livers (from all groups) and gross lesions were histopathologically examined.

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B.6.3.1.2 Oral 90-day toxicity (rat)

Reference I:	Suter, P (1986b). 13-week oral toxicity (feeding) study with CGA 169374 in the rat.
Guideline:	OECD TG 408 (1981) There was some deviation from the revised version (1998). See comments.
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / 94.5%.
Species / Strain:	Rat / SPF Wistar (outbred KFM-Han)
Doses / No. of animals:	0, 40, 250 or 1 500 ppm , corresponding to 0, 3.3, 20 and 121 mg/kg for males and 0, 3.5, 21 and 129 mg/kg for females / 10/dose/sex. An additional 10 animals/sex in satellite groups (0 and 1500 ppm) continued on control diet for a 4-week recovery period after the exposure period.
Administration:	Orally, via the diet
Exposure time / Duration:	13 weeks Satellite groups (0 and 1500 ppm) for an additional 4-week recovery period.
Statistics:	One-way analysis of variance (ANOVA), Dunnett's test (normally distributed data), Steel's test (non-normally distributed) on body weights, food and water consumption, organ weights and clinical laboratory data.

Materials and Methods:

The doses were selected based on the results of a 28-day study (*Suter, 1986a*), in which treatment-related effects were seen at 250 and 1500 ppm and the maximum tolerated dose (MTD) was exceeded at 10 000 ppm.

Results:

General observations

There were no **deaths** or remarkable **clinical signs** in the test. **Body weights** in 1500 ppm animals were significantly lower than control animals from week five and throughout the study. **Food consumption** in the males and females of this group was 9-13% and 10-15% lower than controls from week 5 and 9, respectively.

Water consumption was also reduced in these males (up to 17%) and females (up to 25%) during the treatment phase. Food consumption by controls and 1500 ppm animals was similar by the end of the recovery period.

However, the statistics on food and water consumption are made on a small number of samples (2-4 cages) and should be interpreted with caution.

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Table B.6.3.1.2-1: Body weights, food and water consumption, 13-week feeding study in rats with difenoconazole (means)

Dose level (ppm)	Males				Females			
	0	40	250	1500	0	40	250	1500
Body Weight (g)								
Week 13	433	443	425	375** ↓ 13%	249	240	245	225** ↓ 10%
Week 17 (end of recovery)	456	-	-	413 ↓ 9%	254	-	-	243 ↓ 4%

* = $p < 0.05$, ** = $p < 0.01$, Dunnett test or Steel test

Hearing tests and **ophthalmoscopic** examination of the eyes revealed no treatment-related findings.

Haematology, clinical chemistry and urinalysis

In **males** of the highest dose group (1 500 ppm) **erythrocyte, leukocyte and thrombocyte counts** were lower than in controls at weeks 13 and/or 17. However, the alterations in these blood parameters are within laboratory reference ranges and there is no obvious dose response effect.

Table B.6.3.1.2-2: Haematology, 13-week feeding study in rats with difenoconazole. Means at weeks 13 and 17 (after recovery)

Dose level (ppm)	Males						Females					
	0	40	250	1500	0	1500	0	40	250	1500	0	1500
	study week						13	13	13	13	17	17
erythrocyte count (T/l)	9.8	9.8	9.6	9.5* ↓ 3%	10.2	9.9* ↓ 3%	8.8	8.8	8.8	8.8	8.8	9.0
haemoglobin (mmol/l)	10.0	10.0	9.7* ↓ 3%	9.8	10.3	10.2	9.6	9.4	9.6	9.5	10.0	9.9
haematocrit (%)	44	43	42	43	45	45	42	41	41	42	43	44
leukocyte count (G/l)	8.3	7.4	7.2	7.0* ↓ 16%	7.6	7.2	5.1	5.4	5.4	5.9	5.5	5.8
platelet count (G/l)	953	895	911	838* ↓ 12%	952	928	950	976	989	888	992	1014

* = $p < 0.05$, Dunnett test or Steel test

There were also significant changes in several **biochemistry parameters**. No value however, was beyond the respective tolerance limits provided by the study laboratory for untreated Wistar rats of similar age.

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Table B.6.3.1.2-3: Clinical chemistry, 13-week feeding study in rats with difenoconazole. Means at weeks 13 and 17 (after recovery)

Dose level (ppm) Study week	Males						Females					
	0	40	250	1500	0	1500	0	40	250	1500	0	1500
	13	13	13	13	17	17	13	13	13	13	17	17
urea (mmol/l)	6.62	6.73	6.61	7.30* ↑ 10%	6.15	6.85* ↑ 11%	8.02	7.64	7.88	7.47	8.27	7.80
GT, γ-glutamyl transferase (nkat/l)	44.4	48.3	51.8	59.4* ↑ 34%	64.1	59.7	61.2	63.3	67.7	66.2	63.9	61.2
ALP, alkaline phosphatase (ukat/l)	2.55	2.60	2.81	3.52* ↑ 38%	2.48	2.81* ↑ 13%	1.14	1.27	1.21	1.69* ↑ 48%	1.07	1.27
protein (g/l)	71.3	72.1	71.1	68.7* ↓ 4%	69.9	67.7	73.3	73.1	73.1	71.7	70.9	67.7* ↓ 4%
Albumin (g/l)	35.7	36.9	37.5* ↑ 5%	37.7* ↑ 6%	35.7	36.0	42.8	43.5	41.4	43.0	40.5	37.3* ↓ 8%
A/G ratio	1.01	1.05	1.12	1.23* ↑ 22%	1.05	1.15	1.42	1.47	1.32	1.51	1.34	1.23

* = p < 0.05, Dunnett test or Steel test

The quantitative and qualitative **urinalysis** revealed no toxicologically significant effects.

Pathology, organ weights and histopathology

Analysis of **organ weights** of the animals sacrificed after 13 weeks on test revealed significantly **reduced carcass** weights and **increased liver weights** in the 1 500 ppm animals. These differences were not apparent among the animals sacrificed at the end of the recovery period (at week 17). The difference in the relative organ-to-body weight ratios is most likely attributed to the reduced carcass weights.

Table B.6.3.1.2-4: Organ weights, 13-week feeding study in rats with difenoconazole - means at weeks 13 and 17 (after recovery)

Dose level (ppm) Study week		Males						Females					
		0	40	250	1500	0	1500	0	40	250	1500	0	1500
		13	13	13	13	17	17	13	13	13	13	17	17
body (carcass)	absolute ^a	403.2	415.1	394.4	346.6** ↓ 14%	424.9	387.6	232.3	219.2	219.1	201.2** ↓ 13%	239.2	227.9
brain	absolute	2.07	2.10	2.05	2.03	2.08	2.11	1.92	1.94	1.94	1.89	1.93	1.98
	relative	0.5	0.5	0.5	0.6**	0.5	0.6*	0.8	0.9	0.9	1.0**	0.8	0.9*
heart	absolute	1.17	1.11	1.17	1.04* ↓ 11%	1.10	1.09	0.78	0.75	0.80	0.72	0.75	0.72
	relative	0.3	0.3	0.3	0.3	0.3	0.3*	0.3	0.3	0.4	0.4	0.3	0.3
kidneys	absolute	2.37	2.30	2.42	2.20	2.35	2.15* ↓ 9%	1.35	1.37	1.48	1.28	1.45	1.47
	relative	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.7*	0.6	0.6	0.6
liver	absolute	10.7	11.4	11.7	12.6** ↑ 18%	10.7	10.4	6.4	6.4	7.2	7.8** ↑ 22%	7.6	7.0
	relative	2.7	2.8	3.0* ↑ 11%	3.6**	2.5	2.7	2.8	2.9	3.3** ↑ 18%	3.9**	3.2	3.1
testes/ovaries	absolute	3.43	3.43	3.63	3.45	3.48	3.39	0.09	0.09	0.10	0.10	0.10	0.10
	relative	0.9	0.8	0.9	1.0*	0.8	0.9	0.04	0.04	0.05	0.05**	0.04	0.04

^a All absolute weights in grams

^b Relative organ weights = % of body weight (g/g); * = p ≤ 0.05, ** = p ≤ 0.01, Dunnett test or Steel test

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Macroscopic and **microscopic** examinations at the end of the treatment period did not reveal any treatment-related findings.

Table B.6.3.1.2-5: Selected necropsy findings, 13-week feeding study in rats with difenoconazole (incidence)

Dose level (ppm)	Males						Females					
	0	40	250	1500	0	1500	0	40	250	1500	0	1500
Study week	13	13	13	13	17	17	13	13	13	13	17	17
no remarkable findings	4	3	5	4	9	9	3	9	7	9	10	10
<u>kidney</u>	0	1	0	0	0	0	0	0	1	0	0	0
pelvic dilation												
<u>liver</u>	4	3	2	6	0	0	5	0	0	1	0	0
accentuated lobular pattern												
<u>lymph node, mandibular</u>	2	2	2	0	0	0	1	1	1	0	0	0
enlarged												
<u>thymus</u>	1	0	1	0	0	0	1	0	0	0	0	0
several dark red foci												

Note: **10 animals in each group**

Table B.6.3.1.2-6 : Selected histopathology findings, 13-week feeding study in rats with difenoconazole (incidence)

dose level (ppm)		Males						Females					
		0	40	250	1500	0	1500	0	40	250	1500	0	1500
study week		13	13	13	13	17	17	13	13	13	13	17	17
<u>Heart</u>	N ex.	10	0	0	10	0	10	10	0	0	10	0	10
Myocarditis		1	-	-	4	-	2	1	-	-	0	-	0
<u>Liver</u>	N ex.	10	10	10	10	10	10	10	10	10	10	10	10
bile duct proliferation		5	1	1	2	5	0	4	0	0	0	2	0
Congestion		2	3	1	4	3	4	8	3	4	6	5	1
fatty change		3	2	2	4	0	0	0	0	0	0	0	0
Fibrosis		9	10	10	10	10	10	10	10	10	10	10	10
Kupffer cell proliferation		0	0	0	0	2	0	0	1	1	0	0	1
Necrosis		1	1	0	2	4	0	1	0	2	1	0	3
<u>Testes</u>	N ex.	10	0	0	10	0	10	-	-	-	-	-	-
Calcification		0	-	-	0	-	1	-	-	-	-	-	-
Fibrosis		0	-	-	0	-	1	-	-	-	-	-	-
tubular atrophy		0	-	-	0	-	1	-	-	-	-	-	-
<u>Uterus</u>	N ex.	-	-	-	-	-	-	10	0	1	10	0	10
dilation		-	-	-	-	-	-	4	-	1	3	-	2

Summary:

Exposure to 1 500 ppm difenoconazole via the diet resulted in reduced **food and water intakes** and reduced **body-, carcass- and organ weights** in animals of both sexes. Alterations in blood and biochemistry parameters were within normal ranges for Wistar rats. Reduced food and water consumption in treated animals probably explain part of the observed variations. **Liver** weight was increased in the 1 500-ppm group (18 and 22%, respectively for males and females); however, this change was not accompanied by any significant macroscopic or microscopic changes. Histologically, congestion and bile duct proliferation might explain the raise in the liver specific enzymes ALP and γ -GT in the high-dose animals, but the liver effects are not considered to be adverse since the enzyme values are within normal ranges and the histopathological findings were observed also in controls. Furthermore, after 4 weeks of recovery the liver changes were reversible.

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Conclusion:

The **NOAEL** was set to **250 ppm** (corresponding to 20 and 21 mg/kg bw/day in males and females, respectively) based on reductions in food and water consumption and in body-, carcass- and organ (heart) weight observed at 1500 ppm. The increased relative liver weights observed at 250 ppm (11 and 18%, respectively in males and females) was considered to be an adaptive change since this finding was no longer apparent following a 4-week recovery period. The **NOEL** of the study was **40 ppm** (corresponding to 3.3 and 3.5 mg/kg bw/day in males and females, respectively) based on increased relative liver weight in both sexes and on increased blood albumin concentrations in males at 250 ppm.

Comments:

Deviations from the OECD TG No 408 (1998).

The TG proposes that a pair-fed control group is used when a test substance causes reduced dietary intake, to distinguish between reductions due to palatability and toxicological alterations in the test model. The reduced food consumption in higher dose groups was evident already in the 28-day study above, performed by the same author and might thus have been considered for the present study. Moreover, the dose intervals are somewhat too large (6-fold) as compared to the recommended 2-4 fold. Also, the wet weights of the epididymidis, uterus and spleen were not recorded and histopathology of mammary gland, skin and salivary glands was not performed, and sensory reactivity to external stimuli was not examined, which deviates from the above mentioned TG.

B.6.3.1.3 Oral 90-day toxicity (rat)

Reference II:	Cox RH (1987a). CGA 169374: Subchronic Toxicity/[Metabolism]Study in rats.
Guideline:	US-EPA FIFRA 83-5 (82-1). There were some deviations from the OECD TG No. 408 (1998), see comments .
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / 94.5%
Species / Strain:	Rat / CRL:CD(SD)®
Doses / No. of animals:	0, 20, 200, 750, 1 500 or 3 000 ppm, corresponding to 0, 1.3, 13, 51, 100 and 210 mg/kg/day for males and 0, 1.7, 17, 66, 130 and 270 mg/kg/day for females / <u>Treatment groups:</u> 15/sex, <u>Control group:</u> 20/sex , see comments
Administration:	Orally, via the diet
Exposure time / Duration:	90 days
Statistics:	Analysis of Variance (ANOVA), Dunnett's post hoc test for organ weights and body weight gain, food consumption and clinical pathology (except cell morphology) Trends were analyzed by the Terpstra-Jonckheere non-parametric test.

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Materials and Methods:

Ten animals/sex/group were sacrificed after 13 weeks on trial (at day 90), and the remaining animals were removed from the study at day 119.

Results:

General observations

One female in the 1 500-ppm dose group was found **dead** during week 10 (the cause of death could not be established histopathologically and this death was therefore considered incidental) and one male of the 200- ppm dose group was accidentally killed. There were **sporadic signs of discomfort or ill-health** such as hunched appearance, thinness, soft feces and chromodacryorrhea, however **without a dose response pattern**.

No statistical analyses were performed on the body weights. They tended however, to be lower than control in 3 000-ppm males and in females from 200 ppm, throughout the study. There were **significant negative trends** in body weight **gain** from initiation to termination for both males and females. In males, the weight gain of the 3 000 ppm males was significantly lower than that of the control males, while for females, all groups except for the 20 ppm females had **lower** body weight **gains** than the control females. In males of the 3 000-ppm group, the **body weight gain** was significantly lower than controls. For females, all groups except the 20-ppm females had lower body weight gains than the controls.

Table B.6.3.1.3-1: Body weights and body weight gain, 3-month feeding study in rats with difenoconazole (means)

	Males						Females					
Dose level (ppm)	0	20	200	750	1500	3000	0	20	200	750	1500	3000
Body Weight (g)												
Week 13	532.3	530.6	532.4	516.7	518.9	478.9 ↓10%	294.9	290.7	278.2 ↓6%	275.2 ↓7%	261.5 ↓11%	237.1 ↓20%
Body Weight Gain (g)												
during weeks 0-13 ^a	336.9	339.4	336.4	325.3	325.6	287.0* ↓15%	149.8	146.1	134.0* ↓11%	131.8* ↓12%	115.8* ↓23%	93.3* ↓38%
Trend ^b for change	significant (p< 0.05) negative trend						significant (p< 0.05) negative trend					
^a includes only animals which survived to termination												
^b Terpstra-Jonckheere Test for Trend												
* = p < 0.05, Dunnett test following Terpstra-Jonckheere												

The absolute food consumption differences were not statistically significant. However, there was a significant **negative trend** (p<0.05) across the **female** groups.

Ophthalmoscopic examination of the eyes revealed no treatment-related findings.

Haematology, clinical chemistry and urinalysis

Treatment-related differences between the control and treated animals in the **haematology parameters** were decreases in red cell mass (RBC, haemoglobin, haematocrit) in males from 750 ppm and in females from 1 500

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ppm. However, all values recorded are within normal ranges found in the literature for rats (Falkmer and Waller, 1994).

Table B.6.3.1.3-2: Haematology, 3-month feeding study in rats with difenoconazole (means at week 13)

dose level (ppm)	Males						Females					
	0	20	200	750	1500	3000	0	20	200	750	1500	3000
erythrocyte count (mi/μl)	8.39	8.23	8.25	7.70*	7.59*	7.76*	7.83	8.01	8.01	7.68	7.13*	6.97*
trend ^a for erythrocytes	significant (p < 0.05) negative trend						significant (p < 0.05) negative trend					
haemoglobin (g/dl)	15.8	15.5	15.5	15.0	15.2	15.1	15.2	15.3	15.2	15.2	14.4*	14.0*
trend for haemoglobin	no trend						significant (p < 0.05) negative trend					
haematocrit (%)	45.7	44.6	44.5	41.4*	40.8*	42.2*	43.3	44.6	44.4	42.9	38.2*	37.1*
trend for haematocrit	significant (p < 0.05) negative trend						no trend					

^aTerpstra-Jonckheere Test for Trend

* = p < 0.05, Dunnett test following Terpstra-Jonckheere

There were statistically significant differences between the control and treated animals in some **clinical chemistry parameters**, which suggest altered hepatocellular metabolism and/or secondary nutritional factors. However, the levels of **liver specific enzymes** were not increased in treated animals. Neither was there a dose-response trend. **Globulin** levels were significantly reduced in treated males in the 750 and 1 500-ppm groups but the total protein level (g/dl) was unaffected for these males. There were no internal reference values given by the laboratory to compare the urea nitrogen- and the bilirubin values with. The biological significance of the differences found in serum chemistry parameters is considered to be negligible.

Table B.6.3.1.3-3: Clinical chemistry, 3-month feeding study in rats with difenoconazole (means at week 13)

dose level (ppm)	Males						Females					
	0	20	200	750	1500	3000	0	20	200	750	1500	3000
urea nitrogen (mg/dl)	13	14	15	15	16*	18*	14	14	13	15	14	16
total bilirubin (mg/dl)	0.11	0.11	0.07	0.04	0.06	0.02*	0.13	0.15	0.10	0.08	0.05*	0.04*
globulin (g/dl)	3.0	2.9	2.9	2.7*	2.7*	2.7	2.9	2.8	2.8	2.9	2.7	2.8

* = p < 0.05, Dunnett test

There was an increasing incidence of **ketones in the urine** of the high-dose (3 000 ppm) males, possibly due to reduced food intake (starvation).

Pathology, organ weights and histopathology

Macroscopic examinations at the end of the treatment period did not reveal any treatment-related findings.

Livers of all females and of males exposed to 750 ppm or more were macroscopically **unremarkable**. **Absolute liver weight** was increased in animals of both sexes from 750 ppm. **Carcass weight** was significantly lower than controls in females from 1 500 ppm, whereas it tended to be lower than controls in the corresponding males but the difference was not significant. **Absolute adrenal weight** was decreased in males exposed to 750 and 3 000 ppm (17%, respectively), however, there was no clear dose-response as the adrenal weight of the 1 500-ppm group was unaffected.

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Table B.6.3.1.3-4: Terminal body- and organ weights, 3-month feeding study in rats with difenoconazole (means at week 13)

		Males						Females					
dose (ppm)	level	0	20	200	750	1500	3000	0	20	200	750	1500	3000
body (carcass)	abs. ^a	481.2	499.3	505.5	497.4	477.3	449.2	264.0	268.4	255.8	257.0	246.7* ↓ 7%	200.2* ↓ 24%
Liver	abs.	12.7	13.5	13.3	16.0* ↑ 26%	16.6* ↑ 31%	16.5* ↑ 30%	7.1	8.3	8.3	8.8* ↑ 24%	9.0* ↑ 27%	9.9* ↑ 39%
	rel.	2.64	2.71	2.62	3.19* ↑ 21%	3.48* ↑ 32%	3.70* ↑ 40%	2.69	3.08	3.26* ↑ 21%	3.45* ↑ 28%	3.64* ↑ 37%	4.50* ↑ 67%
testes / ovaries	abs.	5.15	5.34	5.17	5.11	5.08	5.13	0.10	0.10	0.11	0.10	0.10	0.09

^a All absolute weights (abs.) in grams; relative (rel.) organ weights = % of body weight (g/g)
* = p ≤ 0.05, Dunnett test

Histopathology revealed a treatment-related increase of **hepatocellular enlargement** in rats of the two highest dose groups (1 500 and 3 000 ppm). The other liver findings showed no dose-response.

Table B.6.3.1.3-5: Liver histopathology findings, 3-month feeding study in rats with difenoconazole (terminal sacrifice and late accidental deaths, incidence)

Dose level (ppm)	Males						Females					
	0	20	200	750	1500	3000	0	20	200	750	1500	3000
liver n ex.	10	10	10	10	10	10	10	10	10	10	10	10
diffuse hepatocell. enlargement	1	1	0	1	10	10	0	0	1	1	4	10
- minimal	1	1	-	1	9	2	-	-	1	1	4	3
- slight	0	0	-	0	1	8	-	-	0	0	0	7
bile duct hyperplasia	1	1	0	1	0	2	1	1	1	0	0	0
congestion	0	0	2	1	1	2	4	5	4	3	3	3
focal mononuclear infiltration	10	10	10	10	10	10	10	10	10	10	10	10
↑ extramedullary haematopoiesis	0	1	1	1	1	2	3	4	5	5	5	4
necrosis	1	0	0	1	1	0	1	0	1	0	2	0
nonsuppurative pericholangitis	10	10	10	10	10	10	10	10	7	9	7	9

Other histopathological changes that were noted did not give any indication of a treatment-related association.

Summary:

Toxicity was demonstrated in dose groups 750, 1 500 and 3 000 ppm by discomfort, decreased body weights and food consumption as well as decreased red cell mass. There was a treatment-related reduction in carcass weight and an increase in liver weights (24-39%) accompanied by hepatocellular enlargement in these groups.

Conclusion:

The **NOAEL** was set to **750 ppm** (corresponding to 51 and 66 mg/kg bw in males and females, respectively) based on significant reductions in body weight gain correlated with lower body weights (>10%) observed in 1500-ppm females as compared with controls. The **NOEL** was set to **20 ppm** (corresponding to 1.3 and 1.7 mg/kg bw in males and females, respectively) based on reduced body weight gain (11%) in females of the 200-ppm dose group.

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Comments:

The report title suggests that there was a metabolism component to this study. No metabolism component was performed, i.e., it was cancelled by protocol amendment. Only **ten** animals/sex/group were sacrificed and sampled after 13 weeks on trial. Results from the animals removed from the study at day 119 (referred to as the remaining animals) were not reported.

Deviations from the OECD TG No. 408 (1998)

The wet weights of uterus and thymus were not recorded or presented and a mammary gland sample should have been preserved and examined histologically to comply with the requirements of the above TG.

B.6.3.1.4 Oral 90-day toxicity (mouse)

Reference:	Cox RH (1987b). CGA 169374: Subchronic Toxicity/[Metabolism]Study in mice.
Guideline:	US-EPA FIFRA 83-5. The experimental procedures followed the OECD TG No. 408 in principle. However, there were some deviations from the revised version (1998).
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / purity 94.5%
Species / Strain:	Mouse / CD-1®(ICR)
Doses / No. of animals:	0, 20, 200, 2 500 (7 500 or 15 000) ppm , corresponding to 0, 3.3, 34 and 440 mg/kg/day for males and 0, 4.6, 45 and 640 mg/kg/day for females in the 20, 200 and 2500 ppm dose groups, respectively. No data are given by the notifier regarding the doses per kg bw for the two highest dose levels. / Treatment groups:15/sex, Control group:20/sex
Administration:	Orally, via the diet
Exposure time / Duration:	3 months (13 weeks)
Statistics:	The National Cancer Institute Package for cumulative survival, Analysis of Variance (ANOVA) and Dunnett's post hoc test for organ and body weights, food consumption and clinical pathology (except cell morphology), Levene's test for homogeneity of variances; heteroscedastic variances were transformed to achieve homogeneity

Materials and Methods:

Ten animals/sex/group were sacrificed after 13 weeks on trial (at day 90), and the remaining animals were retained on study for a metabolism study according to the original report, **see comments**.

Results:

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General observations

Most animals of the 7 500 and 15 000-ppm dose groups **died** during the first week of treatment, and by the third treatment week, all animals of these dose groups but 2 males of the 7 500-group were dead. These 2 males were then sacrificed for humane reasons. **Thus, the highest surviving dose was 2 500 ppm.**

Table B.6.3.1.4-1: Mortality, 3-month feeding study in mice with difenoconazole.

dose level (ppm)	Males						Females					
	0	20	200	2500	7500	15000	0	20	200	2500	7500	15000
died/moribund sacrifice on days 3-4	0	0	0	0	0	0	0	0	0	0	7	13
days 5-7	0	0	0	0	2	11	0	0	0	0	8	2
days 8-11	0	0	0	0	5	4	0	0	0	0	0	0
days 12-14	0	0	0	0	2	0	0	0	0	0	0	0
days 15-18	0	0	0	0	4	0	0	0	0	1	0	0
days 19-21	0	0	0	0	2 ^a	0	0	0	0	0	0	0
week 5	0	0	0	0	0	0	0	0	1	0	0	0
week 7	1	0	0	0	0	0	0	0	0	0	0	0
week 10	0	1	0	0	0	0	0	0	0	0	0	0
week 13	0	0	0	0	0	0	0	0	1	0	0	0
cumulative	1/20	1/15	0/15	0/15	15/15	15/15	0/20	0/15	2/15	1/15	15/15	15/15

^aanimals not moribund but sacrificed/removed from study

The **cause of death** was not evident in the above mentioned animals, however the deaths were accompanied by **clinical signs** in the males (all at 7 500 ppm and one at 15 000 ppm) such as thinness, hunched posture, langour and tremors. The animals which survived (males) to the first **body weight** measurement (at week 1) had lost approximately 25% of their initial weight. The 7 500- and 15 000-ppm males which survived the first week consumed about 10% and 15% less feed than the control males, respectively. **Dark areas** in the stomach were noted at necropsy in 38% of the 7 500 ppm males, and in >90% of the 7500-ppm females and 15 000-ppm animals, respectively. These findings corresponded to microscopic findings of **erosion/ulceration** of the glandular stomach and/or **hyperkeratosis** in the nonglandular stomach in most of these animals. In addition, **hepatocellular enlargement** was seen in 62% of the 7500-ppm males, and in 67% of the 7 500-ppm females and 15 000-ppm animals, respectively. **Hepatocellular necrosis** was also seen in a few of the early deaths. Other histopathological changes such as erosion/ulceration of the glandular stomach, lymphoid depletion or necrosis of the spleen, lymph nodes and thymus, hypocellularity of the bone marrow were also seen but may be induced by stress.

In the remaining four study groups (0, 20, 200 and 2 500 ppm), **clinical signs** were noted at low frequencies without dose-response pattern, although **tachypnea** was seen in most 2 500-ppm females during the first study week. Body weights of the 2 500-ppm males and females tended to be lower than the control animals throughout the study, although **no statistical analyses were performed**. **Body weight gain however, was significantly lower** (21%) than controls for 2 500-ppm females through weeks 0-13, even though the food consumption for these animals tended (not statistically significant) to be higher than for controls.

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Table B.6.3.1.4-2: Body weights and body weight gain, 3-month feeding study in mice with difenoconazole (means)

Dose level (ppm)	Males				Females			
	0	20	200	2500	0	20	200	2500
Body Weight (g)								
Week 13	37.8	38.4	36.6	36.8	28.7	28.2	28.8	27.1
Body Weight Gain (g)								
Change (weeks 0-13 ^a)	8.1	9.5	8.0	7.4	6.8	6.7	6.8	5.4* ↓ 21%
Food Consumption (g/animal/week)								
Week 8	41.9	41.9	42.7	42.2	44.3	45.1	45.1	49.8
Week 13	39.6	38.9	39.4	39.5	43.6	43.2	41.8	42.4

^aincludes only animals which survived to termination

* = $p < 0.05$, Dunnett test following Terpstra-Jonckheere Test for Trend

Ophthalmoscopic examinations revealed no treatment related findings.

Haematology

There were **no treatment-related differences** between the control and treated animals in the haematology parameters. There was a statistically significant **positive trend in platelet counts** for males, but there was no trend in the females and no other findings which would indicate that this was a meaningful effect.

Pathology, organ weights, and histopathology

Analysis of organ weights of the animals revealed **increased liver weights** in 2 500-ppm males and females. A significant positive trend in **adrenal weights** in treated males and negative trend in **ovary weights** in females had no macroscopic or microscopic correlates. The reduced **heart weights** in 2 500-ppm females could be secondary to reduced body mass since heart-to-body weight ratios were not affected.

Table B.6.3.1.4-3: Organ weights, 3-month feeding study in mice with difenoconazole (means at week 13)

Dose level (ppm)		Males				Females			
		0	20	200	2500	0	20	200	2500
<u>body (carcass)</u>	absolute ^a	34.0	33.2	32.6	32.0	25.1	24.4	25.1	23.0* ↓ 8%
<u>liver with gallbladder</u>	absolute	1.48	1.58	1.62	2.70* ↑ 82%	1.22	1.21	1.36	2.08* ↑ 70%
	relative	4.35	4.75	4.99*	8.44*	4.87	4.97	5.43	9.05*
trend for abs. liver		significant ($p < 0.05$) positive trend				significant ($p < 0.05$) positive trend			
<u>heart</u>	absolute	0.18	0.19	0.18	0.17	0.13	0.14	0.13	0.12* ↓ 8%
	relative	0.53	0.59	0.56	0.52	0.53	0.56	0.54	0.51
<u>testes / ovaries</u>	absolute	0.41	0.40	0.44	0.40	0.028	0.025	0.024	0.021* ↓ 25%
	relative	1.21	1.22	1.34	1.24	0.11	0.10	0.10	0.09
trend for abs. testes/ovaries		no trend				significant ($p < 0.05$) negative trend			

^a All absolute weights in grams; relative organ weights = % of body weight (g/g)

^b Terpstra-Jonckheere Test for Trend

* = $p \leq 0.05$, Dunnett test

Macroscopic examinations revealed **liver enlargement** in 2 500-ppm males (6/10) and females (7/9).

Histopathology also confirmed treatment-related liver changes. **Hepatocellular enlargement** was seen in all 2 500-ppm animals and in nearly all 200-ppm males. **Hepatic vacuolisation** also occurred with greater

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frequency and severity in 2 500-ppm animals. The **coagulative necrosis** observed in four out of nine 2 500-ppm females were **small random foci** and the significance of this finding is uncertain. It should be noted that the activity of the **liver specific enzymes were never investigated** in this study, which makes it hard to make a definite opinion about the significance of the observed liver effects.

Table B.6.3.1.4-4: Liver histopathology findings, 3-month feeding study in mice with difenoconazole (terminal sacrifice and late accidental death, incidence)

dose level (ppm)		Males				Females			
		0	20	200	2500	0	20	200	2500
<u>liver</u>	n ex.	9	9	10	10	10	10	9	9
diffuse hepatocellular enlargement		0	1	1	10	0	0	2	9
- minimal		-	1	0	0	-	-	1	0
- slight		-	0	1	2	-	-	1	1
- moderate		-	0	0	7	-	-	0	8
- moderately severe		-	0	0	1	-	-	0	0
centrilobular hepatocellular enlargement		2	1	9	0	0	0	0	0
- minimal		1	1	8	-	-	-	-	-
- slight		1	0	1	-	-	-	-	-
<u>hepatic vacuolisation</u>		1	3	1	7	0	0	2	7
- minimal		1	3	1	0	-	-	1	6
- slight		0	0	0	6	-	-	1	1
- moderate		0	0	0	1	-	-	0	0
coagulative necrosis		1	0	0	0	0	0	0	4
focal mononuclear infiltration		1	0	1	1	1	1	0	1
foci of acute hepatitis		0	0	0	1	0	0	0	0
necrosis indiv. hepatocytes		0	0	0	1	0	0	0	0

The **other histopathological changes** noted are considered not to be related to treatment.

Table B.6.3.1.4-5: Selected histopathology findings, 3-month feeding study in mice with difenoconazole (terminal sacrifice, incidence)

dose level (ppm)		Males				Females			
		0	20	200	2500	0	20	200	2500
n of terminal sacrifices/late accidental deaths		9	9	10	10	10	10	8/1	9
<u>Heart</u>	n ex.	9	9	10	10	10	10	8	9
degenerative cardiomyopathy		0	1	1	0	0	0	0	0
focal chronic inflammation		0	0	0	1	0	0	0	0
<u>Ovary</u>	n ex.	-	-	-	-	10	10	8	9
cyst(s)		-	-	-	-	0	0	0	1

Summary:

Administration of 15 000 ppm test substance resulted in death of all animals and a dose of 7500 ppm caused death in 13/45 animals. The Maximum Tolerated Dose (MTD) was exceeded at 2500 ppm. Toxicity was demonstrated in animals exposed to 2 500-ppm test substance, by decreased carcass weights, reduced organ (heart and ovary) weights and increased liver weights accompanied by hepatocellular enlargement and hepatocytic vacuolization.

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Conclusion:

The **NOAEL** is set to **200 ppm** (corresponding to 34 and 45 mg/kg bw in males and females, respectively) based on reduced ovary weight (25%) and reduced body weight gain in females of the 2 500-ppm dose group. The increased liver weights observed in males and females of the 2 500-ppm dose group were accompanied by macroscopic liver enlargement and also enlargement, vacuolization and coagulative necrosis of the hepatocytes, which indicate that the liver findings are of adverse nature. However, analyses of clinical chemistry parameters are lacking, which makes it difficult to make a definite opinion about the significance of the observed liver effects. The **NOEL** was set to **20 ppm** (corresponding to 3.3 and 4.6 mg/kg bw in males and females, respectively).

Comments:

A study with unnecessarily high dose levels which resulted in death for many animals. The report title suggests that there was a metabolism component to this study and the results of the metabolism phase are stated to be presented in an addendum to the original report, but are not to be found.

Deviations from the OECD TG No. 408 (1998)

Blood samples were drawn from only 10 animals per sex and group (at week 13) instead of from all animals.

Biochemical determinations were not performed at all. Blood clotting time/potential was not determined.

Terminal pathology examinations were only performed on 10 animals per sex and dose instead on all animals.

The wet weights of the uterus and thymus were not recorded. The female mammary gland was not preserved or examined histologically.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

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B.6.3.1.5 28-week oral toxicity study in dogs

Reference:	O'Connor, D.J., McCormick, G.C. and Green, J.D. (1987). CGA 169374 technical: 26-week oral toxicity study in dogs.
Guideline:	EPA FIFRA 83-1, OECD TG 452 (1981). The procedure followed the OECD TG in principle but there were deviations, see comments .
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / 96.1%
Species / Strain:	Dog / purebred beagle (Marshall Farms, NY)
Doses / No. of animals:	0, 100, 1000, 3000 or 6000 ppm corresponding to 0, 3.6, 31, 97 and 160 mg/kg/day for males and 0, 3.4, 35, 111 and 204 mg/kg/day for females / 3/dose/sex
Administration:	Orally, via the diet
Exposure time / Duration:	28 weeks
Statistics:	Analysis of Variance (ANOVA) and Dunnett's post hoc tests on the quantitative variables. However, due to the low number of animals per group (n=3), results are presented and discussed in terms of meaningful differences rather than statistically significant differences.

Materials and Methods:

Physical examinations, including hearing tests were performed pre-test and during weeks 13 and 28. Eye examinations were conducted on all animals during the pre-test period and every 2-3 weeks thereafter. The eye and auditory examinations are not mandatory by the OECD TG.

Results:

General observations

There were **no deaths** in the test. All animals (except for two animals in the 100-ppm group) lost weight during the first study week. In the 6000-ppm dose group, males and females lost 15 and 18 % of their respective body weight. By week four, the 6000-ppm animals were gaining weight at rates similar to the animals in the other groups, but the initial loss was never recovered. At termination, **body weights** of 6000-ppm animals were approximately **30% lower** than the respective controls but the difference was **only** statistically **significant for males**.

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Table B.6.3.1.5-1: Body weights and body weight change, 6-month feeding study in dogs with difenoconazole (means)

males				females		
Dose level (ppm)	Body weight (kg)		Change (%)	Body weight (kg)		Change (%)
	Day 0	Week 28		Day 0	Week 28	
0	8.57	11.03	+29	7.73	9.33	+21
100	8.67	11.03	+27	7.50	9.37	+25
1000	8.20	10.27	+25	7.80	9.43	+21
3000	8.97	10.13	+13	8.10	8.47	+5
6000	8.67	7.67*	-12**	7.47	6.33	-15*
		↓ 30%			↓ 32%	

The body weight depression correlated with reductions in **food consumption**. Animals of the 6 000-ppm dose group had significantly lower food intake than control throughout the majority of the dosing period. Reductions were also noted for 1000- and 3000-ppm males, but not in the corresponding females.

Table B.6.3.1.5-2: Food consumption, 6-month feeding study in dogs with difenoconazole (g/animal/week, means)

Dose level (ppm)	Males					Females				
	0	100	1000	3000	6000	0	100	1000	3000	6000
Week 1	2012	2069	1501	1015*	260**	1406	1521	1339	1010	316**
				↓50%	↓87%					↓78%
Week 4	2706	2651	1994**	2273**	1475**	2229	2113	2319	2255	1333**
			↓26%	↓16%	↓45%					↓40%
Week 28	2186	2506	2052	2224	1430**	2199	2186	1802	2137	1611
					↓35%					

n=3 dogs per dose group at each interval

Physical/veterinary examinations, including **hearing tests** revealed no treatment-related findings. **Lenticular opacity** was recorded in all 6 000-ppm animals and in one 3 000-ppm female from study week 20.

Ophthalmoscopic examinations performed on dogs at week 11 revealed bilateral lenticular aberrations (**cataracts**) in all 6 000-ppm dogs and in one 3 000-ppm female. Subsequent examinations (every 2-3 weeks) revealed progression (increase in vacuoles, opacities and feathering of the lens suture) of the lenticular aberrations in the affected dogs. In addition, **iridic** changes were manifested in the affected females considered to have occurred from lens-induced uveitis associated with rapidly developing cataracts.

Haematology, clinical chemistry and urinalysis

Platelet counts were significantly elevated in the 6 000-ppm males, but this was considered to be incidental due to the absence of a clear dose-response. The mean value for the 6 000-ppm animals is close to the upper limit for normal range values for dogs ($150-400 \times 10^3/\text{mm}^3$).

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Table B.6.3.1.5-3: Haematology, 6-month feeding study in dogs with difenoconazole (means)

dose level (ppm)	Males					Females				
	0	100	1000	3000	6000	0	100	1000	3000	6000
platelet count (10³/mm³) wk 17	248	289	322	266	398* ↑ 60%	326	232	284	300	481
wk 28	198	329	354	262	437* ↑ 121%	350	311	324	347	460

n=3 dogs per dose group at each interval wk=week

ALP activity was elevated in the 6 000-ppm males (at week 17) and females (at termination). At termination, ALP activity in the 6 000-ppm males was not statistically higher than control, but still above the normal range for dogs in the literature (< 252 U/l). ALP activity was also elevated in the 3 000-ppm females at weeks 17 and 28. (The activity increase in ALP correlated with increased liver weights in 3000-ppm females only. No significant microscopic findings were noted that supported the change in ALP). Decreased **calcium and total protein** were also noted in 6 000-ppm females probably attributed to the reductions in food intake in these animals.

Table B.6.3.1.5-4: Clinical chemistry, 6-month feeding study in dogs with difenoconazole (means)

dose level (ppm)	Males					Females				
	0	100	1000	3000	6000	0	100	1000	3000	6000
<u>ALP (U/l)</u> wk 17	73.0	57.7	80.3	93.0	172.0* ↑ 136%	82.7	63.0	71.7	283.7** ↑ 243%	122.7
wk 28	147.7	51.7	81.7	116.0	262.7 ↑ 78%	72.3	59.7	80.7	401.0** ↑ 455%	279.7* ↑ 287%
<u>calcium (mg/dl)</u> wk 28	10.2	10.1	10.1	9.9	10.1	10.6	10.5	10.3	9.8	9.1*
<u>total protein (g/dl)</u> wk 28	5.97	5.93	5.53*	5.67	5.97	5.97	5.67	6.03	5.97	5.10*
<u>LDH (U/l)</u> wk 28	35.0	44.0	29.0	54.0	56.3	129.0	32.3*	41.3	82.0	35.3

n=3 dogs per dose group at each interval wk=week

Urinalysis revealed no treatment-related changes.

Pathology, organ weights and histopathology

Macroscopic examinations revealed treatment-related bilateral **ocular opacity** in one 3000-ppm female and two 6000-ppm animals (one male, one female). There were no other treatment-related gross findings.

Analysis of organ weights revealed significantly reduced **carcass** weights of animals in the 6 000-ppm dose group. **Liver weights** were increased in 3 000-ppm females only without dose-response pattern. There was a dramatic drop in absolute **uterus and ovary weights** in 6 000-ppm females. However, the change was not significant and there was no dose-response. Similarly, the drop in **prostate weight** of 6 000-ppm males was marked with a tendency for dose-response. The increased brain weight in 3 000-ppm females is considered incidental.

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Table B.6.3.1.5-5: Organ weights, 6-month feeding study in dogs with difenoconazole (means)

Dose level (ppm)		Males					Females				
		0	100	1000	3000	6000	0	100	1000	3000	6000
body (carcass)	absolute ^a	10.90	11.00	10.03	9.93	7.57* ↓ 31%	9.13	9.07	9.20	8.23	6.27* ↓ 31%
liver	absolute	339.5	320.5	294.0	366.6	344.5	269.7	284.6	296.6	342.6** ↑ 27%	310.8
	relative	31.98	29.16	29.49	37.91	46.10	30.09	31.47	32.26	42.33* ↑ 31%	49.62
<u>prostate/uterus</u>	absolute	10.01	7.03	6.30	6.18	3.88** ↓ 61%	6.66	6.03	5.80	8.96	1.12 ↓ 83%
testes/ovaries	absolute	14.6	13.0	14.1	14.2	14.8	0.94	1.09	1.58	1.64	0.49 ↓ 48%
brain	absolute	85.2	79.0	79.9	87.7	80.1	74.6	74.1	77.0	85.1* ↑ 14%	68.9

^a carcass weights in kg; all other absolute weights in grams^b Relative weights = (organ-g/body-g)*100

n=3 dogs per dose group

Histopathologically, cataracts were diagnosed in one 3000-ppm male, all 3000-ppm females and in all 6000-ppm animals; these findings confirmed ophthalmoscopic and macroscopic findings.

Table B.6.3.1.5-6: Ocular histopathology findings, 6-month feeding study in dogs with difenoconazole (incidence)

dose level (ppm)		Males					Females				
		0	100	1000	3000	6000	0	100	1000	3000	6000
cataract lens, left	minimal	0	0	0	0	0	0	0	0	1	0
	moderate	0	0	0	1	2	0	0	0	1	1
	severe	0	0	0	0	1	0	0	0	1	2
cataract lens, right	minimal	0	0	0	1	1	0	0	0	0	1
	severe	0	0	0	0	0	0	0	0	1	2

n=3 dogs per dose group

The other histopathological changes that were noted did not give any indication of a treatment-related association.

Summary:

Toxicity was demonstrated in dose groups 1 000, 3 000 and 6 000 ppm by body weight loss and reduced body weight gain (at 6000 ppm), reduced food consumption (from 1000 ppm), increased liver weights accompanied by increased ALP activity (in 3000-ppm females), increased ALP activity without other significant liver changes (at 6000 ppm). Cataracts were observed from 3000 ppm.

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Table B.6.3.1.5-7: Summary incidence of ophthalmoscopic and microscopic ocular findings 6-month feeding study in dogs with difenoconazole

		Dose (ppm)			
		3000		6000	
		M	F	M	F
Ophthalmoscopic Lens	Cataract				
	unilateral	0/3	0/3	0/3	0/3
	bilateral	0/3	1/3	3/3	3/3
	TOTAL	0/3	1/3	3/3	3/3
Microscopic Lens	Cataract				
	unilateral	0/3	2/3	2/3	0/3
	bilateral	1/3	1/3	1/3	3/3
	TOTAL	1/3	3/3	3/3	3/3

Conclusion:

The **NOAEL** is set to **1 000 ppm** (corresponding to 31 and 35 mg/kg bw in males and females, respectively) based on the **cataract findings** in animals exposed at 3 000 ppm. Furthermore, the increase in absolute liver weight correlated with elevated ALP levels in 3000-ppm females, support adverse effects at this dose level although no histological liver changes were found. Liver finding in 3000-ppm males were negative. The **NOEL** was set to **100 ppm** (corresponding to 3.6 and 3.4 mg/kg bw in males and females, respectively), based on reduced food consumption in males at 1000 ppm.

Comments:

The title of the report implies that the duration of the study was 26 weeks, when in fact it was 28 weeks.

Deviations from the OECD TG No. 452 (1981)

The duration of the study should be at least 12 months according to guideline and there should be 4 animals per sex in each dose group.

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B.6.3.1.6 52-week oral toxicity study in dogs

Reference:	Rudzki, M.W., McCormick, G.C. and Arthur, A.T. (1988). CGA 169374 technical: Chronic toxicity study in dogs.
Guideline:	EPA FIFRA 83-1, OECD TG 452 (1981)
GLP:	Yes, verified by the U.S. EPA
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / purity 96.1%.
Species / Strain:	Dog / purebred beagle (Marshall Farms, NY)
Doses / No. of animals:	0, 20, 100, 500 or 1500 ppm corresponding to 0, 0.71, 3.4, 16 and 51 mg/kg/day for males and 0, 0.63, 3.7, 19 and 44 mg/kg/day for females / 4/dose/sex
Administration:	Orally, via the diet
Exposure time / Duration:	52 weeks
Statistics:	Analysis of Variance (ANOVA) and Dunnett's and Wilcoxon post hoc test on the quantitative variables. However, due to the low number of animals per group (n=4), the meaning and relevance of statistical significance is limited.

Materials and Methods:

The doses were selected in consultation with the US EPA, based on the results of a 6-month feeding study in dogs (O'Connor et al., 1987). Eye examinations were conducted on all animals during the pre-test period and during weeks 11, 27, 39 and 51; eyes of control and 1500-ppm animals were also examined at nine additional time points throughout the study. The auditory and the eye examinations are not required by the OECD TG. All animals were sacrificed on study days 365-367.

Results:

General observations

All animals **survived** the study. Clinical observations revealed sporadic **gastrointestinal symptoms** during the treatment period. However, these observations were also noted for control dogs which make the interpretation uncertain. **Body weights** were **not significantly** affected by treatment, but **tended** to be depressed among the 500- and 1500-ppm females compared to controls.

Food consumption by the 1 500-ppm females was reduced throughout the study and corresponds to reduced body weight gains among these females. Statistically significant reductions were recorded on days 7 (23%), 35 (19%), 70 (24%) and 357 (29%).

Physical/veterinary examinations, including **hearing tests** revealed no treatment-related findings. A single incidence of ocular change (corneal opacity) was seen in one control-group female but no treatment-related

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ophthalmic changes were observed. The cataracts observed to occur at levels $\geq 3\ 000$ ppm in a previous 6-month dog study (O'Connor et al., 1987) were not observed in the present study, grossly or microscopically.

Haematology, clinical chemistry and urinalysis

There were no treatment-related differences among the groups in the **haematology parameters**. The only difference among the groups was a significant decrease in relative eosinophils in 100-ppm females at week 13 and a significant reduction (63%) in reticulocyte count in 1 500 ppm females at week 52. These changes were considered to be inconsistent and not related to treatment.

Statistically significant treatment-related increases in mean alkaline phosphatase (**ALP**) were noted throughout the study in 1 500-ppm males, and in 500-ppm males at week 52. None of the recorded ALP activities is above the normal range for dogs (<252 U/l). Thus, the observation is regarded to be **an effect, but not an adverse effect** of treatment. Furthermore, the ALP activity decreases over time in the respective dose groups.

Table B.6.3.1.6-1: Clinical chemistry, 1-year feeding study in dogs with difenoconazole (means)

dose level (ppm)		Males					Females				
		0	20	100	500	1500	0	20	100	500	1500
ALP, alkaline phosphatase (U/l)	wk 13	88.0	108.0	107.8	94.0	125.0** ↑ 42%	105.0	72.5	98.8	82.8	94.8
	wk 26	62.5	70.0	79.5	89.3	123.5** ↑ 98%	71.0	68.0	76.8	104.3	79.3
	wk 52	41.5	55.3	57.8	64.3* ↑ 55%	100.8** ↑ 143%	52.3	51.8	55.5	77.0	66.8

* = $p \leq 0.05$, ** = $p \leq 0.01$, Dunnett T on values adjusted for differences in baseline values
n=4 dogs per dose group at each interval

There were no treatment-related changes noted in any of the **urinalysis** for the duration of the study.

Pathology, organ weights and histopathology

Macroscopic and microscopic examinations revealed no treatment-related findings. There were no treatment-related changes in mean **organ weight** in any treatment group. The observed alterations in the 20- (adrenal and thyroid/parathyroid weight) and 500-ppm (adrenal weight) animals were considered incidental due to lack of dose-response and lack of correspondence with gross or histological findings.

Summary:

Dietary administration of difenoconazole technical to dogs at dose levels of 20, 100, 500 and 1500 ppm over a 1-year period resulted in reduced food consumption (1 500-ppm females) and in a tendency for reduced body weight gain in females from 500 ppm. Increased ALP activity was noted (in males from 500 ppm) however, no treatment-related macro- or microscopic findings were recorded.

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Conclusion:

The **NOAEL** was $\geq 1\ 500\ \text{ppm}$ (corresponding to 51 and 44 mg/kg bw in males and females, respectively). At 1500 ppm reduced food consumption and an ALP increase were recorded. The latter were within normal range and had no correlates in macro- or micro findings. The **NOEL** was set to **100 ppm** (corresponding to 3.4 and 3.7 mg/kg bw in males and females, respectively) based on increased ALP levels recorded in males at 500 ppm.

B.6.3.2 Percutaneous**B.6.3.2.1 28-day dermal toxicity study in rats**

Reference:	Gerspach, R. (2000). CGA 169374 Technical: 28-Day Repeated Dose Dermal Toxicity Study in Rats.
Guideline:	EEC 92/69 B.9, OECD TG 410
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical / 91.8% Vehicle Distilled water, Carboxymethylcellulose (CMC) (1%), Tween 80 (0.1%)
Species / Strain:	Rat / Wistar HanIbm:WIST (SPF)
Doses / No. of animals:	0, 10, 100 or 1000 mg/kg 10/sex/dose
Administration:	Dermally. The test article was held in place by gauze patches covered with aluminium foil and tape.
Exposure time / Duration:	6 hours/day, 5 days/week for the first 3 weeks and 7 days/week thereafter
Statistics:	Bartlett's test for homogeneity testing of the within group variances. Analysis of Variance (ANOVA) and Dunnett's post hoc test (homogeneity of variances) or Kruskal-Wallis and Dunn's post hoc test (heterogeneity of variances) for in-life and organ weight data. Trend Test Analysis (Armitage/Cochran) provided by PathData System for Evaluating Non-Neoplastic lesions, for the hyperkeratosis findings.

Materials and Methods:

The procedure followed the OECD TG No. 410 (1981) in principle but there were some deviations, **see comments**. Control animals were treated with vehicle only. Neurological examinations were performed following weekly detailed clinical examinations, and included tests for sensorymotor function (approach, touch, vision, audition, pain, vestibular). Eye examinations were performed bilaterally on all animals at pretest and on all surviving animals of the control and 1 000 mg/kg-dose group, respectively. The neurological and the eye examinations are not requested by the OECD TG. The animals were sacrificed on study day 29.

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Results:

General observations

One male control was found **dead** on treatment day 28. No **clinical sign** considered treatment-related was observed during the study and there was no occurrence of visible erythema or oedema (irritation) of the skin at the application site. Mean **food consumption** was significantly lower (10 %) in males of the 1000 mg/kg group than in controls during week 1, but similar thereafter. The **body weight** development of all treatment groups was similar to that of the control groups. **Ophthalmologic** examinations revealed no treatment-related findings.

Haematology, clinical chemistry and urinalysis

Treatment had no effect on the **haematological profile** of the animals. Lower globulin values with an associated increase in the albumin to globulin ratio were recorded for males at 1 000 mg/kg. Also in this group, plasma **bilirubin** and calcium levels were lower than control values. Females of the 10 mg/kg group had an increase (29%) in **ALAT** levels. However, the latter finding lacked dose-dependency.

Table B.6.3.2.1-1: Clinical chemistry, 28-day dermal toxicity study in rats with difenoconazole (means)

dose level (mg/kg)	Males				Females			
	0	10	100	1000	0	10	100	1000
globulin (g/l)	31.61	30.62	29.75	28.99**	30.09	29.47	30.09	29.77
albumin/globulin ratio	1.10	1.12	1.15	1.22**	1.16	1.18	1.14	1.20
total bilirubin (umol/l)	2.05	2.13	1.84	1.30** ↓ 37%	1.90	2.13	1.90	1.58
calcium (mmol/l)	2.81	2.77	2.74	2.71**	2.79	2.73	2.72	2.74
ALAT, alanine aminotransferase (U/l)	39.52	42.75	42.43	45.56	28.99	37.47*	32.42	31.59

* = $p \leq 0.05$, ** = $p \leq 0.01$, ANOVA + Dunnett or Kruskal-Wallis + Dunn

Pathology, organ weights and histopathology

Macroscopic examinations at the end of the treatment period revealed no treatment-related findings. Reddish discoloration of the lachrymal glands was the only finding noted in more than one animal in a dose group (one control group male, two 10 mg/kg males, three 100 mg/kg males, and one 10-mg/kg female). Organ weight analysis revealed increased absolute and relative **liver weights** in 1 000 mg/kg animals of both sexes.

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Table B.6.3.2.1-2: Organ weights, 28-day dermal toxicity study in rats with difenoconazole (means)

dose level (mg/kg)		Males				Females			
		0	10	100	1000	0	10	100	1000
body	absolute ^a	300.6	292.5	288.3	290.4	222.5	214.7	215.6	219.6
	absolute	12.25	12.29	12.07	13.74*	9.74	9.81	10.03	10.65
	relative	4.08	4.20	4.19	4.73**	4.37	4.57	4.63	4.85**
testes/ovaries	absolute	3.31	3.42	3.24	3.40	0.17	0.16	0.16	0.17
	relative	1.11	1.17	1.13	1.18	0.08	0.08	0.08	0.08
epididymides/uterus	absolute	1.18	1.15	1.14	1.24	0.83	0.77	0.91	0.83
	relative	0.40	0.39	0.39	0.43	0.38	0.36	0.43	0.38

^a All absolute weights in grams ^bRelative organ weights = % of body weight (g/g); * = $p \leq 0.05$, ** = $p \leq 0.01$, ANOVA + Dunnett

All other organ weights were comparable among the control and treated animals. **The thyroid gland was not weighed.**

Microscopically, an increase in the number of epidermal cell rows, as well as in thickness of the retained lamellar keratin layer (**hyperkeratosis**) was seen in both male and female 1000 mg/kg animals. These increases (in both males and females) were statistically significant ($p < 0.05$) by the Armitage/Cochran trend test. The change in frequency and grading did not however, occur in a linear relationship to the increase in dose of test substance. Moreover, treatment-related changes in the **liver and thyroid gland** were observed with centrilobular **hepatocellular hypertrophy** present at an increased incidence in 1 000 mg/kg males and females, and **hypertrophy of the thyroid follicular epithelium** in 1 000 mg/kg animals (in males the severity only was increased, and in females both the severity and incidence).

Table B.6.3.2.1-3: Treatment-related histopathological findings, 28-day dermal toxicity study in rats with difenoconazole

dose level (mg/kg)		Males				Females			
		0	10	100	1000	0	10	100	1000
skin application site	N ex.	10	9	10	10	10	10	10	10
	incidence	2	4	2	6	4	7	6	10
	wtd. grade	1.0	1.0	1.0	1.1	1.3	1.0	1.7	1.2
liver	N ex.	10	10	10	10	10	10	10	10
	incidence	2	1	2	7	1	1	1	7
	wtd. grade	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
thyroid gland	N ex.	10	10	10	10	10	10	10	10
	incidence	8	6	9	8	7	6	7	9
	wtd. grade	1.6	1.5	1.7	2.0	1.3	1.3	1.4	1.7

wtd. grade is Σ (severity grade x n with that grade) / total examined

Summary:

Dermal treatment of rats with Difenoconazole resulted in significant findings in 1 000-mg/kg the dose group with transient lower food consumption, increased liver weights and decreased bilirubin levels with corresponding hepatocellular hypertrophy, follicular cell hypertrophy of the thyroid gland and hyperkeratosis at

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the application site. None of the findings are considered adverse but to represent reactions of adaption. The NOAEL is thus 1 000 mg/kg and the NOEL at 100 mg/kg.

Conclusion:

The **NOAEL** is set to **1000 mg/kg bw** and the **NOEL at 100 mg/kg bw** based on the liver findings in males exposed at 1 000 mg/kg bw.

Comments:

Deviations from the OECD TG No. 410 (1981)

According to guideline, careful clinical observations should be made at least once daily and not once weekly as in the present report.

B.6.3.3 Summary and conclusions on short-term toxicity studies

The short term oral toxicity was investigated in rats, dogs and mice. One 28 day study and two 90 day studies were performed in rats. Two studies were performed in dogs, one 6 month study and one 1 year study. A 90 day study was performed in mice. A short term dermal toxicity study was also performed in rats, but a 90-day dermal study was not performed because of the limited effects seen in the 28-day study. All of these studies were considered acceptable. All of the studies were performed according to GLP principles with the exception of the 28 day study in rats. A 90 day dog study was not performed since studies of 6 and 12 months duration, which look at effects at 3 months either side of this timepoint, were conducted. Furthermore, there were no indications that dogs were more sensitive to Difenoconazole than rats. No short-term inhalation toxicity studies were performed because difenoconazole is not volatile (vapour pressure 0.0000332 mPa [25 °C]) and is not used as a fumigant or an aerosol.

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
ORAL:					
28-day in rat					
Suter, P., 1986a	27/27, 156/166 and 914/841 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 156/166 mg kg⁻¹ day⁻¹ 1500 ppm	LOAEL: M/F: 914/841 mg kg⁻¹ day⁻¹ 10 000 ppm	↓ Body weight ↓ Carcass weight ↓ Organ weight 10 000 ppm: Altered clinical chemical parameters Altered blood parameters ↓ PT time Dysproteinemia
90-day in rat					
Suter, P., 1986b (Wistar rats)	0, 3.3/3.5, 19.9/21.4 and 120.9/ 128.5 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 20/21 mg kg⁻¹ day⁻¹ 250 ppm	LOAEL: M/F: 120.9/ 128.5 mg kg⁻¹ day⁻¹ 1500 ppm	↓ Body weight ↓ Carcass weight ↓ Heart weight (11%) ↓ Food consumption 1500 ppm: Altered blood parameters Altered clinical chemistry parameters Dysproteinemia ↑ Liver weight ↑ Serum albumin
Cox, R.H., 1987a (Sprague Dawley rats)	0, 1.3/1.7, 13/17, 51/66, 105/131 and 214/275 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 51/66 mg kg⁻¹ day⁻¹ 750 ppm NOEL: M/F: 1.3/1.7 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 105/131 mg kg⁻¹ day⁻¹ 1500 ppm LOEL: M/F: 13/17 mg kg ⁻¹ day ⁻¹	↓ Body weight ↓ Body weight gain 1500 ppm: ↓ Carcass weight Altered clinical chemistry parameters Hepatocellular enlargement 750 ppm: ↓ RBC parameters dysproteinemia ↑ Liver weight
			20 ppm	200 ppm	↓ Body weight gain (F)
90-day in mouse					
Cox, R.H., 1987b	0, 3.3/4.6, 34.2/45.2 and 440/639 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 34.2/45.2 mg kg⁻¹ day⁻¹ 200 ppm NOEL: M/F: 3.3/4.6 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 440/639 mg kg⁻¹ day⁻¹ 2 500 ppm LOEL: M/F: 34.2/45.2 mg kg ⁻¹ day ⁻¹	↓ Ovary weight ↓ Body weight gain 2500 ppm: ↑ Liver weight Macroscopic liver enlargement Hepatocellular vacuolization

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
	ppm				Hepatocellular coagulative necrosis
			20 ppm	200 ppm	Hepatocellular enlargement
6 months in dog					
O'Connor et al., 1987	0, 3.6/3.4, 31.3/34.8, 96.6/110.6 and 157.8/203.7 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 31.3/ 34.8 mg kg⁻¹ day⁻¹	LOAEL: M/F: 96.6/110.6 mg kg⁻¹ day⁻¹	6000 ppm: ↓ Body weight ↓ Food consumption ↓ Carcass weight ↓ Prostate weight
	0, 100, 1000, 3000 and 6000 ppm		1000 ppm	3000 ppm	Cataract
			NOEL: M/F: 3.6/ 3.4mg kg ⁻¹ day ⁻¹	LOEL: M/F: 96.6/110.6 mg kg ⁻¹ day ⁻¹	6000 ppm: ↓ Ovary weight (n.s.) ↓ Uterus weight (n.s.) ↑ Platelet count ↓ Calcium Dysproteinemia
					3000 ppm: ↑ Liver weight (F) ↑ ALP (F)
			100 ppm	1000 ppm	↓ Food consumption (M)
1-year in dog					
Rudzki et al., 1988	0, 0.71/0.63, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: ≥ 51.2/44.3 mg kg⁻¹ day⁻¹	LOAEL: Could not be established	
	0, 20, 100, 500 and 1500 ppm		NOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	LOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	1500 ppm: ↓ Food consumption ↓ Body weight gain
			100 ppm	500 ppm	↑ ALP (M)
DERMAL:					
28-day in rat					
Gerspach, R., 2000	0, 10, 100 ^a or 1000 mg kg ⁻¹ bw day ⁻¹	Dermal	NOAEL 1 000 kg ⁻¹ bw day ⁻¹	LOAEL >1 000 kg ⁻¹ bw day ⁻¹	
			NOEL: 100 kg ⁻¹ bw day ⁻¹		1000 mg kg ⁻¹ bw day ⁻¹ : ↑ Liver weight ↓ Bilirubin levels Hepatocellular hypertrophy ↓ Food consumption Hypertrophy of thyroid gland Hyperkeratosis of application site
M = male; F = female					

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The body weight was the most sensitive parameter in the oral short term toxicity studies in rats with a NOAEL for body weight reduction at 20 and 21 mg/kg bw for males and females respectively, derived from the 90 day study (Suter 1986b). The liver was identified as target organ in the rat studies and the effects were mostly expressed as increases in relative and absolute liver weights, but these changes were not associated with histological changes except in the second 90 day study where an increased incidence and severity of hepatocellular enlargement were noted at $\geq 100/130$ mg/kg bw for males and females respectively. The liver findings were not considered as adverse in the first 90 days study (Suter 1986b) and were reversible.

The dogs in the 28 day study also responded to Difenconazole treatment by body weight loss (NOAEL 97/111 mg/kg bw males and females respectively). In the 6 month study the target organ was the liver with increased liver weights seen in the 6000 and 3000ppm animals. There was no associated pathological change. The NOAEL was set at 1000 ppm (31/35 mg/kg bw for males and females respectively) based on the findings of cataracts in animals at 3000 ppm. The increase in absolute liver weight correlated with elevated ALP levels in 3000 ppm females which also supports adverse effects at this level although no histological liver changes were found. In the 1 year dog study the only treatment related effects were increased alkaline phosphatase activity in animals given 1500 and 500 ppm in the diet and reduced food consumption at 1500 ppm. There were no effects on liver weight or histopathology, nor any evidence of cataracts.

Dermal application of difenoconazole resulted in a NOAEL of ≥ 1000 mg/kg/day – the highest dose tested. The target organ was again the liver, with increased liver weights and decreased bilirubin levels in animals treated with 1000 mg/kg/day showing associated pathological change manifested as centrilobular hepatocellular hypertrophy. Histopathology revealed hyperkeratosis at the skin application site in the 1000 mg/kg animals, and follicular cell hypertrophy of the thyroid gland was diagnosed in high-dose males and females. None of these changes were considered adverse but to represent reactions of adaptation.

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B.6.4 Genotoxicity (Annex IIA 5.4)

B.6.4.1 *In vitro* studies

B.6.4.1.1 *In Vitro* Mammalian Chromosome Aberration Test - Human Lymphocytes I

Reference:	Strasser F (1985). CGA 169374 tech.: Chromosome studies on human lymphocytes <i>in vitro</i> .
Guideline:	OECD TG 473
GLP:	Yes (certified laboratory)
Acceptability:	As supportive information (see comments)
Test substance / purity:	Difenoconazole (CGA 169374) tech. / 94.5%
Species / Strain:	Human lymphocytes
Doses /Replicates:	0, 2.5, 5.0, 10.0, 20.0 and 40.0 µg/ml With and without microsomal activation
Administration:	Test substance dissolved in DMSO added to the cell suspensions
Exposure time:	3 h

Materials and Methods:

Human blood was obtained from a normal donor and preincubated for 46 h prior to treatment. DMSO was used as solvent for test substance. The final concentration of DMSO in the cell medium was 1%. In the experiments where difenoconazole was metabolically activated, activation mixture (rat liver microsomal fraction S9, NADP and isocitric acid) was added to the cell suspension.

Cytotoxicity test

A preliminary cytotoxicity test was performed with and without metabolic activation where the cells were exposed for 3 h to 14 concentrations of test substance ranging from 0.12 to 1 000 µg/ml. The concentration (40 µg/ml) calculated to produce about 50% suppression of mitotic activity in comparison with control (by counting ≥ 1000 cells/concentration) was used as the highest dose in the mutagenicity experiments.

Mutagenicity test

The lymphocytes were exposed for 3 h to the selected concentrations of test substance or to vehicle (DMSO) or positive controls (mitomycin-C and cyclophosphamide), with and without metabolic activation. After treatment cells were washed and resuspended in chromosome medium and allowed to grow for 43.5 h. Colcemide arrested the metaphase stage before harvesting. Two independent cultures were seeded at each concentration. A number

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of 100 complete metaphase plates altogether from cultures of two growth flasks from each treatment (negative and positive controls and all dose levels, with and without metabolic activation) were examined. This is fewer than recommended (200/concentration) in the OECD TG No. 473, **see comments**.

Results:

The highest concentration selected from the the cytotoxicity test for the mutagenicity test was 40.0 µg/ml. However, the preparations of the two highest concentrations 20.0 and 40.0 µg/ml could not be scored due to cytotoxicity of the test substance.

Table B.6.4.1.1-1: Percent incidence of specific chromosomal aberrations in human lymphocytes

	% of metaphases with specific aberrations		% of metaphases with unspecific aberrations	
	without activation	with activation	without activation	with activation
Solvent control (1% DMSO)	0	0	5	10
CGA 169374 tech.				
2.5 µg/ml	0	3	5	10
5.0 µg/ml	1	1	6	3
10.0 µg/ml	0	3	4	9
20.0 µg/ml	toxic	toxic	toxic	toxic
40.0 µg/ml	toxic	toxic	toxic	toxic
Positive control	7	18	13	14

Conclusion: See comments.

Comments:

Deviations from the OECD TG No 473

According to the guideline, at least 200 well-spread metaphases should be scored per concentration and control, equally divided amongst the duplicate cultures. In the present study, only 100 metaphases per concentration (from 2 cultures) were examined for chromosomal aberrations. Moreover, negative results in the first experiment should be confirmed without activation in a second, confirmatory experiment (OECD TG 473, paragraph 25), but this was not performed here.

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B.6.4.1.2 *In Vitro* Mammalian Cell Gene Mutation Test

Reference:	Dollenmeier P (1986a). CGA 169374 tech.: L5178Y/TK^{+/−} Mouse Lymphoma Mutagenicity Test.
Guideline:	OECD TG 476
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole (CGA 169374 tech.) / 94.5%
Species / Strain:	Mouse lymphoma (L5178Y) heterozygous subline TK
Doses:	<u>Mutagenicity test without activation</u> First experiment: 8-80 µg/l Second experiment: 15-150 µg/l Third experiment: 12-120 µg/l <u>Mutagenicity test with activation</u> First experiment: 5-50 µg/l Second experiment: 3-30 µg/l
Administration:	Test substance dissolved in DMSO (1%) added to the cell suspensions
Exposure time / Duration:	4 h

Materials and Methods:

In a preliminary toxicity test the cells were exposed to 7 concentrations of difenoconazole ranging from 5 to 320 µg/ml for 4 hours - with and without metabolic activation. The activation mixture consisted of NADP, isocitrate, tris(hydrooxylmethyl)aminomethane, MgCl₂ and rat liver microsomal S9-fraction. **In the mutagenicity test**, the cells (at a density of 3x10⁵/ml) were exposed for 4 h to 7 concentrations of difenoconazole ranging from 5 to 320 µg/ml -without and with metabolic activation. An untreated, a solvent and a positive control (ethylmethansulfonate (EMS) and dimethylnitrosoamine (DMN)) were also included. The treatment was terminated by washing with medium. The cells were resuspended in growth medium and allowed to grow for 3 days in order to express the induced TK^{−/−} forward mutants. For mutant selection, cultures were set up in tubes containing 4x10⁵ cells in 5 ml of a semi-solid agar cloning medium with BUdR at a final concentration of 50 µg/ml. The tubes were incubated for 14 to 16 days. For viability control, cultures of 200 cells were set up in cloning medium without BUdR and incubated for 12 days. After that, the number of colonies was determined by an electronic colony counter.

A test substance is considered to be mutagenic in this test system if the mutant colony count exceeds that of the solvent control by a factor of 2.5 at any concentration.

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Results:*Cytotoxicity test*

For the mutagenicity experiments, 80 and 50 µg/ml, respectively were selected as highest concentrations, without and with metabolic activation. These concentrations caused approximately 85% reduction in the relative suspension growth in comparison with control after 4 h of exposure.

Mutagenicity tests

In the first experiments without and with activation there was no marked increase in mutant frequencies, indicative of a mutagenic effect, at any concentration.

Table B.6.4.1.2-1: Mutant frequency observed in the mouse lymphoma test with difenoconazole, original experiment

Without metabolic activation				With metabolic activation			
Treatment	Relative suspension growth (% of control)	Mutant frequency (x10 ⁻⁶)	Mutant factor	Treatment	Relative suspension growth	Mutant frequency (x10 ⁻⁶)	Mutant factor
Solvent control	100.0	15.1		Solvent control	100.0	45.1	
Negative control	93.7	13.9		Negative control	105.7	43.0	
Positive control EMS 0.75 µl/ml	16.0	326.1	23.5	Positive control DMN 8.0 µl/ml	37.3	223.3	5.19
Difenoconazole				Difenoconazole			
8 µg/ml	98.5	15.9	1.05	5 µg/ml	77.1	41.3	0.92
16 µg/ml	101.1	17.1	1.13	10 µg/ml	66.4	55.9	1.24
32 µg/ml	79.3	18.0	1.19	20 µg/ml	32.7	45.0	1.0
48 µg/ml	65.7	18.9	1.25	30 µg/ml	0.4	*	
64 µg/ml	43.7	19.0	1.26	40 µg/ml	0.2	*	
72 µg/ml	45.9	17.3	1.15	45 µg/ml	0.2	*	
80 µg/ml	40.9	10.9	0.72	50 µg/ml	0.2	*	

A test substance is considered to be mutagenic in this test system if the mutant colony count exceeds that of the solvent control by a factor of 2.5 at any concentration.

Relative suspension growth was reduced to 40.9% at the highest concentration without metabolic activation. Because this reduction is less (59.1%) than the desired reduction (85%), the experiment was repeated with a lower concentration range (15-150 µg/ml). With metabolic activation, relative suspension growth at 20 µg/ml was 32.7% of the control. Higher concentrations were *toxic and therefore, the experiment was repeated.

In the second experiment without activation, an increase in mutant frequency was registered at 120 µg/ml. However, at this concentration toxicity was still excessive and relative growth reduced to only 1.6% of that of the control. Therefore this concentration had to be excluded from evaluation. The next lower concentrations showed no increase in mutant frequency. **In the experiment with activation**, no marked increase in mutant frequencies was observed.

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Table B.6.4.1.2-2: Mutant frequency observed in the mouse lymphoma test with difenoconazole, first confirmatory experiment

Without metabolic activation				With metabolic activation			
Treatment	Relative suspension growth	Mutant frequency (x10 ⁻⁶)	Mutant factor	Treatment	Relative suspension growth	Mutant frequency (x10 ⁻⁶)	Mutant factor
Solvent control	100.0	21.7		Solvent control	100.0	35.4	
Negative control	104.0	27.9		Negative control	131.7	37.7	
EMS 0.75 µl/ml	10.8	3020.8	108	DMN 8.0 µl/ml	14.9	1360.0	36.1
Difenoconazole				Difenoconazole			
15 µg/ml	88.5	19.5	0.90	3 µg/ml	121.5	28.4	0.80
30 µg/ml	98.0	31.5	1.45	6 µg/ml	77.8	26.0	0.73
60 µg/ml	55.4	29.9	1.38	12 µg/ml	69.8	17.8	0.50
90 µg/ml	14.1	35.6	1.64	18 µg/ml	35.6	22.2	0.63
120 µg/ml	1.6	71.3	3.29	24 µg/ml	13.3	38.0	1.07
135 µg/ml	0.1	*		27 µg/ml	4.0	46.0	1.30
150 µg/ml	0.1	*		30 µg/ml	0.3	*	

In the third experiment without metabolic activation no increase was observed in mutant frequencies.

Relative suspension growth was reduced to 3.8% of the control at 72 µg/ml and higher concentrations were toxic.

Table B.6.4.1.2-3: Mutant frequency observed in the mouse lymphoma test with difenoconazole, second confirmatory experiment

Without metabolic activation			
Treatment	Relative growth	Mutant frequency (x10 ⁻⁶)	Mutant factor
Solvent control	100.0	30.0	
Negative control	79.0	36.3	
EMS 0.75 µl/ml	25.2	566.6	15.6
Difenoconazole			
12 µg/ml	59.7	34.3	1.14
24 µg/ml	66.7	33.7	1.12
48 µg/ml	13.6	35.5	1.18
72 µg/ml	3.8	35.6	1.19
96 µg/ml	0.0	*	
108 µg/ml	0.0	*	
120 µg/ml	0.0	*	

* No data since the number of cells was insufficient for mutant-selection

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Conclusion:

An increased incidence of mutant frequencies was registered in all experiments with the positive controls. Under the conditions of this assay, no evidence of induction of gene mutations was found in mouse lymphoma L5178Y/TK+/- cells.

Comments:

It is not transparent whether single or duplicate cell cultures have been exposed at each dose level.

B.6.4.1.3 Bacterial Reverse Mutation Test

Reference:	Ogorek B. (1990). CGA 169374 tech.: <i>Salmonella</i> and <i>Escherichia</i>/mammalian-microsome mutagenicity test.
Guideline:	OECD TG 471
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole (CGA 169374) tech. / 91.8%. Vehicle: Dimethylsulfoxide (DMSO)
Species / Strain:	<u>For mutagenicity tests:</u> <i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535 and TA 1537 (histidine-auxotrophic) and <i>E. coli</i> WP2uvrA (tryptophan-auxotrophic)
Doses:	340-5447 µg/0.1 ml (original and first confirmatory mutagenicity test) 85-1362 µg/0.1 ml (second confirmatory mutagenicity test)
Administration:	Test substance dissolved in DMSO (1%) added to the cell cultures
Exposure time:	48 h

Materials and Methods:

A preliminary toxicity test with concentrations ranging from 20 to 5000.0 µg/0.1 ml determined the highest concentration applied (5447 µg/0.1 ml) in the following mutagenicity tests. Four lower concentrations spaced by a factor of 2 were then tested. Two sets of independent studies were performed (original and confirmatory studies) with and without metabolic activation with all strains. Because strong growth-inhibiting effects occurred with strains TA 98 and TA 1537 at the upper concentrations, a second confirmatory experiment with a lower concentration range was performed on these strains. After preparation, 3 plates per concentration were incubated for about 48 hours at $37 \pm 1.5^\circ\text{C}$ in darkness. Thereafter, they were evaluated by counting the number of colonies and determining the background lawn. Relevant positive controls were included in the tests. DMSO served as negative control. Statistical analyses were not performed.

Criteria for a positive response

The test substance is considered to be positive if **a**) at least a reproducible doubling of the mean number of revertants per plate above negative control is determined for strains TA 98, TA1535, TA 1537 and *E. coli*

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WP2uvrA b) a reproducible increase of the mean number of revertants per plate for any concentration above negative control by ≥ 1.5 is determined for strain TA 100. A concentration-related effect should (generally) be demonstrable.

Results:

From the results of the cytotoxicity test the highest concentration for the first mutagenicity study was set to 5000 $\mu\text{g}/0.1\text{ ml}$. However, on request of the sponsor the concentration of the test material was related to 100% purity of the test substance and therefore, 5447 $\mu\text{g}/0.1\text{ ml}$ was used as the highest concentration.

Treatment with difenoconazole did not lead to an increase in the incidence of either histidine- or tryptophanprototrophic mutants in comparison to negative control.

Table B.6.4.1.3-1: Gene mutation assay with difenoconazole in *S. typhimurium* strains, number of revertant colonies (mean of 3 plates), original experiment.

Strain	TA 100		TA 1535		TA 98		TA 1537		WP2 uvrA	
Metabolic activation	without	with	without	with	without	with	without	with	without	with
Solvent control	191	156	16	11	15	25	11	7	21	25
<u>Difenoconazole</u>										
340 $\mu\text{g}/\text{plate}$	166	137	7	16	9	14	7	5	20	21
681 $\mu\text{g}/\text{plate}$	168	150	10	12	6	19	2	3	17	23
1362 $\mu\text{g}/\text{plate}$	159	143	4	4	4	7	1	4	13	15
2723 $\mu\text{g}/\text{plate}$	168	145	3	6	4	25	4	4	13	16
5447 $\mu\text{g}/\text{plate}$	155	136	1	3	2	18	2	1	14	11
Positive control ^a	917	2191	806	196	1504	2347	2773	205	129	1311

Table B.6.4.1.3-2: Gene mutation assay with difenoconazole in *S. typhimurium* strains, number of revertant colonies (mean of 3 plates), first confirmatory experiment.

Strain	TA 100		TA 1535		TA 98		TA 1537		WP2 uvrA	
Metabolic activation	without	with	without	with	without	with	without	with	without	with
Solvent control	134	116	7	16	21	32	7	10	16	22
<u>Difenoconazole</u>										
340 $\mu\text{g}/\text{plate}$	113	107	8	13	16	28	4	9	16	27
681 $\mu\text{g}/\text{plate}$	50	61	9	10	12	19	4	5	20	17
1362 $\mu\text{g}/\text{plate}$	18	49	7	9	9	14	4	5	11	14
2723 $\mu\text{g}/\text{plate}$	22	24	9	10	7	17	1	4	7	9
5447 $\mu\text{g}/\text{plate}$	17	18	6	9	4	8	0	3	6	7
Positive control	630	1674	121	626	1119	1303	1787	365	836	844

A growth inhibiting effect of the test substance occurred with strains TA 98 and TA 1537 at the upper concentrations in the first confirmatory experiment and therefore, a second confirmatory experiment with a lower concentration range was performed on these strains. At concentrations $\geq 681\text{ } \mu\text{g}/0.1\text{ ml}$ the test substance precipitated in soft agar.

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Table B.6.4.1.3-3: Gene mutation assay with difenoconazole in *S. typhimurium* strains, number of revertant colonies (mean of 3 plates), second confirmatory experiment

Strain	TA 100		TA 1535		TA 98		TA 1537		WP2 uvrA	
Metabolic activation	without	with	without	with	without	with	without	with	without	with
Solvent control					23	28	9	7		
<u>Difenoconazole</u>										
85 µg/plate					17	32	6	5		
170 µg/plate					19	25	4	7		
340 µg/plate					17	35	6	10		
681 µg/plate					19	30	5	5		
1362 µg/plate					16	24	5	7		
Positive control ^a					620	1844	1296	229		

^a Compound and concentration see Material and methods above

Conclusion:

There was no evidence of induction of point mutations by difenoconazole or its metabolites in the strains of *S. typhimurium* and *E. coli* used in these experiments.

B.6.4.1.4 DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells

Reference:	Hertner T (1992). CGA 169374: Autoradiographic DNA repair test on rat hepatocytes (OECD-conform) <i>in vitro</i>.
Guideline:	OECD TG 482
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	CGA 169374 (difenoconazole) tech. / 92.2%.
Species / Strain:	Primary hepatocytes from male TIF: RAIf (SPF) rats
Doses	Original and confirmatory DNA-repair experiments: 0.46, 1.39, 4.17, 12.5, 25 and 50 µg/ml
Administration:	20 µl test solution added to cell compartments. Each treatment was performed on 4 cultures.
Exposure time:	16-18h

Materials and Methods:

Test substance was dissolved in dimethylsulfoxide (DMSO). DMSO and 2-acetylaminofluorene were used as negative and positive controls, respectively in all experiments except where indicated. Primary hepatocytes were prepared by *in situ* collagenase perfusion of rat livers. The hepatocytes were then cultured accordingly, and freshly isolated cells (4×10^5) were seeded in compartments of multi-hole plates containing gelatinised cover slips.

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Preliminary toxicity tests

Part I (cytotoxicity): The hepatocytes were incubated in their compartments for 16 to 18 h with test-article solution (20 µl) at 11 different concentrations increasing by a factor of two from 0.98 to 1000 µg/ml. After washing and staining, the cells were fixed and the number of viable (unstained) cells was determined by counting 100 cells. Part II (DNA-synthesis, allows the detection of a possible DNA-synthesis inhibiting activity of the test substance): Treatment was the same as described in part I. Hydroxyurea (HU, 10 mM) served as positive control. One hour after addition of the test substance, ³H-thymidine was added to each culture. After washing the cells, nuclei were isolated and washed. Half of the nuclear suspension was used for determination of radioactivity in a liquid scintillation counter. The other half of the suspension was used for determination of DNA content by measuring fluorescence after staining. From the results of the preliminary toxicity test, the highest usable concentration was selected and 5 further lower concentrations were determined.

Original DNA-repair test

The hepatocytes were incubated with the test article (0.46 - 50 µg/ml) and ³H-thymidine for 16-18 hours. After removal of the medium, washing, fixation and staining of the hepatocytes, autoradiographs were prepared. From each treatment group, 150 nuclei from three coded slides (50 cells/slide) were scored and the number of silver grains was determined. Two independent experiments were performed (original and confirmatory experiments, respectively). Silver grain over the nucleus and cytoplasm of the hepatocytes were counted using an electronic counter (gross values). The net values were calculated by subtracting the grain count over the cytoplasm from the grain count over the nucleus. The percentage of nuclei in repair were calculated (both for gross and net grain counts) and defined as those nuclei with grain counts exceeding the 90th percentile value of the grain count distribution of the negative control. Cells with > 120 silver grains/nucleus were considered to be in the DNA-synthesis phase (S-phase) and were excluded.

The significance of differences was assessed by Dunnett's one-tailed t test. **Level of significance (alpha) was 0.01.**

Criteria for a positive response

The test substance is considered to be active in the DNA repair test if at least one of the following conditions is reproducibly met: a) the mean gross and the mean net number of silver grains per nucleus in relation to their respective vehicle controls show a statistically significant difference at any concentration and the mean net value is at least 2.0, b) the percentage of nuclei in repair, with respect to their gross and their net numbers of silver grains, show a statistically significant difference at any concentration as compared to their respective vehicle controls.

Criteria for a negative response

The test substance is considered to be inactive in the DNA repair test if the following conditions are reproducibly met: a) the mean gross and net numbers of silver grains per nucleus as well as the percentage of nuclei in repair,

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with respect to their gross and their net numbers of silver grains, in relation in to the vehicle control are not statistically different at all concentrations and no concentration dependence can be seen.

Results:

Part I (cytotoxicity):

The number of viable cells was not determinable due to insufficient adhered cells as a consequence of cytotoxicity at 62.5 µg/ml and above. At 31.3 µg/ml, 31 % viable cells were determined. **Thus, a maximum concentration of 50 µg/ml was considered to be suitable for the main original and confirmatory experiment.** With the DMSO control the viability was 88 %.

Part II (DNA-synthesis):

At the highest concentration (50 µg/ml) nearly complete inhibition of DNA synthesis occurred. At all other concentrations (0.46, 1.39, 4.17, 12.5 and 25 µg/ml), DNA-synthesis was reduced by 47-64%, although without obvious concentration-dependency.

Original DNA-repair test

Neither the evaluation of the mean gross number of silver grains per nucleus nor the net values of grains per nucleus revealed a statistically significant difference from the respective vehicle control.

Table B.6.4.1.4-1: UDS test on rat hepatocytes *in vitro* with difenoconazole (original experiment)

Treatment	Total (gross) nuclear grain counts	Cytoplasm (gross) grain counts	net nuclear grain counts	% cells in S-phase
DMSO, negative control	2.05	1.36	0.69	0.66
2-acetylaminofluorene, 45 µM, positive control	11.47	2.30	9.17	0.66
<u>Difenoconazole tech.</u>				
50 µg/ml	1.96	1.12	0.84	0
25 µg/ml	2.44	1.43	1.01	0
12.5 µg/ml	2.76	1.55	1.21	0
4.17 µg/ml	2.62	1.68	0.94	0
1.39 µg/ml	2.49	1.42	1.07	0
0.46 µg/ml	2.45	1.76	0.69	0.66

At the concentrations of 25 µg/ml and above, precipitates in the culture medium were observed.

Confirmatory DNA-repair test

Evaluation of the mean gross number of silver grains per nucleus revealed a statistically significant increase in comparison to the negative control at the concentration of 4.17 µg/ml. Evaluation of the net number of silver grains per nucleus revealed a statistically significant increase at the concentrations of 4.17 and 12.5 µg/ml.

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Table B.6.4.1.4-2: UDS test on rat hepatocytes *in vitro* with difenoconazole (confirmatory experiment)

Treatment	total nuclear grain counts	cytoplasm grain counts	net nuclear grain counts	% cells in S-phase
DMSO, negative control	1.92	1.52	0.40	1.32
2-acetylaminofluorene, 45µM, positive control	12.49	3.24	9.26	1.32
Difenoconazole tech..				
50 µg/ml	1.95	1.25	0.70	0
25 µg/ml	2.23	1.58	0.65	0
12.5 µg/ml	2.49	1.33	1.17***	1.96
4.17 µg/ml	2.91***	1.83	1.08***	2.60
1.39 µg/ml	2.53	1.90	0.63	1.96
0.46 µg/ml	2.71	1.79	0.92	0

However, these increases were neither reproducible nor did they show a concentration dependency and may be due to the low variance of the test values and the low negative control counts. P values < 0.01 were considered significant.

Conclusion:

Under the present test conditions, the test substance or its metabolites did not induce a biologically significant increase in the DNA-repair activity of primary rat hepatocytes.

B.6.4.1.5 *In Vitro* Mammalian Chromosome Aberration Test - Chinese Hamster Ovary (CHO) Cells I

Reference:	Lloyd M (2001). CGA 169374 tech.: Induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells.
Guideline:	OECD TG 473
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole (CGA 169374) tech. / 94.3%
Species / Strain:	cultured Chinese hamster ovary (CHO) cells
Doses:	Cells were incubated with 18 doses ranging from 9.007 to 400.0. Analyses for chromosome aberrations were performed on selected concentrations.
Administration:	Test substance dissolved in DMSO added to the cells in culture medium
Exposure time:	3 h

Materials and Methods:

The test substance was dissolved in dimethyl sulfoxide (DMSO). Duplicate cultures of CHO cells were used at all dose levels and for positive controls in two independent experiments. Four cell cultures per experiment were used for the negative controls (DMSO). Treatment was for 3 h followed by a 17-hour recovery period.

Chromosome aberrations were analysed at 3 dose levels in the first experiment, and in the second experiment at 3 and 4 dose levels in the absence and presence of metabolic activation (S-9), respectively. Colchicine arrested dividing cells in metaphase. The cell numbers were determined using a Coulter counter. **In experiment 1**, Cells

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were examined for mitotic index (at least 1000 cells counted). From selected concentrations, 200 metaphases were scored for chromosome aberrations. The highest concentration scored was selected to give close to 50% reduction in cell number and/or mitotic index.

Results:

In the first experiment concentrations of 42.95 µg/ml (without metabolic activation) and 67.11 µg/ml (with metabolic activation) and above, caused strong cytotoxicity. In the experiment without metabolic activation, the highest concentration (34.36 µg/ml) revealed an incidence of 14% of cells with chromosomal aberrations. When this concentration was re-analysed, however, the frequency of cells with aberrations was 6% only and fell within the historical negative control ranges. The highest concentration (67.11 µg/ml) tested in the part with metabolic activation revealed 30% of cells with chromosomal aberrations. When this concentration was re-analysed, a similar incidence of aberrant cells was observed (33%). This value differs significantly from that of the concurrent negative control and **exceeds the historical negative control ranges**. At this concentration the mitotic index was reduced to 41% of that of the negative control. The effect was not repeated in the confirmatory second experiment.

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Table B.6.4.1.5-1: Mitotic Index (% of control) and percent incidence of specific chromosomal aberrations in CHO cells treated with difenoconazole

	Experiment 1				Experiment 2			
	-		+		-		+	
Metabolic Activation								
Treatment	3		3		3		3	
(h)								
Recovery	17		17		17		17	
(h)								
	Mean aberrant cells, excluding gaps	Mean mitotic Index	Mean aberrant cells, excluding gaps	Mean mitotic Index	Mean aberrant cells, excluding gaps	Mean mitotic Index	Mean aberrant cells, excluding gaps	Mean mitotic Index
Negative control	1		1		2		0	
CGA								
169374								
tech..								
21.99	0	94	--		0	80	--	
µg/ml								
27.49	0	88	--		3	65	--	
µg/ml								
34.36	6 (14*)	48	0	100	3	54	4	100
µg/ml								
53.69	--		3	67			3	63
µg/ml								
67.11	--		33* (30*)	41	--		0	55
µg/ml								
83.89	--		--		--		8	43
µg/ml								
Positive control	38* ^a		75* ^b		34* ^a		70* ^b	
200 cells with well spread metaphase figures were scored, except for positive controls where 50 cells were scored								
-- not scored								
^a 4-Nitroquinoline-1-oxide, 0.125 µg/ml								
^b Cyclophosphamide, 6.25 µg/ml								
* numbers highlighted exceed historical negative control range								
() numbers in brackets represent results of the original analysis								

In the second experiment without metabolic activation, concentrations of 42.95 µg/ml and higher caused strong toxicity. In the part with metabolic activation, considerable cytotoxicity (M.I. 10%) was observed at the highest concentration only (104.90 µg/ml). In the second experiment, treatment of cultures in the absence and in the presence of metabolic activation resulted in frequencies of cells with structural aberrations which were similar to those in concurrent negative controls and fell within historical negative control ranges.

Mitotic indices for the positive controls were not reported.

Conclusion:

In the presence of metabolic activation, the incidence of chromosome aberration was increased in Chinese hamster cells exposed to 67.11 µg Difenoconazole/ml. The increase exceeds the historical control range, but might be due to cytotoxicity (M.I. 59% reduction). The effect was not repeated in the confirmatory experiment under corresponding conditions.

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B.6.4.1.6 *In Vitro* Mammalian Chromosome Aberration Test – Human Lymphocytes II

Reference:	Fox V (2001). CGA 169374 tech.: <i>In vitro</i> cytogenetic assay in human lymphocytes.
Guideline:	OECD TG 473
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole (CGA 169374) tech. / 94.3%
Species / Strain:	Human lymphocytes
Doses:	1-75 µg/ml With and without microsomal activation
Administration:	Test substance dissolved in DMSO added to the cell suspensions
Exposure time:	3 h

Materials and Methods:

Cultures were established from heparinized blood samples obtained from healthy non-smoking donors. Mitomycin C, a mutagen not requiring metabolic activation, and cyclophosphamide, which requires metabolic activation, were used as positive controls. Two independent experiments were carried out. **In experiment 1**, cultures were exposed for 3 h with and without metabolic activation (S-9). **In experiment 2**, cultures were exposed for 3 h with metabolic activation and 20 h without metabolic activation. Colcemide arrested the cells in metaphase prior to harvesting. The cells were resuspended, fixed and dropped on microscope slides, which were air dried, stained and mounted with cover slips in DPX. Where appropriate, the percentage of mitotic suppression was determined by evaluating 1000 cells from each culture. Where possible, two hundred metaphase figures from altogether two cultures (100 metaphases per replicate culture) in the vehicle and positive control and in the treated groups were scored. The cells were exposed to 12 concentrations ranging from 0.1-75 µg/ml. The following concentrations were selected for chromosomal aberration analysis based on reductions in mitotic activity and cytotoxic effects on the chromosomes.

Data Evaluation

The Fisher Exact Probability test (one-sided) was used to statistically evaluate the percentage of metaphases showing aberrations. Data is interpreted as **negative** if a) no statistically significant increase is present in the percentage of aberrant cells above concurrent solvent control values b) a statistically significant increase in the percentage of aberrant cells above concurrent solvent control values is present but falls within the laboratory solvent control range. Data is interpreted as **positive** if a) an increase in the percentage of aberrant cells, at least at one concentration, is present, which is substantially greater than the laboratory historical solvent control range. (A statistically significant increase in the percentage of aberrant cells above concurrent solvent control values, and at the historical solvent control range upper value may require further evaluation).

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Table B.6.4.1.6-1

Experiment 1		Experiment 2	
With activation 3-hour exposure	Without activation 3-hour exposure	With activation 3-hour exposure	Without activation 20-hour exposure
62 µg/ml	75 µg/ml	50 µg/ml	10 µg/ml
30 µg/ml	30 µg/ml	30 µg/ml	5 µg/ml
5 µg/ml	5 µg/ml	5 µg/ml	1 µg/ml

Results:

Significant reductions in mitotic activity (compared to the solvent control values) were observed in cultures from both experiment 1 (56% -S9 mix, 62% +S9 mix) and experiment 2 (53% -S9 mix, 56% + S9 mix) treated with the highest concentrations of CGA 169374 tech. selected for chromosomal analysis.

The increase in the percentage of aberrant cells observed in cultures treated with 5 µg/ml in experiment 2, in the presence of S9 mix, was within the laboratory historical control range, not concentration related and not reproducible in experiment 1. It is therefore considered to be of no biological significance.

Table B.6.4.1.6-2: Mitotic index (% of control) and percent incidence of chromosomal aberrations in human lymphocytes treated with difenoconazole

	Experiment 1				Experiment 2			
	-	+	-	+	-	+	-	+
Metabolic activation	-	+	-	+	-	+	-	+
Treatment (h)	3	3	3	3	20	20	3	3
Recovery (h)	17	17	17	17	0	0	17	17
	Mean aberrant cells, excluding gaps	Mean mitotic Index	Mean aberrant cells, excluding gaps	Mean mitotic Index	Mean aberrant cells, excluding gaps	Mean mitotic Index	Mean aberrant cells, excluding gaps	Mean mitotic Index
Negative control CGA 169374 tech.	0		0		3		0.5	
75 µg/ml	0	44 ^d	--		--		--	
62 µg/ml	--		0	38 ^d	--		--	
50 µg/ml	--		--		--		2	44 ^d
40 µg/ml	--		--		--		--	
30 µg/ml	0	60 ^d	0	69 ^d	--		1	63 ^d
20 µg/ml	--		--		--		--	
10 µg/ml	--		--		1.5	47 ^d	--	
5 µg/ml	0	102	0.5	108	3	48 ^d	4*	118
2.5 µg/ml	--		--		--		--	
1 µg/ml	--		--		76		--	
0.5 µg/ml	--		--		--		--	
0.1 µg/ml	--		--		--		--	
Positive control	20** ^a	68	22** ^c	72	48** ^b	85	40** ^c	55

--not scored or no culture at this concentration

^aMitomycin-C, 0.5 µg/ml

^bMitomycin-C, 0.2 µg/ml

^cCyclophosphamide, 50 µg/ml

^dcytotoxic effects on the chromosomes

* p < 0.05; ** p < 0.01 using Fisher's Exact Test (one-sided).

Conclusion:

It is concluded that, under the conditions of this assay, difenoconazole tech. is not clastogenic to cultured human lymphocytes treated *in vitro* in either the presence or absence of metabolic activation.

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B.6.4.1.7 *In Vitro* Mammalian Chromosome Aberration Test - Chinese Hamster Ovary Cells II

Reference:	Ogorek B (2001). CGA 169374 tech.: Cytogenetic test on Chinese hamster cells <i>in vitro</i>.
Guideline:	OECD TG 473
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole (CGA 169374) tech. / 94.3%.
Species / Strain:	Chinese hamster ovary cells (ATCC CCL61)
Doses:	<u>cytotoxicity evaluation:</u> 2.3 - 200.0 µg/ml <u>Original experiment without metabolic activation:</u> 26.3, 39.5 and 59.3 µg/ml <u>Confirmatory experiment without metabolic activation:</u> 2.3, 5.2 and 11.7 µg/ml <u>Original experiment with metabolic activation:</u> 11.7, 17.6 µg/ml, deviates from guideline, see comments. <u>Confirmatory experiment with metabolic activation:</u> 7.8, 11.7, 17.6 µg/ml
Administration:	Test substance dissolved in DMSO added to the cells in culture medium
Exposure time:	3 h, except in the confirmatory experiment without metabolic activation where it was 21 h. See comments.

Materials and Methods:

The cells were incubated *in situ* on glass slides in culture dishes with several concentrations of the test article. Mitomycin C (0.2 µg/ml), a mutagen not requiring metabolic activation, and cyclophosphamide (20.0 µg/ml), which requires metabolic activation, were used as positive controls. Quadruplicate cultures were prepared for each group in each assay. Colcemide arrested the cells in metaphase prior to harvesting. The cells were fixed, air dried and stained. A cytotoxicity test (measurement of mitotic index) was performed as an integral part of the mutagenicity test. The percentage of mitotic suppression was determined by evaluating at least 2000 cells from each slide. Whenever possible two hundred well spread metaphase figures with 19 to 21 centromeres from two cultures (100 metaphases per replicate culture) in the vehicle control and in the treated groups were scored for chromosomal aberrations. At least fifty metaphases were scored in the positive controls (25 per replicate culture). The slides were examined for specific and unspecific structural aberrations. The numbers of specific aberrations were statistically evaluated by a chi-square test.

Criteria for a positive response:

Under the conditions of the test laboratory, a test item is considered to be active in the Chinese hamster cells if a) the percentage of metaphases containing specific aberrations in a dose group is higher than 6.0 (based on historical negative control range) and produces a statistically significant increase compared with the respective value of the the negative control b) a concentration-related response is demonstrable.

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Criteria for a positive response:

Under the conditions of the test laboratory, a test item is considered to be inactive in the Chinese hamster cells if

a) the percentage of metaphases containing specific aberrations in all dose groups is less than or equal to 6.0 (based on historical negative control range) and does not produce a statistically significant increase compared with the respective value of the negative control.

The percentage of metaphases showing specific chromosomal aberrations should be less than 6.0.

Results:

The concentrations from 200 µg/ml (solubility limit) down to 88.9 µg/ml caused strong cytotoxicity. The lowest concentration causing > 50% suppression in the mitotic activity (compared to the control group) was selected as the highest for the analysis of chromosome aberrations together with two lower concentrations.

In the original experiment without metabolic activation (experiment 1) at the concentration of 59.3 µg/ml a first analysis revealed a statistically significant increase in the number of metaphases with specific chromosomal aberrations (6.0%). A re-examination showed that the majority of the chromosome deletions and gaps were located in the X chromosome, at one and the same position. It has been reported by several authors that certain chemicals may preferentially induce undercondensed or achromatic regions, gaps or deletions in the long, heterochromatic arm of the X chromosome of Chinese hamster cells *in vitro*. CGA 169374 tech. appears to cause such localised effects at the X chromosome of CHO cells. These X chromosome aberrations are considered to be of no relevance for the assessment of the clastogenic properties of the test substance and were therefore excluded from the aberration scoring. The resulting frequency of specific aberration at the concentration of 59.3 µg/ml was then 2.5%. In the confirmatory experiment without metabolic activation (experiment 3) there was no statistically significant increase in aberrant metaphases.

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Table B.6.4.1.7-1: Mitotic index (% of negative control) and percent incidence of specific chromosomal aberrations in CHO cells treated with difenoconazole

Experiment	Original study				Confirmatory study			
	1	2	3	4	3	4	3	4
Metabolic activation	-	+	-	+	-	+	-	+
Treatment (h)	3	3	21	3				
Recovery (h)	18	18	-	18	-	-	-	-
	Mean aberrant cells	Mean mitotic Index	Mean aberrant cells	Mean mitotic Index	Mean aberrant cells	Mean mitotic Index	Mean aberrant cells	Mean mitotic Index
Negative control	2.0		2.0		1.5		1.0	
<u>CGA 169374 tech.</u>								
2.3 µg/ml	--	--	2.0	92	--	--	--	--
5.2 µg/ml	--	--	2.5	48	--	--	--	--
7.8 µg/ml	--	--	-		0.5	117		
11.7 µg/ml	--	4.5	64	45	2.5	52		
17.6 µg/ml	--	5.0	44	--	6.5*** (7.0***)	100		
26.3 µg/ml	1.5	104	--	--	--	--		
39.5 µg/ml	3.0	88	--	--	--	--		
59.3 µg/ml	2.5 (6.0**)	37	--	--	--	--		
Positive control	46.0 ^a		46.0 ^a		62.0 ^a		50.0 ^a	
200 cells with well spread metaphase figures were scored, except for positive controls where 50 cells were scored								
-- not scored								
^a Mitomycin-C, 0.2 µg/ml								
^b Cyclophosphamide, 20 µg/ml								
** p ≤ 0.01; *** p ≤ 0.001 (Chi-square test)								
() numbers in brackets represent results of the original analysis, i.e., including localised aberrations observed at the X chromosome.								

In the part with metabolic activation (experiment 4) at the concentration of 17.6 µg/ml, a statistically significant increased number of metaphases with specific chromosomal aberrations (7.0%) was observed. After re-examination of the slides from this experiment with regard to the possible presence of lesions at the X chromosome, one localised X chromosome aberration was found at the concentration of 17.6 µg/ml and the frequency of aberrant metaphases was reduced to 6.5%, which exceeds the historical negative control range (0.0 - 5.0%) and meets the criteria for a positive response. However, no positive response was observed in the original experiment (experiment 2) so **the result is considered to be equivocal.**

In experiments 2 and 3, no statistically significant increase in metaphases with chromosomal aberrations was observed, but only two analysable exposure concentrations were reported in experiment 2, see comments.

Conclusion:

A clastogenic effect of uncertain relevance was obtained in Chinese hamster ovary cells *in vitro* treated with CGA 169374 tech. at a concentration of 17.6 µg/ml in the presence of S9 mix. There was no sign of cytotoxicity at this concentration but the effect was not repeated in a confirmatory experiment.

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Comments:*Deviations from the OECD TG No 473*

Only two analysable concentrations are reported in the original experiment with metabolic activation (41.7 and 17.6 µg/ml). According to the OECD TG No. 473, at least three analysable exposure concentrations should be used for the chromosome aberration evaluation.

B.6.4.2 In vivo studies**B.6.4.2.1 Mammalian Erythrocyte Micronucleus Test**

Reference:	Ogorek, B. (1991). CGA 169374 tech.: Micronucleus test, mouse, <i>in vivo</i> study.
Guideline:	OECD TG 474, there were some deviations from this guideline, see comments.
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance / purity:	Difenoconazole (CGA 169374 tech.): 91.8 % Vehicle: Arachis oil
Species / Strain:	Tif: MAGf (SPF) mice
Doses / No. of animals:	400, 800 and 1600 mg/kg bw 8/dose/sex
Administration:	Oral gavage
Exposure time / Duration:	single dose, 16 to 48 h

Materials and Methods:

The animals were obtained from the animal farm at least one day prior to being used in the test. This is shorter than recommended, see comments. After a preliminary tolerability (limit) test using a dose of 2 000 mg/kg, a high dose (1600 mg/kg), a negative (arachis oil) and a positive (cyclophosphamide (CPA), 64 mg/kg) control dose were orally administered (10 ml/kg), as single doses, to groups of mice with 8 animals per sex (**part 1**). The groups treated with Difenoconazole and the negative control groups were killed after 16, 24, and 48 h. The positive control group was killed after 24 hours, see comments.

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Table B.6.4.2.1-1

Treatment	Sacrificed at 16 hours	Sacrificed at 24 hours	Sacrificed at 48 hours
Part 1			
Difenoconazole (1600 mg/kg)	X	X	X
negative control (arachis oil)	X	X	X
positive control (CPA, 64 mg/kg bw)		X	
Part 2			
Difenoconazole (1600 mg/kg)		X	
Difenoconazole (800 mg/kg)		X	
Difenoconazole (400 mg/kg)		X	
negative control (arachis oil)		X	
positive control (CPA, 64 mg/kg bw)		X	

In a second part, three groups of 8 male and 8 female mice were administered a low (400 mg/kg), an intermediate (800 mg/kg) and a high dose (1600 mg/kg) and killed 24 h after administration. Bone marrow was harvested and smears of five animals/sex/dose (showing good differentiation between mature and polychromatic erythrocytes (PCE)) were scored. In order to detect any disturbance of erythropoiesis the ratio of polychromatic to normochromatic erythrocytes was determined and 1000 polychromatic erythrocytes were scored for micronuclei from each animal. This is too few cells according to the OECD TG, see comments.

The significance of differences was assessed by the Chi-Square test ($p < 0.05$).

Criteria for a positive effect

The test substance is considered to be active in this test system if at any group treated with the test substance the mean number of micronucleated polychromatic erythrocytes exceeds 0.20%, and if there is a statistically significant difference in the number of micronucleated polychromatic erythrocytes in comparison with the control. If these criteria are not met, the test substance is considered inactive.

Results:

In the tolerability test, mortality was noted at 2000 mg/kg in one of four animals. Accordingly, 1600 mg/kg was used as high dose. In the definitive study, the animals exposed to 1600, 800 and 400 mg/kg bw showed clinical symptoms of ataxia, laterocumbency and piloerection, but no mortality.

In the first part of the micronucleus test, no significantly increased incidence of micronucleated polychromatic erythrocytes was noted after treatment with 1600 mg/kg at any sampling time (16, 24, and 48 h).

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Table B.6.4.2.1-2: Mouse micronucleus test on bone marrow cells: Percentage of micronucleated PCE, Part 1

Test article and concentration	Sex	16 hours	24 hours	48 hours
Difenoconazole (1600 mg/kg)	females	0.02	0.00	0.02
	males	0.08	0.04	0.02
	mean	0.05	0.02	0.02
Negative control (bidistilled water)	females	0.08	0.04	0.04
	males	0.02	0.04	0.00
	mean	0.05	0.04	0.02
Positive control Cyclophosphamide (64 mg/kg)	females		1.14*	
	males		1.54*	
	mean		1.34*	

* $p \leq 0.05$ (Chi-square test)

Similarly, in the second part of the micronucleus test, no increase in the incidence of micronucleated polychromatic erythrocytes was noted. The ratio of polychromatic to normochromatic erythrocytes after treatment with difenoconazole indicated no cytotoxic effects on blood forming cells.

Table B.6.4.2.1-3

Test article and concentration	Sex	24 hours
Difenoconazole (1600 mg/kg)	females	0.02
	males	0.02
	mean	0.02
Difenoconazole (800 mg/kg)	females	0.00
	males	0.02
	mean	0.01
Difenoconazole (400 mg/kg)	females	0.02
	males	0.00
	mean	0.01
Negative control (arachis oil)	females	0.02
	males	0.00
	mean	0.01
Positive control Cyclophosphamide (64 mg/kg)	females	0.78*
	males	1.44*
	mean	1.11*

* $p \leq 0.05$ (Chi-square test)

Conclusion:

Under the given experimental conditions no evidence for clastogenic or aneugenic effects was obtained in mice.

Comments:

Deviations from the OECD TG No 474

The animals were acclimatized for 1 day or longer before they were used in the experiments. According to the OECD TG the acclimatisation period should be at least 5 days (OECD TG 474, 1997). According to the OECD TG No. 474 (1997), bone marrow collecting should not start earlier than 24 h after treatment, earlier sampling times should be justified. From each animal, 1000 polychromatic erythrocytes were scored for micronuclei, which is fewer than the 2000 immature erythrocytes recommended in the OECD TG No. 474 (1997)

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B.6.4.3 Summary and conclusions on genotoxicity testing

In vitro, difenoconazole was negative in both bacterial and mammalian cell assays for gene mutation, negative for chromosomal damage in cytogenetic assays using isolated human lymphocytes and negative for DNA damage/repair in the unscheduled DNA synthesis assay. *In vivo*, difenoconazole was negative for chromosomal damage in the mouse bone marrow micronucleus assay. Increases in chromosomal aberrations were reported in CHO cells treated *in vitro* with difenoconazole, but only at high concentrations inducing cytotoxicity and they were not clearly reproducible either between repeat examinations of the same slides, between experiments or across studies. These observations are not considered of significance in light of the negative results in the other genotoxicity assays, including other *in vitro* and *in vivo* cytogenetic assays.

Table B.6.4.3-1: Survey of mutagenicity studies performed with difenoconazole

Study	Concentrations/ Dose levels	Results	Reference
Salmonella/E. coli <i>in vitro</i>	0 to 5447 µg/plate, +/-activation	negative	Ogorek, 1990
Gene mutation in mouse lymphoma L5178Y/TK ^{+/+} cells <i>in vitro</i>	0 to 150 µg/ml, - activation 0 to 50 µg/ml, + activation	negative	Dollenmeier, 1986a
Cytogenetic test on Chinese hamster cells <i>in vitro</i>	0 to 105 µg/ml, - activation 0 to 105 µg/ml, + activation	equivocal and non-reproducible positive response without and with metabolic activation at cytotoxic concentrations	Lloyd, 2001
Cytogenetic test on Chinese hamster cells <i>in vitro</i>	0 to 200 µg/ml, - activation 0 to 200 µg/ml, + activation	non-reproducible positive response with metabolic activation at one concentration	Ogorek, 2001
Cytogenetic test in human lymphocytes <i>in vitro</i>	0 to 40 µg/ml, - activation 0 to 40 µg/ml, + activation	negative	Strasser, 1985
Cytogenetic test in human lymphocytes <i>in vitro</i>	0 to 75 µg/ml, - activation 0 to 75 µg/ml, + activation	negative	Fox, 2001
DNA repair on rat hepatocytes <i>in vitro</i>	0 to 50 µg /ml	negative	Hertner, 1992
Micronucleus test mouse bone marrow <i>in vivo</i>	0, 400, 800, 1600 mg/kg bw	negative	Ogorek, 1991

From the results obtained in these tests *in vitro* and *in vivo* it is concluded that difenoconazole is not genotoxic.

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B.6.5 Long-term toxicity and carcinogenicity (Annex IIA 5.5)

B.6.5.1 Combined Chronic Toxicity/Carcinogenicity Study in rats

Reference:	Cox, RH. (1989a). Combined chronic toxicity and oncogenicity study of CGA-169374 technical in rats.
Report supplement:	Saunders, S (1992). Historical control data, SD rats, 104-week studies. Hazleton Washington, Vienna, VA, USA, supplement to report 483-249.
Guideline:	US-EPA FIFRA 83-5. This study deviates from the OECD TG No. 453 (1981) in some respects, see comments .
GLP:	Yes, verified by the U.S. EPA by means of inspections.
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical, batches: 851406 (weeks 1-20) and 861408 (weeks 21-106) / 94.5% and 95%, respectively. Vehicle: Acetone
Species / Strain:	Rat / (Sprague Dawley)® CRL: CD
Doses / No. of animals:	0, 10, 20, 500 or 2500 ppm corresponding to 0, 0.5, 1.0, 24.1 and 124 mg/kg/day for males and 0, 0.6, 1.3, 32.8 and 170 mg/kg/day for females / 80 and 90 animals/sex in the control and the 2 500-ppm groups, respectively
Administration:	Orally via the diet
Exposure time / Duration:	2 years (104 weeks) / Interim sacrifices at weeks 53 and 57 (including 4 weeks recovery) and terminal sacrifice at week 105.
Statistics:	Cumulative survival data were analysed using the National Cancer Institute Package. Levene's test checked homogeneity of variances for the data and Analysis of Variance (ANOVA) and Dunnet's post hoc test assessed significant differences amongst treatment groups and control.

Materials and Methods:

The doses were selected based on the results of two 3-month feedings studies in the rat (Suter, 1986b, Cox, 1987a). Viral serology screening for nine common infections was performed on 10 randomly selected animals/sex prior to treatment initiation. Ophthalmoscopic examinations (not mandatory) were performed on all animals prior to study initiation and on control and high-dose animals during weeks 28, 52, 78 and 104. Haematology, clinical chemistry and urinalysis were performed on 20 animals/sex at weeks 27, 52, 78 and 104. **Ten** animals/group/sex were sacrificed at week 53 (interim) and 10 animals/sex from the control and 2 500-ppm groups were placed on basal diet between weeks 53 and 56 (recovery) and then sacrificed, **see comments**.

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Results:

Diet analyses performed on each diet batch (prepared bi-weekly) revealed that the test item was present at the targeted concentrations with the exception of the week 7-8 mix at 500 ppm (44% of target) and week 47-48 mix at 10 ppm (77% of target). Homogeneous distribution in the diet mixes was demonstrated for the first set of diet mixes and stability for at least 16 days at room temperature was determined on 10 ppm samples.

General observations

There were **no difference in survival** between control and the treated groups at study termination. **Clinical observations** were without marked differences among the groups and considered to be incidental to treatment. Malocclusion (teeth not aligned properly), swelling of various body locations, lacrimation, alopecia, sores, thinness, soft faeces and hunched posture were the most frequent observations and occurred at all dose levels and in controls. **Body weights** of the 500 and 2500-ppm animals tended to be lower than in the control animals throughout the study and significant differences were recorded at weeks 52, 76 and 104.

Table B.6.5.1-1: Body weights, chronic toxicity/oncogenicity study in rats with difenoconazole (means)

Males						Females				
Dose level (ppm)	0	10	20	500	2500	0	10	20	500	2500
Body Weight (g)										
Week 0	262.1	270.4	269.7	268.7	269.1	182.9	181.0	182.2	178.6	178.5
Week 52	684.9	686.7	684.7	666.0	608.7* ↓ 11%	414.5	414.9	419.6	387.7* ↓ 6.5%	317.1* ↓ 23%
Week 76	673.9	675.0	666.9	665.1	619.6* ↓ 8%	434.1	442.6	438.2	415.9	336.3* ↓ 23%
Week 104	596.6	607.2	617.4	625.8	561.6	430.8	446.7	452.1	416.6	334.9* ↓ 22%
Body weight gain (g)										
Week 13	286.7	276.3	271.4* ↓ 5%	265.3* ↓ 7%	222.4* ↓ 22%	114.3	111.3	112.4	102.2* ↓ 11%	76.7* ↓ 33%
Week 24	350.7	340.7	339.0	325.7* ↓ 7%	273.0* ↓ 22%	150.6	154.4	151.2	136.0* ↓ 10%	101.8* ↓ 32%
Week 52	423.0	416.5	415.5	397.4* ↓ 6%	339.2* ↓ 20%	231.5	233.9	237.4	209.1* ↓ 10%	138.8* ↓ 40%
Week 104	331.8	339.5	352.4	358.9	293.8* ↓ 11%	250.9	270.3	268.7	238.1	157.8* ↓ 37%

* = p < 0.05. ANOVA + Dunnett

Significantly lower values for **body weight gain** were noted from week 13 in the 2 500-ppm animals and by study termination, the weight gains of males and females of this group were 11% and 37% lower than the control male and females, respectively. The significant reduction in weight gain for the 20- and the 500-ppm animals did not persist at termination (week 104). The mean values for **food consumption** were generally lower for the animals in the 2 500-ppm group than for the control group and statistical differences were recorded at weeks 52, 76 and 104. For the males however, the significant reductions were < 8%. The time points for this finding correspond to the observations of reduced body weight and lower body weight gain in the 2500-ppm dose group. There were no records over the water intakes.

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Table B.6.5.1-2: Food consumption, chronic toxicity/oncogenicity study in rats with difenoconazole (means)

	Males					Females				
Dose level (ppm)	0	10	20	500	2500	0	10	20	500	2500
Food Consumption (g/animal/week)										
Week 1	189.8	188.1	188.8	186.8	166.0	143.0	143.5	142.5	135.7	131.4
Week 52	166.1	163.9	162.8	161.5	154.2* ↓ 7%	131.8	133.1	133.4	125.7	114.1* ↓ 13%
Week 76	184.2	180.6	180.5	180.1	175.6* ↓ 5%	159.0	158.2	156.0	155.3	141.2* ↓ 11%
Week 104	165.5	173.9	175.1	168.7	163.4* ↓ 1%	155.7	156.3	150.5	148.4	134.3* ↓ 14%

* = p < 0.05. ANOVA + Dunnett [no statistics calculated week 1]

Ophthalmoscopic examinations of the control and 2500 ppm animals revealed no treatment-related findings.

Haematology, clinical chemistry and urinalysis

There was a **decrease in red cell mass** (RBC count, Hb, Hct), especially early in the study and especially for females of the 2 500-ppm group. All red cell values however, **are within normal range** for rats in the literature (Falkmer and Waller, 1994) and in most cases the red cell parameters were not significantly affected at study termination.

Table B.6.5.1-3: Haematology, chronic toxicity/oncogenicity study in rats with difenoconazole (means)

		Males					Females				
Dose level (ppm)		0	10	20	500	2500	0	10	20	500	2500
RBC (mi/μl)	wk 53	7.4	7.7	7.9	7.6	6.9	7.5	7.3	7.3	7.2	6.7* ↓ 10%
	wk 105	6.9	7.7	7.5	7.6	7.8	7.4	7.2	7.3	7.4	7.1
Hb (g/dl)	wk 28	14.4	14.9	15.5* ↑ 8%	14.7	14.1	15.3	15.2	14.7* ↓ 4%	14.6* ↓ 5%	14.1* ↓ 8%
	wk 53	14.7	15.1	15.4	15.1	14.3	15.1	15.0	15.1	14.8	14.1* ↓ 7%
	wk 105	12.5	14.2	13.3	13.6	13.7	14.6	14.0	14.5	14.4	13.4
Hct (%)	wk 53	40.5	41.8	42.3	41.0	36.4* ↓ 10%	43.0	42.4	42.5	41.5	37.2* ↓ 13%
	wk 105	36.5	40.9	38.7	39.6	39.0	42.4	40.5	42.2	41.8	38.8* ↓ 8%
Platelets (th/μl)	wk 53	1123	1061	1063	994* ↓ 20%	855* ↓ 24%	944	910	949	878	833
	wk 105	1512	1328	1409	1419	1222* ↓ 19%	1113	1105	1038	1043	1024
WBC (th/μl)	wk 105	12.8	11.9	12.1	10.3	9.0* ↓ 30%	8.5	7.5	8.6	6.2	5.4* ↓ 36%
segmented neutrophils (th/μl)	wk 105	6.5	-	-	-	4.7* ↓ 28%	4.7	-	-	-	2.4* ↓ 49%
lymphocytes (th/μl)	wk 105	5.8	-	-	-	4.0* ↓ 31%	3.7	-	-	-	2.9

* = p < 0.05, ANOVA + Dunnett

Platelet counts were significantly lower than control values for males in the 500-ppm and the 2 500-ppm groups. However, the recorded platelet values are all **within normal literature range** for rats. **Leukocyte counts** were depressed for 2 500 ppm males and females at week 105, resulting from lower absolute segmented

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neutrophil (neutropeni) and lymphocyte (lymfopeni) counts. Also for the total WBC count, the recorded values are **within normal range** for rats in the literature (Falkmer and Waller, 1994). Other statistically significant differences are considered to be of no toxicological importance due to inconsistency and lack of dose-response.

Changes in clinical chemistry parameters included **increased albumin** and **decreased globulin** levels in 2 500-ppm males, resulting in **increased A/G ratios** throughout the study. Serum albumin levels were elevated in 2 500-ppm females in the beginning of the study only. **Total protein** levels however, were **unaffected** and within normal range for rats (Falkmer and Waller, 1994). Thus, the albumin increase is probably an effect of dehydration.

Table B.6.5.1-4: Clinical chemistry, chronic toxicity/oncogenicity study in rats with difenoconazole (means)

Dose level (ppm)		Males					Females				
		0	10	20	500	2500	0	10	20	500	2500
total protein (g/dl)	wk 105	6.8	6.9	7.0	6.8	7.1	7.4	7.5	7.5	7.4	7.7
albumin (g/dl)	wk 53	3.7	3.7	3.7	3.7	3.9*	4.6	4.6	4.7	4.7	4.8
	wk 105	3.8	4.1	4.0	3.9	4.6* ↑ 5% ↑ 21%	5.0	4.8	4.8	5.2	5.3
globulin (g/dl)	wk 53	3.1	3.1	3.2	3.1	2.8* ↓ 10%	3.1	3.0	3.1	3.0	3.0
	wk 105	3.0	2.8	3.0	2.9	2.5* ↓ 17%	2.4	2.7	2.7	2.2	2.3
A/G ratio	wk 53	1.18	1.23	1.17	1.20	1.39*	1.49	1.57	1.52	1.58	1.61
	wk 105	1.28	1.48	1.43	1.44	1.90*	2.28	1.87	1.99	2.56	2.59
Alanine aminotransferase, ALAT (ukat/l)	wk 53	33	34	31	47* ↑ 42%	71* ↑ 115%	41	35	33	30	28* ↓ 32%
	wk 105	40	43	40	50	42	58	36	35	50	38
glucose (mg/dl)	wk 28	124	119	114	115	109* ↓ 12%	111	113	112	109	102* ↓ 8 %
cholesterol (mg/dl)	wk 28	97	93	102	112	119* ↑ 23%	114	109	122	129	146* ↑ 28%
	wk 105	119	128	128	117	176* ↑ 48%	146	133	134	145	166
total bilirubin (mg/dl)	wk 28	0.16	0.14	0.17	0.12	0.09* ↓ 44%	0.12	0.14	0.15	0.09	0.04* ↓ 67%
	wk 53	0.19	0.19	0.17	0.16	0.14	0.26	0.28	0.28	0.21	0.07* ↓ 73 %
	wk 105	0.11	0.09	0.13	0.10	0.08	0.11	0.12	0.13	0.09	0.08

* = p < 0.05, ANOVA + Dunnett

Alanine aminotransferase (ALAT) activities were increased in 500 and 2 500-ppm males at week 53 but decreased in 500 and 2500-ppm females at week 28 and 2 500-ppm females at week 53. The inconsistency of these changes suggests that there was no relationship to treatment. There was a transient **decrease in glucose** (males and females) and a transient **increase in total cholesterol** (females) at week 28 in the 2 500-ppm group possibly **reflecting the nutritional status** of these animals. The **decreases in total bilirubin** in 2 500-ppm males at week 28 and 2500-ppm females at week 28, 53 and 79 could reflect reductions in Hb-values as bilirubin is a degradation product of haem-proteins including Hb. Moreover, the statistical change was not consistent at termination of the study.

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There was an increase in urine **ketone bodies** and decrease in pH in 2 500-ppm males at week 28, which would be consistent with the diminished nutritional status of these animals.

Gross pathology, organ weights and histopathology

Macroscopic examinations did not reveal any treatment-related findings. The **terminal body weights** (carcass) of the 2 500-ppm animals were lower than controls at weeks 53 (males and females) and 105 (females). There was no difference in carcass weight between the groups after the 4-week recovery period. Absolute **liver weights** were **unaffected** among the groups during the study. The relative **liver weights** for the 2 500-ppm animals however, were higher than control values at week 53 (interim sacrifice) and at termination of the study (week 105), but were similar to control values following the 4-week recovery period. The time points and dose groups of the reductions in carcass weights and increase in relative liver weights correspond.

Table B.6.5.1-5: Selected organ weights, chronic toxicity/oncogenicity study in rats with difenoconazole (means)

		Males					Females				
Dose level (ppm)		0	10	20	500	2500	0	10	20	500	2500
Week 53 (Interim Sacrifice, n=10 per group)											
Carcass weight (g)		651.7	653.7	665.9	629.4	582.2* ↓ 11%	401.7	403.3	377.3	364.5	317.1* ↓ 21%
liver	abs.	16.97	16.84	17.84	16.83	17.36	9.82	10.23	8.82	10.11	11.36
	rel.	2.61	2.57	2.69	2.66	2.98* ↑ 14%	2.45	2.52	2.34	2.79	3.63* ↑ 48%
adrenals	abs.	0.07	0.06	0.07	0.06	0.05* ↓ 24%	0.12	0.10	0.09	0.10	0.09
brain	rel.	0.36	0.36	0.34	0.36	0.38	0.53	0.51	0.55	0.58	0.66*
heart	rel.	0.26	0.26	0.27	0.26	0.27	0.31	0.30	0.31	0.33	0.36*
kidney	rel.	0.55	0.57	0.59	0.58	0.60	0.62	0.61	0.56	0.65	0.80*
Week 57 (Recovery Sacrifice, n=10 per group)											
Carcass (g)		636.5	-	-	-	593.8	391.2	-	-	-	352.5
Liver	abs.	15.16	-	-	-	14.35	9.25	-	-	-	9.12
	rel.	2.40	-	-	-	2.43	2.43	-	-	-	2.61
Spleen	abs.	0.86	-	-	-	0.87	0.61	-	-	-	0.50* ↓ 18%
Week 105 (Terminal Sacrifice, n=9 or 10 per group^a)											
Carcass (g) ^a		576.5	578.4	594.6	599.8	545.1	401.6	415.5	433.3	390.3	313.2* ↓ 22%
Liver	abs.	14.83	15.68	14.02	14.78	16.26	11.82	9.75	10.28	10.91	12.53
	rel.	2.63	2.50	2.52	2.37	3.09	2.53	2.41	2.44	2.88	3.63* ↑ 43%
Brain	rel.	0.40	0.36	0.43	0.37	0.43	0.45	0.51	0.49	0.59	0.61*
Ovaries	abs.	-	-	-	-	-	0.10	0.12	0.10	0.19	0.19* ↑ 89%
	rel.	-	-	-	-	-	0.022	0.031*	0.024	0.046*	0.051*

Note: All absolute weights (abs.) in grams; relative (rel.) organ weights = % of body weight (g/g*100), * = p < 0.05, ANOVA + Dunnett, ^aTerminal body weight means presented are based on ALL animals at terminal sacrifice

The other statistically significant differences were considered not to be toxicologically relevant; decreased absolute **adrenal weights** at week 53 in 2 500-ppm males and **spleen weights** at week 57 in 2 500-ppm females were not seen at other intervals, and the increased **ovary weights** at week 105 (500 n.s. and 2 500 ppm) were attributable to one animal in each group with massive ovarian cysts.

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Histopathological examinations revealed an increased incidence and severity of **hepatocellular hypertrophy** in 500 and 2 500 ppm animals **at study termination**. For males, the incidence was 65 and 89%, respectively in the 500 and 2 500-ppm dose groups. Corresponding values for females were 34 and 84%. These changes were not evident at the interim sacrifice at week 53.

Table B.6.5.1-6: Liver histopathology findings, chronic toxicity/oncogenicity study in rats with difenoconazole (incidence)

		Males										Females									
Dose level (ppm)		0		10		20		500		2500		0		10		20		500		2500	
liver		n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex
		Treatment-Related Effect																			
hepatocyte hypertrophy	U	0	30	0	39	0	30	1	27	0	26	0	38	0	35	0	44	3	29	4	32
	I	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10
	R	0	10	0	-	0	-	0	-	0	10	0	10	0	-	0	-	0	-	0	10
	T	7	40	5	31	8	40	28	43	39	44	4	32	0	35	0	26	14	41	32	38
minimal severity		7		4		5		24		7		4		-		-		10		18	
slight severity		0		1		3		3		27		0		-		-		4		8	
moderate severity		0		0		0		1		5		0		-		-		0		6	
n = number of animals with finding, ex = number of animals examined																					
\$: U = unscheduled deaths I = interim sacrifice week 53 R = recovery sacrifice week 57 T = terminal sacrifice week 79-80																					

The other liver findings, including neoplasia, were considered to be incidental due to the lack of a dose response and/or the low incidence. The **other histopathological changes** that were noted did **not** give any indication of a **treatment-related** association and there was no compound-related increase in neoplasia in treated animals.

Table B.6.5.1-7: Ovary histopathology findings, chronic toxicity/oncogenicity study in rats with difenoconazole (incidence at terminal sacrifice week 79-80)

		Males					Females				
Dose level (ppm)		0	10	20	500	2500	0	10	20	500	2500
Number of animals examined		-	-	-	-	-	32	34	26	41	38
number of animals with finding											
Paraovarian cyst		-	-	-	-	-	1	4	1	2	3
Ovarian cyst		-	-	-	-	-	2	6	3	7	6
Medullary tubular hyperplasia		-	-	-	-	-	19	17	14	14	14
Benign granulosa/Theca cell tumor		-	-	-	-	-	0	1	0	0	1

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Table B.6.5.1-8: Neoplastic Findings (no relation to treatment), chronic toxicity/oncogenicity study in rats with difenoconazole (incidence)

dose level (ppm)	Males					Females				
	0	10	20	500	2500	0	10	20	500	2500
total examined ⁿ	90	80	80	80	90	90	80	80	80	90
hepatocellular adenoma	4	1	0	0	0	3	1	0	0	0
hepatocellular carcinoma	1	3	1	1	1	0	0	0	0	0
histiocytic sarcoma	1	1	0	0	3	1	0	0	1	1
leukaemia, granulocytic	0	0	0	0	0	0	0	1	0	0
malign. lymphoma, histiocytic	0	0	0	1	0	1	0	0	0	0
malign. lymphoma, lymphocytic	1	0	1	0	3	0	1	0	0	0
mesothelioma	0	0	0	0	0	0	0	1	0	0
paraganglioma	0	0	0	0	1	0	0	0	0	0

Summary:

Dietary administration of difenoconazole technical to rats resulted in a decrease (8-23%) in absolute body weight values in the 2 500-ppm dose group and a dose-related decrease (6-40%) in body weight gain in the 500 and 2 500-ppm dose groups. Mean food consumption was consistently lower (1-14%) for the 2 500-ppm animals as compared with controls. There was a negative effect on the red cell mass in the 2 500-ppm females however the effect is **not regarded to be adverse**. Relative liver weight was increased (14-48%) in the 2 500-ppm animals at weeks 53 and 105, but in the 2 500-ppm recovery animals it was similar to the control animals, indicating that **the liver enlargement is adaptive** during exposure and that the effect is reversible after exposure cessation. There were **no treatment-related macroscopic findings**; increased incidence and severity of hepatocellular hypertrophy were noted in 500 and 2 500-ppm males and females. **No treatment-related increases in neoplastic findings occurred during the study.**

Conclusion:

The **NOAEL** (No-Observable-Adverse-Effect Level) was considered to be **20 ppm** (1.0 and 1.3 mg/kg bw per day for males and females, respectively) based upon reductions in body weight gain and absolute body weight at 500 and 2500 ppm. The **NOEL** (No-Observable-Effect Level) was also considered to be 20 ppm based on reductions in body weight, body weight gain, Hb (females) and platelet count (males) and increase in ALAT levels and hepatocellular hypertrophy observed at the 500 ppm-level. The reductions in body weight gain (males) and in Hb (females) observed at 20 ppm were early observations that did not persist at term and were therefore considered to be negligible.

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Comments:*Deviations from the OECD TG No. 453*

According to the TG, each treatment and control group not intended for earlier sacrifice than terminal sacrifice should contain at least 50 animals of each sex. Satellite groups should consist of **20** and 10 animals per sex in treatment and control groups, respectively. If interim sacrifices are included, the initial number of animals should be increased by the number of animals scheduled for the interim sacrifices. Thus, this may be interpreted so that the number of animals sacrificed at interim time points (at weeks 53 and 57) is too low, for thorough biological and statistical evaluations.

B.6.5.2 Oncogenicity study in mice

Reference:	Cox, R.H. (1989b). CGA 169374: Oncogenicity study in mice.
Guideline:	US-EPA FIFRA 83-2. There were deviations from the OECD TG No 451 (1981), see comments.
GLP:	Yes, verified by the U.S. EPA by means of inspections.
Acceptability:	Yes
Test substance / purity:	Difenoconazole technical batches 851406 (weeks 1-20) and 861408 (weeks 21-80) / 94.5 and 95%, respectively.
Species / Strain:	Mouse / CD-1®(ICR)
Vehicle:	Acetone
Doses / No. of animals:	0, 10, 30, 300 , (3000 ppm 1 st two weeks reduced to 2500 ppm the remaining weeks) and 4500 ppm corresponding to 0, 1.5, 4.7, 46.3, 423 and 819 mg/kg per day for males and 0, 1.9, 5.6, 57.8 and 513 mg/kg per day for females in the 10, 30, 300, 2500 (and 4500 ppm dose groups for males), respectively / 60 per sex per group or 70 per sex in the control-, 3000- and 4500-ppm groups.
Administration:	Orally, via the diet
Exposure time / Duration:	18 months
Statistics:	Cumulative survival data were analyzed by the National Cancer Institute Package. Levene's test checked homogeneity of variances for data and Analysis of Variance (ANOVA) with Dunnet's post hoc test assessed significant differences amongst treatment groups and control.

Materials and Methods:

Animals were approximately 8 weeks of age at initiation, **see comments**. The test material was given to 5 groups of mice (groups 2-6) and an additional control group received basal diet mixed with vehicle (group 1). The treatment began with 60 CD-1®(ICR) mice per sex in each dose group plus an additional 10 animals per sex in the control-, the 3 000- and the 4 500-ppm groups (for a recovery phase of 4 weeks after 12 months of treatment), see table. The doses were selected based on the results of a 3-month study (Cox, 1987b).

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However, all of the 4 500-ppm females and 16 (out of 70) of the 3 000-ppm females died within the first 2 weeks. **The following changes were therefore made:** (a) the 4 500-ppm female group was considered terminated; (b) the 3 000-ppm dose was lowered to 2 500 ppm for both males and females, (c) the 10 female control-recovery animals were moved to the 2 500-ppm group (kept on study 2 weeks longer at termination), (d) no control females were sacrificed as recovery animals, see table. There were sacrifices at weeks 53 (interim), 57 (recovery) and 79-80 (termination).

Eye examinations (not mandatory) were conducted on all animals during the pre-test period and on all control and high-dose animals at 6-month intervals thereafter. Haematology investigations consisted of differential counts and cell morphology on (a) 10 animals/sex during pre-test, (b) 10 control and high-dose animals/sex at 52 and 78 weeks, (c) all (10/sex) recovery animals at week 57 and (d) all animals sacrificed moribund, **see comments**. Clinical chemistry investigations (not mandatory) were also performed on (a) 10 animals/sex during pre-test (animals not assigned to the study), (b) 10 control and high-dose animals/sex at 52 and 78 weeks and (c) all (10/sex) recovery animals at week 57. Ten animals/sex/ group were sacrificed during week 53. Ten additional females from the 2 500-ppm group and 10 males from the control-, the 2 500- and the 4 500-ppm dose groups were placed on control diet from weeks 53-56 and sacrificed during week 57. The remaining surviving animals were sacrificed during week 79 or 80. Viral serology screening for 12 common infections was performed on 10 animals/sex prior to treatment.

Results:

Mortality

All (70) females in the 4 500-ppm dose group died or were sacrificed in a moribund condition during the first 2 weeks. Eleven males (out of 70) in the 4 500-ppm dose group died or were sacrificed for the same reason during the first 3 weeks of the study. At the next lower dose, 3 000 ppm, 15 (out of 70) females died or were sacrificed during the first week, which lead to a reduction in dose to 2 500 ppm for both sexes of this dose group, beginning at week 2 of the study. After the lowering of dose, one additional female died during the second week of experiment and 3 out of 10 replacement animals (from the control group arriving at beginning of week 3) were sacrificed due to moribund during their first week of exposure to 2 500 ppm. The early deaths were accompanied by clinical signs of thinness, hunched posture and rough hair coat.

Survival for 4 500-ppm males was **significantly less** than for controls. Females from this dose group were excluded from the analysis since all died during the first 2 weeks.

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Table B.6.5.2-1: Mortality, oncogenicity study in mice with difenoconazole

	Males						Females					
Dose level (ppm)	0	10	30	300	2500 ^a	4500	0	10	30	300	2500 ^a	4500
n at initiation	70	60	60	60	70	70	70	60	60	60	70	70
died/moribund sac. days 1-9	0	0	0	0	0	3	0	0	0	0	16	52
moribund sac. day 10	0	0	0	0	0	1	0	0	0	0	0	18
re-assignment day 10							-10					
died/moribund sac. days 11-21	0	0	0	0	0	7	0	0	0	0	3	0
died/moribund sac. wks 4-52	2	2	2	4	0	3	3	3	4	4	2	0
interim sacrifice week 53	10	10	10	10	10	10	10	10	10	10	10	0
post-recovery sacrifice week 57	9 ^b	0	0	0	10	10	0	0	0	0	10	0
died/moribund sac. wks 53-68	9	8	8	6	2	7	10	5	6	6	1	0
died/moribund sac. wks 69-80	9	8	13	16	14	13	13	7	11	5	9	0
terminal sacrifice wks 79-80	31	32	27	24	34	16	24	35	29	35	29	0
% survival to termination^c	62	64	54	40	68	32	48	70	58	70	36	0

^adose level 3000 ppm through day 21, ^bone recovery-group animal died during recovery, ^cexcluding interim and post-recovery sacrifices

General observations

Thinness and hunched appearance were noted more frequently in the highest dose groups, 2 500 and 4500 ppm compared to controls and the incidence of reduced motor activity was increased for the 4 500-ppm males when compared with control. There was a dose-dependent **reduction in body weight** of treated animals. Males of the two highest dose groups (2 500 and 4500 ppm) had significantly lower body weights (below and about 10%, respectively) than control from week 1 throughout to week 56. Females of the highest dose group (2500 ppm) had significantly lower body weight ($\leq 10\%$) throughout the study period.

Table B.6.5.2-2: Body weights, Oncogenicity study in mice with difenoconazole (means)

	Males						Females					
Dose level (ppm)	0	10	30	300	2500 ^a	4500	0	10	30	300	2500 ^a	4500 ^b
Body Weight (g)												
initiation	30.8	31.1	30.8	30.9	30.7	30.9	24.6	24.2	24.5	24.5	24.6	24.7
Week 56	40.2	41.2	39.8	38.7	37.8*	37.2*	34.5	33.7	33.4	34.0	32.3*	-
Week 60	39.7	40.5	40.0	38.6	38.1	38.4	34.9	34.1	34.4	34.9	32.5*	-
Week 72	39.1	40.2	38.5	38.9	38.0	37.8	35.3	34.7	34.8	34.9	32.6*	-
Termination week 76	39.3	40.5	38.7	38.4	38.3	37.3	36.2	35.1	34.7	35.3	33.4*	-
Body weight gain (% of control)												
Weeks 0-52	-	105	95	85*	79*	68*	-	92	98	92	77*	-
Weeks 0-72	-	106	98	99	90	77	-	99	98	98	77	-

^adose level 3000 ppm through day 21

^ball animals died or sacrificed by week 3

* = $p < 0.05$, ANOVA+Dunnett

Dose-related significantly lower cumulative **body weight gain** was recorded from 300 ppm, but there were no statistically significant differences in weekly food consumption among the groups for weeks 1 to 76. Body weight and food consumption values for recovery animals at week 56 were similar to the pre-recovery weights at week 52.

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No ocular finding at **ophthalmoscopic examination** was considered attributable to treatment.

Haematology and clinical chemistry

In the differential counts parameters, the percent of **segmented neutrophil** count was increased (14%) and percent **lymphocytes** decreased (30%) in 2 500-ppm females at week 79, but the biological significance of this is unclear. The absolute leukocyte differential count was not recorded.

Table B.6.5.2-3: Relative differential counts, Oncogenicity study in mice with difenoconazole (means)

Dose level (ppm)	Males				Females			
	0		4500		0		2500	
	study week	53	79	53	79	53	79	53
segment neutrophils	66	63	69	73	66	61	65	75* ↑ 14%
lymphocytes	32	33	29	26	33	37	33	23* ↓ 30%
band (immature) neutrophils	0	0	1	0	0	0	1	0

Note: 10 animals per group

* = p < 0.05, ANOVA+Dunnett

There were statistically significant increases in **liver enzyme values** in the highest dose groups at weeks 53 and 79 indicating disturbed permeability of the hepatocytes and cholestasis. After a 4-week recovery period the values approximated control values indicating that the changes are at least in part reversible.

Table B.6.5.2-4: Clinical chemistry, Oncogenicity study in mice with difenoconazole (means)

Dose level (ppm)		Males						Females				
		0	10	30	300	2500 ^a	4500	0	10	30	300	2500 ^a
Alanine aminotransferase, ALAT (U/L)	wk 53	30	32	22	76	104* ↑247%	114* ↑280%	52	25	23	57	45
	wk 57 recovery	34	-	-	-	56	47	-	-	-	-	30
	wk 79	54	52	31	30	89	222* ↑311%	29	28	24	56	182* ↑528%
Alkaline phosphatase, ALP (U/L)	wk 53	36	41	33	42	39	66	40	39	48	42	39
	wk 57 recovery	40	-	-	-	31	47	-	-	-	-	52
	wk 79	72	45	34	41	59	392* ↑444%	71	57	53	49	88
Sorbitol dehydrogenase, SDH (U/L)	wk 53	45	46	41	89* ↑98%	179* ↑298%	215* ↑378%	72	31* ↓57%	26* ↓64%	51	71
	wk 57 recovery	49	-	-	-	68	73	-	-	-	-	39
	wk 79	51	51	34	43	115* ↑125%	231* ↑353%	43	36	27	56	112* ↑160%

^a dose level 3000 ppm through day 21

* = p < 0.05, ANOVA+Dunnett

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Organ weights, pathology and histopathology

Lower **body/carcass weight** was recorded for females of the highest dose-group at termination. Treatment-related changes were noted in the two highest dose groups in absolute and relative **liver weight** values, at both the interim and terminal sacrifices. Liver weights from recovery-group males (week 57) were lower than the weights for the 53-week sacrifice animals, indicating reversibility. The other statistically significant differences were not corroborated by macro- or microscopic findings and not considered to be toxicologically relevant due to inconsistency or no dose-response pattern. **Note that no control females were sacrificed as recovery animals, making statistical analysis impossible.**

Table B.6.5.2-5: Organ weights, Oncogenicity study in mice with difenoconazole (means)

Dose level (ppm)			Males						Females				
			0	10	30	300	2500 ^a	4500	0	10	30	300	2500 ^a
organ	wk	\$											
body (carcass)	53	Absolute	33.7	32.3	34.6	32.8	32.5	31.3	28.9	28.1	29.5	29.7	27.7
	57 recovery	Absolute	35.5	-	-	-	32.1* ↓ 10%	30.7* ↓ 14%	-	-	-	-	27.7
	79	Absolute	32.5	34.4*	33.0	32.5	32.6	30.6	29.9	29.4	29.3	28.8	27.4* ↓ 8%
liver/gall bladder	53	Absolute	1.49	1.42	1.56	1.61	1.99* ↑ 34%	2.43* ↑ 63%	1.38	1.38	1.39	1.66* ↑ 20%	1.94* ↑ 41%
	53	Relative	4.42	4.35	4.53	4.89	6.09* ↑ 38%	7.83* ↑ 77%	4.77	4.90	4.74	5.59* ↑ 17%	6.99* ↑ 47%
	57 recovery	Absolute	1.08	-	-	-	1.04	1.18	-	-	-	-	1.01
	79	Absolute	1.64	0.52	1.61	1.65	2.36* ↑ 44%	3.48* ↑ 112%	1.41	1.27	1.48	1.70 ↑ 21% n.s.	2.56* ↑ 82%
	79	Relative	5.08	4.52	4.73	4.98	7.04* ↑ 39%	11.25* ↑ 121%	4.89	4.32	5.06	5.92	9.71* ↑ 99%
brain	53	Absolute	0.51	0.53	0.50	0.50	0.50	0.48* ↓ 6%	0.53	0.51	0.53	0.51	0.50
	57 recovery	Absolute	0.36	-	-	-	0.32	0.32	-	-	-	-	0.34
	79	Absolute	0.49	0.51	0.50	0.55	0.49	0.48	0.50	0.51	0.51	0.46	0.51
kidneys	79	Absolute	0.63	0.77*	0.72	0.72	0.70	0.56	0.49	0.51	0.49	0.52	0.45
	79	Relative	1.97	2.28*	2.13	2.16	2.11	1.81	1.71	1.74	1.68	1.80	1.68
testes / ovaries	53	Absolute	0.43	0.41	0.41	0.33* ↓ 23%	0.39	0.38	0.06	0.05	0.05	0.04	0.03
	57 recovery	Absolute	0.31	-	-	-	0.31	0.25	-	-	-	-	0.31
	79	Absolute	0.36	0.34	0.37	0.34	0.33	0.32	0.13	0.04	0.25	0.05	0.06

\$: Relative = organ/body weight ratio (g/g*100), ^adose level 3000 ppm through day 21, * = p ≤ 0.05, ANOVA + Dunnett test

Macroscopic examinations of the interim, recovery and terminal sacrifice animals, as well as the unscheduled deaths, revealed treatment-related **findings in the liver** including enlargement, pale areas and masses. For unscheduled deaths of high-dose (4 500 ppm) males, the incidence of enlargement and masses was 41 and 29%, respectively. At terminal sacrifice the incidence of **masses** was **up to 44%** in high-dose (4 500 ppm) males. Other findings were considered to be spontaneous.

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Table B.6.5.2-6: Necropsy findings in liver, Oncogenicity study in mice with difenoconazole (incidence)

Dose level (ppm)		Males										Females											
		0		10		30		300		2500 ^a		4500		0		10		30		300		2500 ^a	
liver		n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex
enlarged	Early	0	2	0	2	0	2	0	4	0	0	2	14	0	3	0	3	1	4	0	4	1	11 ^b
	Interim	0	10	0	10	0	10	1	10	0	10	1	10	0	10	0	10	0	10	0	10	0	10
	Recovery	0	9	-	-	-	-	-	-	0	10	0	10	-	-	-	-	-	-	-	-	0	10
	Late	2	18	3	16	6	21	2	22	4	16	12	20	2	23	0	12	1	17	4	11	4	10
	Terminal	5	31	0	32	0	27	0	24	8	34	8	16	0	24	3	35	0	29	1	35	13	29
		16%								24%		50%										45%	
pale/pale area	Early	0	2	0	2	0	2	0	4	0	0	6	14	0	3	0	3	0	4	1	4	3	11
	Interim	0	10	0	10	0	10	0	10	1	10	2	10	0	10	0	10	0	10	0	10	1	10
	Recovery	0	9	-	-	-	-	-	-	0	10	1	10	-	-	-	-	-	-	-	-	0	10
	Late	0	18	0	16	2	21	0	22	3	16	8	20	1	23	0	12	4	17	1	11	3	10
	Terminal	1	31	2	32	1	27	1	24	12	34	9	16	0	24	5	35	0	29	3	35	12	29
mass	Early	0	2	0	2	0	2	1	4	0	0	1	14	0	3	0	3	0	4	0	4	0	11
	Interim	0	10	1	10	2	10	0	10	1	10	2	10	0	10	0	10	0	10	0	10	1	10
	Recovery	0	9	-	-	-	-	-	-	1	10	3	10	-	-	-	-	-	-	-	-	0	10
	Late	0	20	3	16	3	23	1	26	10	16	9	34	0	23	1	12	0	17	0	11	4	10
	Terminal	3	31	5	32	5	27	3	24	5	34	7	16	0	24	0	35	1	29	1	35	8	29
		10%		16%		19%		13%		15%		44%						3%		3%		28%	

\$: Early = early unscheduled deaths (weeks 1-52), Interim = interim sacrifice week 53, Recovery = recovery sacrifice week 57, Late = late unscheduled deaths (weeks 53-79), Terminal = terminal sacrifice week 79-80, n = number of animals with finding, ex = examined, ^adose level 3000 ppm through day 21, ^bno necropsy on 10 early deaths (animals 'replaced' by animals from control group).

Histopathology also revealed treatment-related changes in the liver. Effects were seen in 300, 2 500 and 4500-ppm males and the 2 500-ppm females. Non-neoplastic changes in the liver included necrosis, hypertrophy, fatty change and bile stasis. The incidences of necrosis, hypertrophy and fatty change were lower in the recovery-group animals than in the interim sacrifice animals, indicating partial recovery.

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Table B.6.5.2-7: Treatment-related histopathology findings in liver, Oncogenicity study in mice with difenoconazole (incidence)

Dose level (ppm)		Males												Females											
		0		10		30		300		2500 ^a		4500		0		10		30		300		2500 ^a			
	\$	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex	n	ex
liver single cell necrosis	Early	0	2	0	2	0	2	1	4	0	0	14 100%	14	0	3	0	3	0	4	0	4	9 82%	11		
	Interim	0	10	1	10	1	10	3	10	10 100%	10	10 100%	10	0	10	0	10	0	10	3	10	10 100%	10		
	Recovery	1	9	-	-	-	-	-	-	6 60%	10	3 30%	10	-	-	-	-	-	-	-	-	0	10		
	Late	0	18	1	16	0	21	2	22	11 69%	16	17 85%	20	0	26	0	14	0	21	1	15	0	10		
	Terminal	4	31	3	32	1	27	7 29%	24	25 74%	34	9 56%	16	3	24	0	35	0	29	2	35	8 28%	29		
focal/multifocal necrosis	Early	0	2	0	2	1	2	1	4	0	0	6 43%	14	0	3	0	3	0	4	0	4	1	11		
	Interim	0	10	0	10	0	10	2	10	1	10	0	10	0	10	0	10	0	10	1	10	0	10		
	Recovery	0	9	-	-	-	-	-	-	0	10	0	10	-	-	-	-	-	-	-	-	0	10		
	Late	1	18	1	16	3	21	2	22	5	16	5	20	1	26	1	14	0	21	0	15	2	10		
	Terminal	3	31	1	32	0	27	1	24	5	34	5 31%	16	3	24	1	35	0	29	6 17%	35	3 10%	29		
hepatocyte hypertrophy	Early	0	2	0	2	0	2	1	4	0	0	14 100%	14	0	3	0	3	0	4	0	4	6 55%	11		
	Interim	0	10	1	10	0	10	1	10	10 100%	10	10 100%	10	0	10	0	10	0	10	0	10	10 100%	10		
	Recovery	1	9	-	-	-	-	-	-	4 40%	10	2 20%	10	-	-	-	-	-	-	-	-	1 10%	10		
	Late	4	18	9	16	7	21	13 59%	22	15 94%	16	6 18%	34	1	26	1	14	1	21	3	15	80%	10		
	Terminal	12 39%	31	6	32	8	27	11 46%	24	32 94%	34	11 69%	16	1	24	6	35	1	29	4	35	28 97%	29		
fatty change	Early	0	2	0	2	0	2	1	4	0	0	2	14	0	3	0	3	0	4	0	4	1	11		
	Interim	2	10	1	10	0	10	2	10	5	10	9 90%	10	0	10	0	10	2	10	1	10	4 40%	10		
	Recovery	0	9	-	-	-	-	-	-	0	10	1	10	-	-	-	-	-	-	-	-	0	10		
	Late	0	18	0	16	0	21	0	22	1	16	13	20	0	26	0	14	0	21	0	15	0	10		
	Terminal	0	31	0	32	0	27	1	24	7	34	7 44%	16	0	24	1	35	0	29	3	35	4 14%	29		
bile stasis	Early	0	2	0	2	0	2	0	4	0	0	1	14	0	3	0	3	0	4	0	4	0	11		
	Interim	0	10	0	10	0	10	0	10	3 30%	10	10 100%	10	0	10	0	10	0	10	0	10	6 60%	10		
	Recovery	0	9	-	-	-	-	-	-	9 90%	10	6 60%	10	-	-	-	-	-	-	-	-	9 90%	10		
	Late	0	18	0	16	0	21	0	22	12 75%	16	20 100%	20	0	26	0	14	0	21	3 20%	15	6 60%	10		
	Terminal	1	31	0	32	0	27	3	24	32 94%	34	13 81%	16	0	24	0	35	0	29	0	35	29 100%	29		
hepatocellular adenoma	Early	0	2	0	2	0	2	0	4	0	0	0	14	0	3	0	3	0	4	0	4	0	11 ^b		
	Interim	0	10	1 10%	10	2 20%	10	0	10	1 10%	10	2 20%	10	0	10	0	10	0	10	0	10	1 10%	10		
	Recovery	0	9	-	-	-	-	-	-	0	10	3 30%	10	-	-	-	-	-	-	-	-	0	10		
	Late	0	18	3 19%	16	1 5%	21	2 9%	22	9 56%	16	6 30%	20	0	26	0	14	0	21	0	15	5 50%	10		
	Terminal	4 13%	31	6 19%	32	5 19%	27	7 29%	24	3 9%	34	9 56%	16	0	24	0	35	0	29	1	35	10 34%	29		
hepatocell. carcinoma	Early	0	2	0	2	0	2	0	4	0	0	0	14	0	3	0	3	0	4	0	4	0	11		

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Dose level (ppm)		Males						Females					
		0	10	30	300	2500 ^a	4500	0	10	30	300	2500 ^a	
	Interim	0	10	0	10	0	10	0	10	0	10	0	10
	Recovery	0	9	-	-	-	-	1	10	1	10	0	10
	Late	0	18	0	16	1	21	0	22	1	16	4	20
	Terminal	1	31	0	32	0	27	0	24	3	34	6	16

\$: Early = early unscheduled deaths (weeks 1-52), Interim = interim sacrifice week 53, Recovery = recovery sacrifice week 57, Late = late unscheduled deaths (weeks 53-79), Terminal = terminal sacrifice week 79-80, n = number of animals with finding, ex = examined, ^adose level 3000 ppm through day 21, ^bno histopathology on 10 early deaths (animals 'replaced' by animals from control group)

Statistical analysis of liver adenomas and carcinomas revealed significant increases for males of the 2 500- and the 4 500-ppm dose groups, and for females of the 2 500-ppm dose group. The incidence of adenomas and/or carcinomas was already elevated in the 4 500 ppm males at the interim and recovery sacrifices. No other microscopic lesions observed in other organ tissues were considered attributable to treatment with the test substance.

Summary:

Dietary administration of difenoconazole to mice for 18 months resulted in 100% **mortality/morbidity** among the 4 500-ppm females during the first study weeks and decreased survival to termination for the 4 500-ppm males. Terminal **body weights** were significantly reduced compared to controls in the 2 500-ppm females (8%). **Liver enzyme levels** were elevated in the 4 500-ppm males and in the 2 500-ppm animals. **Liver weights** were increased in the 4 500-ppm males, the 2 500-ppm animals and in the 300-ppm females. Treatment-related **macroscopic findings** were seen in the livers of the 4 500-ppm males and the 2 500-ppm animals. Treatment-related **microscopic findings** found in the livers of the 4 500-ppm males, the 2 500-ppm animals and in the 300-ppm males included necrosis, hypertrophy, fatty change and bile stasis. The incidence of **hepatocellular adenomas and carcinomas** was significantly increased in the 4 500-ppm males and in the 2 500-ppm animals.

Conclusion:

LOAEL/LOEL (Lowest-Observable-(Adverse)-Effect Level) could be established from the present study corresponding to **300 ppm** (46.3 and 57.8 mg/kg bw for males and females, respectively) based on adverse effects on liver (increased liver weight and necrosis in females and increased SDH levels and necrosis in males). The **NOAEL/NOEL** was considered to be **30 ppm** (4.7 mg/kg body weight for males and 5.6 mg/kg for females) based on reduced body weight and body weight gain and liver findings at 300 ppm. The Maximum Tolerated Dose (MTD) was exceeded at 2 500 ppm.

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Comments:

Deviations from the OECD TG No. 451 (1981)

According to the guideline, dosing of the rodents should preferably begin before the animals are 6 weeks old.

The animals in this study are older (8 weeks) at initiation of treatment. A differential blood count is to be performed on samples of **all** animals in the highest dosage group and the control, according to guideline. In the present study, only 10/sex were sampled in the respective dose groups.

B.6.5.3 52-week oral toxicity study in dogs

[See section B.6.3.1.6.](#)

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B.6.5.4 Summary and conclusions on long-term toxicity and carcinogenicity

A combined chronic toxicity and carcinogenicity study was performed in rats and an oncogenicity study was performed in mice. A one year study in dogs was also performed and is presented under B.6.3.1.6.

Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
ORAL:					
2-year combined chronic toxicity/oncogenicity in rat					
Cox, 1989a	0, 0.5/0.6, 1.0/1.3, 24.1/32.8 and 124/170 mg kg ⁻¹ day ⁻¹ (M/F)	Orally, via the diet	NOAEL: M/F: 1.0/1.3 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 24.1/32.8 mg kg ⁻¹ day ⁻¹	2500 ppm: ↓ Body weight ↓ Body weight gain ↓ Food consumption (F) ↓ Carcass weight
Report Supplement: Saunders, 1992	0, 10, 20, 500, 2500 ppm		20 ppm	2500 ppm	
			NOEL: M/F: 1.0/1.3 mg kg ⁻¹ day ⁻¹	LOEL: M/F: 24.1/32.8 mg kg ⁻¹ day ⁻¹	500 ppm: ↓ Body weight gain 2500 ppm: ↓ RBC parameters, ↓ WBC parameters Dysproteinemia Altered clinical chemistry parameters 2500 ppm
			20 ppm	500 ppm	500 ppm: ↓ Body weight (F) ↓ Hb (F) ↓ Platelet count (M) ↑ ALAT (M) Hepatocellular hypertrophy
18 months oncogenicity study in mice					
Cox, 1989b	0, 1.5/1.9, 4.7/5.6, 46.3/57.8 and 423/513 mg kg ⁻¹ day ⁻¹ (M/F)	Orally, via the diet	NOAEL: M/F: <46.3 /57.8 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 46.3 /57.8 mg kg ⁻¹ day ⁻¹	4500 ppm: ↑ ALP (M)
	and 819 mg kg ⁻¹ day ⁻¹ for males at 4500 ppm		30 ppm	300 ppm	2500 ppm: ↑ ALAT ↑ Liver weight Hepatocellular carcinoma
	0, 10, 30, 300, 2500 (3000 1 st two weeks)				300 ppm: ↑ Liver weight (F) ↑ Sorbitaldehydrogenas (SDH) (M) Hepatocellular necrosis (M, F)

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
			NOEL: M/F: 4.7/5.6 mg kg ⁻¹ day ⁻¹ 30 ppm	LOEL: M/F: 46.3 /57.8 mg kg ⁻¹ day ⁻¹ 300 ppm	4500 ppm: ↓ Brain weight (6%) ↓ Testis weight (no dose- response) 2500 ppm: ↑ Carcass weight (not at term.) Altered WBC parameters Macroscopic hepatocellular enlargement Macroscopic hepatocellular masses Bile stasis Hepatocellular fatty change ↓ Body weight ↓ Body weight gain ↑ Liver weight (only females at interim) Hepatocellular hypertrophy Hepatocellular adenoma
<i>1-year in dogs</i>					
Rudzki et al., 1988	0, 0.71/0.63, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg kg ⁻¹ day ⁻¹ (M/F) 0, 20, 100, 500 and 1500 ppm	Orally via the diet	NOAEL: M/F: ≥ 51.2/44.3 mg kg⁻¹ day⁻¹ ≥ 1500 ppm	LOAEL: Could not be established	
			NOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹ 100 ppm	LOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹ 500 ppm	1500 ppm: ↓ Food consumption ↓ Body weight gain ↑ ALP (M)
M = male; F = female					

Dietary administration of difenoconazole technical to rats resulted in a decrease (8-23%) in absolute body weight values in the 2 500-ppm dose group and a dose-related decrease (6-40%) in body weight gain in the 500 and 2 500-ppm dose groups. Mean food consumption was consistently lower (1-14%) for the 2 500-ppm animals as compared with controls. There was a negative effect on the red cell mass in the 2 500-ppm females however the effect is **not regarded to be adverse**. Relative liver weight was increased (14-48%) in the 2 500-ppm animals at weeks 53 and 105, but in the 2 500-ppm recovery animals it was similar to the control animals, indicating that **the liver enlargement is adaptive** during exposure and that the effect is reversible after exposure cessation. There were **no treatment-related macroscopic findings**; increased incidence and severity of hepatocellular hypertrophy were noted in 500 and 2 500-ppm males and females. **No treatment-related increases in neoplastic findings occurred during the study**. The dietary concentration of 20 ppm was considered to be the NOEL/NOAEL.

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Dietary administration of difenoconazole technical to mice for up to 18 months at dose levels of 0, 10, 30, 300, 2500 and 4500 ppm resulted in **100% mortality/morbidity** among the 4500 ppm females and **high mortality** among the 4500 ppm males during the first study weeks: survival to termination was therefore decreased for the 4500 ppm males. **Body weight losses** were noted for the high-dose animals during the first study weeks; body weight gains thereafter approached control values, but terminal body weights were reduced. **Liver enzyme levels** were elevated in the 4 500-ppm males and in the 2 500-ppm animals. **Liver weights** were increased in the 4 500-ppm males, the 2 500-ppm animals and in the 300-ppm females. Treatment-related **macroscopic findings** were seen in the livers of the 4 500-ppm males and the 2 500-ppm animals. Treatment-related **microscopic findings** found in the livers of the 4 500-ppm males, the 2 500-ppm animals and in the 300-ppm males included necrosis, hypertrophy, fatty change and bile stasis. The incidence of **hepatocellular adenomas and carcinomas** was significantly increased in the 4 500-ppm males and in the 2 500-ppm animals. The NOAEL was considered to be 30 ppm.

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B.6.6 Reproductive toxicity (Annex IIA 5.6)

B.6.6.1 Multigeneration study -Two generation reproductive toxicity in the rat

Reference:	Giknis MLA (1988). CGA-169374 technical: A two generation reproductive study in albino rats.
Guideline:	US EPA FIFRA 83-4
GLP:	Yes, verified by the U.S. EPA by means of periodic inspections.
Acceptability:	Yes, but limited results see comments
Test substance / purity:	Difenoconazole (CGA 169374) technical / 97.4%
Species / Strain:	Sprague Dawley rats (CRCD VAF/PLUS)
Doses / No. of animals:	0, 25, 250 and 2500 ppm Corresponding to an overall mean of 1.75, 17.3 and 178.0 mg/kg/day for males and females 30/sex/dose
Administration:	Orally via the diet
Exposure time / Duration:	The treatment period was continuous 7 days/week for 2 parental generations of animals and their offspring.

Materials and Methods:

F₀-animals were 37-38 days old at receipt and allowed to acclimatize for one week before initiation of treatment. After an 11-week pre-mating period, animals were paired 1:1 within each dose group until there was evidence of positive mating or for 21 days, whichever occurred first, **see comments**. Litters were culled to 4/sex, where possible, on day 4 post partum. Paired testes and ovaries of the parental animals were weighed.

Histopathological examinations were performed on specified sex organs and the pituitary from the control and 2500-ppm animals, as well as any gross lesion in any animal, **see comments**.

After weaning, 30 F₁-animals per sex and dose were selected as the second parental generation; pups not selected for mating were sacrificed and necropsied. Feeding and mating followed the same procedures as the F₀ generation except that the pre-mating period was 14 weeks long. Sacrifice, necropsy and **histopathology** were performed as for the F₀ generation.

Ophthalmoscopic examinations were performed on F₀-animals prior to and after 10 weeks of exposure.

Furthermore, F₁-animals were examined at 20-28 days of age and F₁-parents were again examined 2 weeks prior to mating.

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F0-males were sacrificed on study day 141. F0-females were sacrificed after weaning of the F1-pups, also on study day 141. F2-pups were sacrificed on lactation day 21 and 5/sex/dose was submitted to necropsy.

Bartlett's test was used for testing the homogeneity of variance and one-way analysis of variance, with **Dunnett's post hoc test**, assessed statistical differences in the continuous parameters between the treatment groups. The **Healy Analysis** assessed F1 and F2-pup body weights. Reproductive parameters were assessed by the **Chi-square** or the **Mantel's trend tests**.

Results:

General observations

The F0 generation

There were two unscheduled **deaths**, not considered to be related to treatment; one control male (on day 109) and one 25-ppm female (on day 118). There were no treatment-related **clinical signs** or **ophthalmologic findings**. There were statistically significant, treatment-related reductions in **food consumption** for males (5-14%) and females (9-15%) of the 2500-ppm dose group throughout the **premating period**, with the exception for females on study days 7-21. Similarly, food consumption was significantly reduced (15-17%) in the 2500-ppm females during the first 14 days of **gestation**.

Mean **body weights** of F₀ males in the 2500-ppm group were significantly lower than controls during **premating** days 7-77 (<10%), **mating** (8-9%) and **post-mating phases** (8-9%) also including **terminal necropsy**. Likewise, **body weight gains** by these males were significantly lower (12-32%) than the controls during most study weeks.

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Table B.6.6.1-1: Body weight development F₀ animals, Multigeneration study in rats with difenoconazole (means)

		males				females			
Timepoint/phase	study day(s)	0	25	250	2500	0	25	250	2500
body weight (g)									
Treatment start	0	175.3	175.6	176.7	178.7	144.3	145.3	143.8	146.4
	7	233.1	230.0	233.1	222.8* ↓ 4%	173.4	173.4	171.6	166.0* ↓ 4%
	42	437.3	435.2	435.0	402.7* ↓ 8%	259.2	255.5	255.7	232.2* ↓ 10%
End premating	77	542.6	540.7	549.7	500.7* ↓ 8%	303.9	295.2	297.0	258.3* ↓ 15%
	Begin gestation ^a	79/98				297.9	295.7	298.6	260.1* ↓ 13%
End mating	98	568.7	567.7	577.9	520.2* ↓ 9%				
End gestation ^a	101/118					430.6	427.3	422.3	379.7* ↓ 12%
Begin lactation ^a	102/119					337.6	335.4	336.5	294.3* ↓ 13%
End lactation ^a	123/140					346.5	342.7	341.4	300.8* ↓ 13%
Termination	141	610.5	614.3	618.1	554.4* ↓ 9%	324.7	317.4	313.6	284.6* ↓ 12%
body weight change (g)									
Overall during Premating	0 to 77	367.3	365.1	373.0	322.0* ↓ 12%				
Gestation (gestation day 0-20)	~79 to ~118					132.7	131.6	123.7	119.6
Lactation (lactation day 0-21)	~102 to ~140					8.9	7.3	4.9	6.6
Overall body weight gain ^b	0 to 141	434.9	438.6	441.4	375.7* ↓ 14%				

^adays variable based on when each dam started/ended the phase
^bterminal body weight – day 0 body weight
 * = p ≤ 0.05, ** = p ≤ 0.01, ANOVA + Dunnett

Similarly, mean **body weights** of F₀ females in the 2500-ppm group were significantly lower than controls during **prematuring** days 7-77 (4-15%), **gestation-** (12-15%) and **lactation phases** (13-16%) also including **terminal necropsy**. **Body weight gains** by these females were significantly lower than the female controls during **prematuring** weeks 1 (33%), 3 (26%), 7 (40%), 8 (56%) and 11 (67%). During **gestation**, body weight gains by the 2500-ppm females were significantly lower than the controls during the first week, 34%. During **lactation**, these females gained less weight (52%) than the controls during the second week but lost less weight during the third week.

The F1 generation

There were no treatment-related clinical observations or malformations on the F1-generation pups. **F1 pup weights** were significantly reduced for both sexes of the highest dose group (2500 ppm) on all assessment occasions (lactation days 0, 4, 7, 14 and 21) except for females on lactation day 0. Furthermore, the pup weight in the 250-ppm dose group tended to be lower during the lactation period and the difference was statistically significant on lactation day 21.

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Table B.6.6.1-2: Litter data of the F₁ generation, Multigeneration study in rats with difenoconazole

Parameter	dose level (ppm)	0		25		250		2500	
Number of litters		26		25		29		29	
Mean pup weight		males	females	males	females	males	females	males	females
- on day 0		6.58	6.15	6.71	6.46	6.35	6.06	6.20* ↓ 6%	5.82
- on day 4	4	10.00	9.38	9.83	9.55	9.46	9.11	8.71* ↓ 13%	8.33* ↓ 11%
- on day 4	4	10.07	9.42	9.84	9.60	9.52	9.10	8.71* ↓ 14%	8.37* ↓ 11%
- on day 7	7	16.54	15.43	16.30	15.68	15.48	14.73	12.81* ↓ 23%	12.28* ↓ 20%
- on day 14	14	32.49	30.67	32.07	31.30	30.93	29.82	23.56* ↓ 27%	22.65* ↓ 26%
- on day 21	21	52.44	49.00	51.54	49.45	48.82* ↓ 7%	46.76	36.85* ↓ 30%	35.00* ↓ 29%

* = p ≤ 0.05, Healy Analysis (weighted for variation in litter size)

There were no treatment-related **clinical signs**, **ophthalmologic findings** or treatment-related **mortality** among the F₁ parental animals. Three females did not survive the lactation phase (one 25-ppm, and two 2500-ppm dams) but the timing and clinical observations (dystocia, hypothermia and delivery of a non-viable litter) suggest that the deaths were related to difficulties in labour. **Food consumption** by the 2500-ppm animals was significantly lower (9-21%) than control animals throughout the premating phase. During gestation, the females of this group continued to consume between 12 and 22% less feed than the control females. Food consumption by the females of the next lower dose group (250 ppm) was significantly lower than control females during pre-mating days 0-7 (7%) and during the second week of gestation (10%), but this difference was considered to be incidental.

Mean **body weights** of F₁ males in the 2500-ppm group were significantly lower than controls during **pre-mating** days 0-98 (17-29%), **mating-** (16%) and **post-mating phases** (16%) also including **terminal necropsy**. Males of the 250-ppm dose group had significantly lower (<10%) mean body weights during the first month of the pre-mating phase. Reductions in mean body weight in the 2500-ppm males were accompanied by statistically significant reductions in the overall **body weight gain** and during the pre-mating phases 0-7 (16%), 28-35 (24%), 63-70 (36%).

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Table B.6.6.1-3: Body weight development F₁ parental animals, Multigeneration study in rats with difenoconazole (means)

		males				females			
Timepoint/phase	study day(s)	0	25	250	2500	0	25	250	2500
body weight (g)									
Treatment start	0	237.0	235.5	217.6* ↓ 8%	169.0* ↓ 29%	178.2	177.3	166.5	138.2* ↓ 22%
	7	304.3	298.7	280.8* ↓ 8%	225.9* ↓ 26%	206.9	208.3	194.0	163.2* ↓ 21%
	49	525.4	517.6	504.0	432.8* ↓ 18%	298.6	289.1	288.5	241.3* ↓ 19%
End premating	98	641.6	619.5	611.7	534.3* ↓ 17%	346.2	329.1	333.1	268.8* ↓ 22%
Begin gestation ^a	101/121					331.7	325.1	321.1	263.5* ↓ 21%
End mating	119	665.0	639.2	631.5	560.4* ↓ 16%				
End gestation ^a	121/143					452.6	439.3	446.8	375.6* ↓ 17%
Begin lactation ^a	122/144					371.3	365.0	358.3	290.3* ↓ 22%
End lactation ^a	144/165					360.1	358.5	341.6	294.4* ↓ 18%
Termination	145/169 (m/f)	683.1	653.0	647.6	572.6* ↓ 16%	375.2	354.8	361.4	291.3* ↓ 22%
body weight change (g)									
Overall during Premating	0 to 98	404.6	384.1	394.1	365.3* ↓ 10%				
Gestation days 0-20	~110 to ~132					120.9	114.2	125.7	112.2
Lactation days 0-21	~132 to ~155					-11.2	-7.3	-16.7	1.7
Overall body weight gain	0 to 145	446.1	417.6	430.0	403.6* ↓ 10%				

^adays variable based on when each dam started/ended the phase

* = p ≤ 0.05, ** = p ≤ 0.01, ANOVA + Dunnett

Similarly, mean **body weights** of F₁ females in the 2500-ppm group were significantly lower (18-22%) than controls during the **premating** phase, **gestation-** (17-21%) and **lactation phases** (18-22%) also including **terminal necropsy**. Mean **body weight gains** by these females were significantly lower than the female controls during **premating** days 21-28 (26%), 42-49 (33%) and 77-84 (84%). During **gestation**, body weight gains by the 2500-ppm females were significantly lower than the controls during the first week, 30%. During **lactation**, these females gained less weight (52%) than the controls during the second week but lost less weight during the third week.

The F₂ generation

There were no compound-related clinical signs or malformations in pups of the F₂-generation. Treatment-related reductions in **body weight** were observed in the 2500-ppm F₂ pups throughout the entire postnatal (lactation) period (days 0, 4(pre-and post-cull), 7, 14 and 21).

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Table B.6.6.1-4: Litter data of the F₂ generation, Multigeneration study in rats with difenoconazole

Parameter	dose level (ppm)	0		25		250		2500	
Number of litters		19		25		21		23	
Mean pup weight		males	females	males	females	males	females	males	females
- on day 0		6.61	6.25	6.77	6.46	6.42	6.15	6.07*	5.79*
								↓ 8%	↓ 7%
- on day 4	4	9.99	9.47	10.23	9.70	9.58	9.29	8.58*	8.23*
precull								↓ 14%	↓ 13%
- on day 4	4	10.07	9.51	10.22	9.71	9.57	9.36	8.61*	8.19*
postcull								↓ 14%	↓ 14%
- on day 7	7	16.07	15.21	15.66	15.12	15.46	15.06	12.77*	12.18*
								↓ 21%	↓ 20%
- on day 14	14	31.76	30.34	30.75	30.15	30.80	30.02	23.45*	22.58*
								↓ 26%	↓ 26%
- on day 21	21	50.99	48.35	50.11	49.02	52.00	50.63	34.22*	32.99*
								↓ 33%	↓ 32%

^aPercent of total born pups which were alive

^bPercentage of live-born pups that survived to day 4

^cPercentage of post-cull pups (day 4) that survived to day 21

* = p ≤ 0.05, Healy Analysis (weighted for variation in litter size)

Reproductive Parameters

The F₀ generation

The **pre-coital interval**, duration of **gestation** (approximately 23 days) and the other **reproductive indices** were comparable between controls and treated animals. Two males (one control and one 25 ppm) failed to mate and five females (three control, one 25 ppm and one 250 ppm) mated but were not pregnant.

Table B.6.6.1-5: Reproductive parameters, F₀ parental animals, Multigeneration study in rats with difenoconazole

dose level (ppm)	0	25	250	2500
Females placed with males (n)	30	30	30	30
Males placed with females (n)	30	30	30	30
Pairs failed to mate (n)	1	1	0	0
Days until evidence of mating (mean/median)	2.5 / 2.0	1.9 / 2.0	3.9 / 2.0	2.9 / 1.5
Females pregnant (n)	26	28	29	30
Duration of gestation (days)	23.2	23.3	23.2	23.3
Females with implants only (n)	0	3	0	1
Females delivering with liveborn pups (n)	26	25	29	29

The F₁ generation

There were no treatment-related effects on the F₁ **litter size** or on the **pup sex ratio**. **Survival indices** were comparable between treated groups and control except for an incidental decrease in percent survival of male pups from day 0 to day 4 (precull).

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Table B.6.6.1-6: Litter data of the F₁ generation, Multigeneration study in rats with difenoconazole

Table 1. Dose level of litter data of the F1 generation, F1 male generation study in rats with diethylenetriamine										
Parameter	dose level (ppm)		0	25		250		2500		
Number of litters			26	25		29		29		
Total pups born			346	342		389		365		
Total stillbirths (pups/litters)			5/5	9/6		11/7		4/2		
Mean litter size day 0 (live births)			13.12	13.32		13.03		12.45		
Live birth index ^a			98.6	97.4		97.2		98.9		
Sex ratio (% males day 0)			51.3	48.6		50.5		47.6		
	males	females		males	females	males	females	males	females	
Viability index (day 0-4) ^b			98.7	97.6	100.0	98.2	99.6	98.8	95.2#	95.3
Viability index (day 4-21) ^c			99.0	100.0	98.2	100.0	100.0	99.1	97.3	100.0

^a Percent of total born pups which were alive^b Percentage of live-born pups that survived to day 4^c Percentage of post-cull pups (day 4) that survived to day 21# = $p \leq 0.05$, Mantel's test for trend

The **pre-coital interval**, duration of **gestation** (approximately 23 days) and the other **reproductive indices** were comparable between controls and treated F₁-parental animals. The mean duration of gestation was approximately 23 days in all groups. One control-group female and two 250-ppm animals did not deliver but were subsequently found to be pregnant; one 2500-ppm female delivered a litter with all pups stillborn.

Table B.6.6.1-7: Reproductive parameters, F₁ parental animals, Multigeneration study in rats with difenoconazole

dose level (ppm)	0	25	250	2500
Females placed with males (n)	30	30	30	30
Males placed with females (n)	30	30	30	30
Pairs failed to mate (n)	5	2	3	0
Days until evidence of mating (mean/median)	3.4 / 3.0	4.1 / 3.0	3.1 / 2.0	2.4 / 2.5
Females pregnant (n)	20	25	23	24
Duration of gestation (days)	23.4	23.6	23.3	23.2
Females with implants only (n)	1	0	2	0
Females with all pups stillborn (n)	0	0	0	1
Females delivering with liveborn pups (n)	19	25	21	23

The F₂ generation

There were no treatment-related effects on the F₂ **litter size** or on the **pup sex ratio**. **Survival indices** were comparable between treated groups and control.

Table B.6.6.1-8: Litter data of the F₂ generation, Multigeneration study in rats with difenoconazole

Parameter	dose level (ppm)		0	25	250	2500
Number of litters			19	25	21	23
Total pups born			234	254	297	281
Total stillbirths (pups/litters)			5 / 5	30 / 9	9 / 6	29 / 9
Mean litter size day 0 (live births)			12.05	8.96	13.71	11.39
Live birth index ^a			97.9	88.2	97.0	93.2
Sex ratio (% males day 0)			44.1	56.3	47.9	51.9
	males	females	males	females	males	females
Viability index (day 0-4) ^b			98.3	94.2	91.4	96.0
Viability index (day 4-21) ^c			98.6	98.6	100.0	98.8

^a Percent of total born pups which were alive^b Percentage of live-born pups that survived to day 4^c Percentage of post-cull pups (day 4) that survived to day 21

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Gross pathology, organ weights and histopathology

The F0 generation

There were no treatment-related **gross or histological findings** in F0-animals. The relative (percentage of terminal body weight) testes weight were statistically increased (9%) in 2500-ppm males due to the decreased terminal weight (-9% as compared to controls) in this group. Ophthalmoscopic examinations of the F0-animals were unremarkable.

The F1 generation

There were no treatment-related **necropsy** findings in the **F1-generation pups** culled on lactation day 4 or sacrificed after weaning. There were no ocular changes attributable to treatment in the F1 pups examined at approximately 20-28 days of age. No histopathology was performed on F1 pups at this stage.

There were no treatment-related **gross or histological findings** in **F1-parental** animals. The relative (percentage of terminal body weight) testes and ovary weights were statistically increased (14 and 33%, respectively) in 2500-ppm animals due to the decreased terminal body weights (-16 and -22%, respectively as compared to controls) in this group. Ophthalmoscopic examinations of these animals two weeks prior to mating were unremarkable.

The F2 generation

There were no compound-related gross or microscopic alterations in pups of the F2-generation.

Conclusion:

There were no compound-related mortalities or clinical observations during treatment of the F0-generation. Reductions in mean body weight and weight gain were observed at 2500-ppm F0 and F1 animals during the pre-mating period and persisted into gestation and lactation for females. Treatment-related reductions in food consumption were also noted in both sexes during pre-mating and in females during gestation.

Mating and fertility indices were comparable for the control and treated groups of both generations. There were no treatment-related effects on any of the reproductive parameters. Absolute ovary and testes weights were comparable among treated and control groups.

Necropsy and histological examinations of the F0 and F1 parental generation animals and F2 pups did not reveal any effects attributable to treatment.

Litter parameters (size, survival, sex ratio) of the F1 and F2 generation were unaffected by treatment. There were no treatment-related clinical observations, necropsy observations or malformations. However, the body weights of the F1 and F2 pups were reduced in the highest dose group (2500 ppm) throughout the lactation period.

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Based on these results, it is concluded that CGA 169374 Technical does not cause any impairment of reproductive performance in rats fed up to 2500 ppm for two consecutive generations. The No-observed-adverse-effect-level (NOAEL) was considered to be 250 ppm based on body weight reduction in parental animals as well as in pups.

Comments:

Reproductive organ weights were only presented for ovaries and testes in this study. In addition a mating period of 14 days is recommended in the OECD TG 416. However, the period was longer for the F0 generation than recommended and is therefore not considered to compromise the scientific value of the study. According to the same guideline, full histopathology should be performed on reproductive organs and target organs for all high-dose and control parental animals. Histopathology was performed on pituitary, uterus, cervix, vagina, prostate, seminal vesicles, ovary, epididymis, testis, coagulating gland for all high dose and control animals of the F0 and F1 generation and F2 pups of all groups (5 animals/group). No target organs were investigated, only organs with gross changes. Therefore, no investigations were made on liver which was identified as a target organ in the short-term toxicology studies. Thus a full assessment on parental toxicology and possible enhancement of toxicity through the generations is not possible. However, body weight was identified as a sensitive parameter in the short-term rat and thus the parental NOAEL of the F0 group is considered valid since the NOAEL derived from the 90 day studies for the same effect equals this value, but the NOAEL for the F1 parents is considered uncertain. Also, target organs from at least one pup/sex/litter from both the F1 and F2 generation, which have not been selected for mating shall be fixed for histopathological examination. This was not done for the F1 pups. Sperm parameters are not evaluated and reports of pathological examination in target organs other than testes and ovaries are short.

WARNING: This document forms part of an EC evaluation data package and should not be used in isolation. Registration must not be granted on the basis of this document.

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B.6.6.2 Developmental toxicity study - Teratology study in rabbits

Reference:	Hummel HE, Yourenneff MA, Giknis MLA and Yau ET (1987). CGA-169374 technical: A teratology (Segment II) study in rabbits.
Report addendum:	Luempert, LG (1992). Analysis of covariance on body weight of male and female foetuses; covariable-number of foetuses.
Guideline:	US EPA FIFRA 83-3
GLP:	Yes, verified by the U.S. EPA by means of periodic inspections
Acceptability:	Yes
Test substance / purity:	Difenoconazole (CGA 169374) technical / 95.7% Vehicle: 3% aqueous cornstarch containing 1.0% Tween 80.
Species / Strain:	New Zealand white (NZW) Rabbits
Doses / No. of animals:	0, 1, 25 or 75 mg/kg 19 females/group, inseminated with semen from 4 NZW rabbit males
Administration:	Orally, by gavage
Exposure time / Duration:	Days 7-19 of presumed gestation

Materials and Methods:

Females were artificially inseminated with semen from males of the same strain. The day of insemination was designated as day 0. The dams were treated between days 7 and 19 of presumed gestation, the approximate period of organogenesis in the rabbit, see comments. Ophthalmoscopic examinations were performed pre-test and at the end of the dosing period on all animals. The surviving dams were sacrificed on day 29. At necropsy, dams were examined for gross pathological changes. The ovaries were examined and the *corpora lutea* in each ovary were counted. The uteri, including their contents and ovaries, were weighted. Each uterus was examined for live and dead foetuses and resorption sites. Implantations were numbered. Viable foetuses were weighed, numbered, sexed, examined externally and then sacrificed. Each viable foetus was examined visceraally using a modification of the Staples technique. Following visceral examination, the foetuses were cleared, stained with alizarin red-S and examined for skeletal alterations.

Bartlett's test was used for testing the homogeneity of variance and one-way analysis of variance (ANOVA), with **Dunnet's post hoc test**, assessed statistical differences in the continuous parameters between the treatment groups. The **Healy Analysis** assessed foetal body weights. Reproductive parameters and incidence of clinical and necropsy observations were assessed by the **Mantel's trend tests**.

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Results:

General observations

Three dams died during the study, one control and one low-dose (1 mg/kg) female were found dead on days 15 and 16, respectively, apparently due to dosing accidents, and one high-dose (75 mg/kg) female was found dead on day 18, following a period of anorexia. In addition, two other high-dose females aborted on gestational days 18 and 24, respectively and were subsequently sacrificed. **The two abortions and the death of the dams in the high-dose group were considered attributable to the exposure of CGA 169374 Technical.**

There was an increase in the stool variations (decreased/soft/no) in 7 out of 19 and 12 out of 19 dams in the 25- and 75 mg/kg dose groups, respectively, compared to two or three in the 0- and 1 mg/kg groups, although this was thought to be secondary to variations in food consumption.

There were no statistically significant differences in mean maternal body weights when treated groups were compared with controls. However, there were significant treatment-related **reductions in body weight gain** or even **body weight losses** in the high-dose (75 mg/kg) group on several observation periods.

Table B.6.6.2-1: Body weight development, teratogenicity study in rabbits with difenoconazole

Day	Time point	Parameter	0		1		25		75	
			mean	n	mean	n	mean	n	mean	n
0	Study start	body weight (kg)	3.26	16	3.31	14	3.32	16	3.35	15
7	Treatment start	body weight	3.53	16	3.55	14	3.52	16	3.57	15
20	Treatment end	body weight	3.73	15	3.74	13	3.70	16	3.56	13
29	Terminal sacrifice	body weight	3.87	15	3.86	13	3.80	16	3.77	13
29	Terminal sacrifice	body weight – (uterus+contents)	3.45	15	3.40	13	3.30	16	3.38	12
7-10	Treatment	body weight change (kg)	0.01	16	0.03	14	0.00	16	-0.11*	15
10-14	Treatment	body weight change	0.06	16	0.06	14	0.05	16	0.01*	15
14-20	Treatment	body weight change	0.13	15	0.09	13	0.12	16	↓ 83%	13
20-29	Post-treatment	body weight change	0.15	15	0.12	13	0.10	16	0.17	12
0-29	Full study	body weight change	0.61	15	0.54	13	0.48	16	0.40*	12
									↓ 34%	

* = $p \leq 0.05$, ANOVA + Dunnett

The reductions in body weight gain were correlated with reduced feed consumption in the 75 mg/kg group and indicative of toxicity. The food consumption was significantly reduced with mean values about 50% lower than the controls during the first week of dosing. Food consumption by the dams in the control, 1 and 25 mg/kg groups tended to decrease after the end of dosing, while the 75 mg/kg animals continued to increase food consumption.

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Table B.6.6.2-2: Food consumption, teratogenicity study in rabbits with difenoconazole

dose level (mg/kg)		0		1		25		75	
Days	Phase	mean	SD	mean	SD	mean	SD	mean	SD
5-6	pre-treatment	188	14	207	36	205	21	211	31
9-10	treatment	195	28	197	26	190	19	100*	70
								↓ 49%	
10-11	treatment	191	20	184	27	179	19	95*	69
								↓ 50%	
11-12	treatment	176	28	185	40	171	28	96*	72
								↓ 45%	
12-13	treatment	179	38	178	36	157	39	90*	66
								↓ 50%	
13-14	treatment	186	35	179	35	164	37	97*	74
								↓ 48%	
14-15	treatment	189	36	190	28	172	48	107*	87
								↓ 43%	
15-16	treatment	192	31	192	28	172	48	123*	92
								↓ 36%	
16-17	treatment	203	24	194	25	187	50	132*	99
								↓ 35%	
17-18	treatment	204	27	197	28	188	45	133*	100
								↓ 35%	
18-19	treatment	208	30	189	27	189	39	153*	89
								↓ 26%	
21-22	post-treatment	200	25	167	33	155	56	180	77
22-23	post-treatment	193	20	159	36	144*	55	186	42
								↓ 25%	
23-24	post-treatment	181	21	124*	43	126*	52	185	32
				↓ 31%		↓ 30%			
26-27	post-treatment	160	35	105*	67	102*	55	145	68
				↓ 34%		↓ 36%			
28-29	post-treatment	141	35	108	65	126	43	129	80

Note: only pregnant animals included in means

* = $p \leq 0.05$, ANOVA + Dunnett

During the last study week the 1 and 25 mg/kg dams consumed significantly less feed than the control dams, but this is consistent with the normal pattern of lower food consumption by pregnant animals late in gestation and not due to the toxicity of the test material.

Ophthalmoscopic examinations did not reveal any treatment-related ocular changes.

Reproductive parameters

Fifteen dams were found not to be pregnant at caesarean section: three controls, five at 1 mg/kg, three at 25 mg/kg and four at 75 mg/kg. Caesarean section data indicated that the mean numbers of **corpora lutea**, **implantation sites** and **live foetuses** were comparable among the groups. There were no dead foetuses. There was an increase in the number and percent of resorptions per litter (mainly early) among the 75 mg/kg animals, but these differences were not statistically significant.

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Table B.6.6.2-3: Reproductive parameters, teratogenicity study in rabbits with difenoconazole

dose level (mg/kg)	0	1	25	75
Pregnancy Status				
Dams pregnant / inseminated (n/n)	16/19	14/19	16/19	15/19
Dams died (n)	1	1	0	1
Dams aborted (n)	0	0	0	2
Dams with total implant loss (n)	0	0	0	0
Dams with viable foetuses (n)	15	13	16	12
C-Section Data^a				
Corpora lutea (mean n per dam± SD)	10.1 ± 2.1	11.0 ± 3.	10.8 ± 2.0	10.3 ± 2.7
Implantation sites (mean n per dam± SD)	6.9 ± 2.7	8.0 ± 3.3	9.0 ± 2.0	7.3 ± 2.9
Foetuses (=live foetuses) (mean n per dam± SD)	6.4 ± 2.8	7.5 ± 3.6	8.5 ± 2.4	6.3 ± 2.9
dead foetuses (total n)	0	0	0	0
Early resorptions (mean n per dam± SD)	0.3 ± 0.5	0.4 ± 1.1	0.4 ± 0.5	0.6 ± 0.7
Late resorptions (mean n per dam± SD)	0.2 ± 0.6	0.1 ± 0.3	0.1 ± 0.3	0.3 ± 0.7
No. resorptions (mean n per dam± SD)	0.5 ± 0.7	0.5 ± 1.1	0.5 ± 0.6	0.9 ± 1.1
Prenatal death (mean ± SD)	0.07 ± 0.1	0.08 ± 0.2	0.07 ± 0.09	0.13 ± 0.1
% post-Implantation loss (mean % of implants per dam)	7.4 ± 11.0	7.8 ± 22.1	6.6 ± 9.4	12.9 ± 14.4
Foetal Data^b				
Sex ratio (% males of total)	57.3	53.1	50.0	51.3
Pup body weight (g, mean males± SD)	47.3 ± 1.6	42.7 ± 1.7	41.3 [*] ± 1.5	41.9 [*] ± 1.8
Pup body weight (g, mean females± SD)	45.6 ± 1.5	41.0 [*] ± 1.6	39.1 [*] ± 1.4	41.4 ± 1.7

^aχ² tests performed; no significant differences.

^bMantel's trend test for sex ratios; Healy analysis for foetal weights; * see discussion below

Foetal sex ratios were not affected by treatment.

Foetal weights of male and female foetuses were not significantly different among the groups, although statistical differences were described in the original report (10-14% foetal weight reductions in all treated groups as compared to controls). It was subsequently determined that the statistical routine used for generating the report tables ran incorrectly, i.e., post-hoc comparisons were inappropriately performed even though the overall F statistics were not significant (p=0.072 for males, p=0.104 for females). It was also suggested in the original report that variations in litter size could account for apparent differences in foetal weights. To this end, the data were subsequently analysed by analysis of covariance with litter size as the covariate (**Luempert, 1992**). These analyses were also not significant at the p ≤ 0.05 level for both males and females. Moreover, it was stated in the original report that the weights for the control-group foetuses were exceptionally high; the mean historical control values were 43.4 g for male foetuses and 42.2 g for female foetuses. The weights for the foetuses in the treated groups were closer to these values than the control group in this study. Above statement is described and verified in the report addendum by Luempert (1992).

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Gross pathology, organ weights and histopathology

Maternal necropsy examinations did not reveal any treatment-related findings. Two abnormal specimens were examined histopathologically, the liver of one 75 mg/kg dam [which aborted] and the lung of one 25 mg/kg dam. The liver specimen received a diagnosis of acute toxic hepatitis, possibly due to toxæmia of pregnancy. The lung specimen was diagnosed as indicative of acute pneumonia and pleuritis, possibly due to aspiration. Neither was considered related to the toxicity of difenoconazole.

There were no compound-related effects on the incidences of foetal external, visceral or skeletal variations or malformations. One 25 mg/kg foetus was found during external examination to have a raised, discoloured area on the ventral thoracic region which may have resulted from technical manipulation and was considered to be incidental. The only soft tissue findings were a horseshoe kidney in one 75 mg/kg foetus, microencephaly in one 25 mg/kg foetus and partial cryptophthalmos in one 75 mg/kg foetus. These findings were considered to be spontaneous and not related to treatment.

Skeletal alterations were seen in approximately 80-90% of the foetuses in all of the litters. There were no statistically significant or biologically meaningful differences among the groups (by foetus or by litter) in the incidence of any finding.

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Table B.6.6.2-4: Foetal Skeletal variations, teratogenicity study in rabbits with difenoconazole

dose level (mg/kg)	0				1				25				75			
	Foetus		Litter		Foetus		Litter		Foetus		Litter		Foetus		Litter	
Observation evaluated	95		15		98		12		136		16		76		12	
n	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
ANY SKELETAL FINDINGS	86	91	15	100	81	83	12	100	118	87	16	100	68	89	12	100
<u>HYOID</u>																
bowed	1	1	1	7	0	0	0	0	0	0	0	0	1	1	1	8
not completely ossified	0	0	0	0	0	0	0	0	3	2	1	6	1	1	1	8
<u>CENTRUM/VERTEBRAE</u>																
additional	3	3	1	7	4	4	1	8	2	1	1	6	2	3	1	8
<u>RIBS</u>																
fully formed (13 th)	50	53	15	100	48	49	10	83	53	39	14	88	49	64	12	100
floating rudimentary (13 th)	8	8	6	40	3	3	3	25	5	4	5	31	4	5	4	33
rudimentary (13 th)	32	34	13	87	18	18	9	75	38	28	14	88	13	17	8	67
localized thickening	0	0	0	0	1	1	1	8	0	0	0	0	0	0	0	0
<u>STERNEBRAE</u>																
bipartite	2	2	2	13	2	2	1	8	0	0	0	0	1	1	1	8
misaligned	2	2	2	13	0	0	0	0	0	0	0	0	0	0	0	0
not completely ossified	18	19	9	60	7	7	6	50	25	18	11	69	11	14	7	58
not ossified	22	23	9	60	19	19	8	67	24	18	9	56	26	34	8	67
fused	0	0	0	0	1	1	1	8	0	0	0	0	0	0	0	0
<u>FOREPAW</u>																
middle phalange-not completely ossified	0	0	0	0	1	1	1	8	1	1	1	6	0	0	0	0
middle phalange-not ossified	2	2	1	7	1	1	1	8	3	2	1	6	1	1	1	8
proximal phalange-not completely ossified	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	8
metacarpal-not completely ossified	1	1	1	7	0	0	0	0	0	0	0	0	0	0	0	0
metacarpal-not ossified	0	0	0	0	0	0	0	0	0	0	0	0	3	4	1	8
<u>HINDLEG</u>																
patella-not ossified	4	4	1	7	15	15	6	50	18	13	8	50	5	7	2	17
patella-not completely ossified	20	21	10	67	14	14	7	58	37	27	11	69	5	7	4	33
<u>HINDPAW</u>																
middle phalange-not ossified	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	8
tarsus-not ossified	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	8

Note: χ^2 test on normal scores or Mantel's Trend test performed; **no statistically significant differences found**

Conclusion:

There was no indication of embryotoxic, fetotoxic, or teratogenic potential.

Maternal toxicity was seen in female rabbits treated with 75 mg difenoconazole technical per kg per day during the period of organogenesis, evidenced by **reduced body weight gain, reduced food consumption, abortions (two animals) and death following anorexia (one animal)**.

There were no differences in reproductive parameters among the groups. No treatment-related external, visceral or skeletal abnormalities were seen.

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No effects were seen in dams treated with 1 mg/kg or in foetuses from dams treated with 1, 25 or 75 mg/kg. The **maternal NOEL** (No-Adverse-Observable-Effect Levels) was 25 mg/kg based on reductions in body weight gain and food consumption, abortion and death at 75 mg/kg per day. The foetal NOEL (No-Observable-Effect Levels) was 25 mg/kg in this study, based on the increased number of resorptions at 75 mg/kg.

Comments:

The period of organogenesis for rabbits is approximately between days 6 and 18 in the gestation according to the OECD TG No. 414 (2001).

B.6.6.3 Developmental toxicity study -Teratology study in rats

Reference:	Lochry EA (1987). Developmental toxicity study of CGA-169374 technical (FL-851406) administered orally via gavage to CrI:COBS®CD®(SD)BR presumed pregnant rats.
Guideline:	US EPA FIFRA 83-3
GLP:	Yes, verified by the U.S. EPA by means of periodic inspections.
Acceptability:	Yes, see comments
Test substance / purity:	Difenoconazole (CGA 169374) Technical / 95.7%. <u>Vehicle:</u> 0.5% carboxymethylcellulose (CMC) in purified water. CGA 169374 first combined with HiSil using acetone as a solvent to reduce to a powder.
Species / Strain:	CrI:COBS®CD®(SD)BR rats
Doses / No. of animals:	0, 2, 20, 100 or 200 mg/kg Doses were selected by the sponsor on the basis of a pilot study (Argus research Project 203-005P) 25/sex/group
Administration:	Orally, by gavage Control animals received vehicle.
Exposure time:	Days 6 to 15 of presumed gestation, see comments

Materials and Methods:

Female were cohabited with males (1:1) for a maximum of 5 days. The day successful mating was established (presence of a vaginal plug or sperm in a vaginal smear) was designated as day 0. The dams were sacrificed on gestational day 20. *Corpora lutea* in each ovary were identified and counted. Each uterus was examined for pregnancy, number and placement of implantations, early and late resorptions and live and dead foetuses. Following removal from the uterus, foetuses were weighed, numbered examined externally and sexed, **see comments**. Approximately 50% of the foetuses in each litter were examined for visceral alterations using a modification of Wilson's sectioning technique. The remaining foetuses in each litter were examined for skeletal

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alterations. Bartlett's Test assessed the homogeneity of variances and parametric analysis of variance (ANOVA) or non-parametric Kruskal-Wallis with post hoc tests Dunnett's and Dunn's tests, respectively assessed significant differences between treated groups and control.

Results:

Analysis of the dosing suspensions used in the study indicated that the **suspensions were consistently lower than the nominal concentrations and somewhat variable** under the conditions of the test. Actual concentrations were 71%, 78%, 85% and 86% of the target concentrations for the 2, 20, 100 and 200 mg/kg doses, respectively.

General observations

There were no mortalities among the adult animals during the course of the study. The incidence of **excess salivation** was significantly increased in 14 out of 23 (61%) and 19 out of 25 (76%) dams, respectively in the two highest dose-groups (100 and 200 mg/kg). Mean **body weight of the dams** in the highest (200 mg/kg) dose group was significantly lower (although <10%) than controls from exposure day 8 throughout to gestational day 19. At termination (gestational day 20), body weights were comparable among all groups. **Weight loss** occurred for these dams following the first dosage (at GD 6) and persisted until gestational day 8 and 10 for the 100 and 200 mg/kg dams, respectively. The overall (GD 0-20) body weight gain in the 200 mg/kg dams were 12% lower than controls.

Table B.6.6.3-1: Body weight development, teratogenicity study in rats with difenoconazole (means)

Day	Time point	Parameter	dose level (mg/kg)				
			0	2	20	100	200
		n	25	25	24	23	24
0	Study start	Mean body weight (g)	264.1	264.6	265.1	262.7	265.3
6	Treatment start	Mean body weight	283.7	285.8	283.5	282.2	283.7
8	Treatment mid	Mean body weight	288.3	290.7	286.8	280.4	247.7** ↓ 5%
10	Treatment mid	Mean body weight	295.9	298.9	295.0	287.5	276.6** ↓ 7%
15	Treatment end	Mean body weight	321.5	325.3	321.2	311.2	300.4** ↓ 7%
19	Post treatment	Mean body weight	369.8	374.6	372.5	364.5	354.3* ↓ 4%
20	Terminal sacrifice	Mean body weight	393.0	398.8	396.8	390.3	378.4
6-15	Treatment	Mean body weight change (g)	37.8	39.5	37.7	29.0## ↓ 23%	16.7## ↓ 56%
16-20	Post-treatment	Mean body weight change	61.7	63.6	64.5	68.6	70.1
0-20	Overall	Mean body weight change	128.9	134.2	131.7	127.6	113.0** ↓ 12%

** = $p \leq 0.05$, ANOVA + Dunnett; ## = $p \leq 0.05$, ANCOVA + T-test

However, after cessation of dosing (at GD 16), mean body weight gains in the 100 and 200 mg/kg animals tended to be greater than in controls (12-19%).

Food consumption was significantly reduced in the 100 and 20 mg/kg groups during the treatment period (days 6-15). However, during the last four days of gestation it was greater than the control animals.

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Table B.6.6.3-2: Maternal Food Consumption, teratogenicity study in rats with difenoconazole (means)

Days	Time point	Parameter n	dose level (mg/kg)				
			0	2	20	100	200
0-6	Study start	gram/day	25	25	24	23	24
6-7	Treatment start	gram/day	21.8	22.5	21.7	21.7	22.1
			22.0	22.1	21.6	18.3** ↓ 17%	16.7** ↓ 24 %
7-8	Treatment start	gram/day	21.6	22.0	20.7	17.2** ↓ 20%	12.7** ↓ 41 %
8-9	Treatment mid	gram/day	22.0	22.4	22.6	18.8** ↓ 15%	12.3** ↓ 44 %
9-10	Treatment mid	gram/day	22.5	23.4	22.1	20.2	15.4** ↓ 32%
11-12	Treatment mid	gram/day	23.6	24.6	23.4	21.0** ↓ 11%	20.5* ↓ 13%
12-13	Treatment mid	gram/day	24.7	26.0	26.0	23.2	22.3* ↓ 10%
14-15	Treatment end	gram/day	24.3	25.0	23.7	22.6	20.1** ↓ 17%
15-16	Treatment end	gram/day	25.2	26.3	26.7	24.4	22.5* ↓ 11%
17-18	Post treatment	gram/day	26.0	26.5	26.9	26.7	28.4** ↑ 9%
18-19	Post treatment	gram/day	25.6	26.2	26.0	27.3	29.2** ↑ 14%
19-20	Post treatment	gram/day	22.9	24.2	24.2	23.4	25.9** ↑ 13%

** = $p \leq 0.05$, ANOVA + Dunnett

Reproductive parameters

Pregnancy incidences were comparable in all dose groups. One 20 mg/kg dam and two 100 mg/kg dams showed signs of positive mating but were not pregnant. The highest dose (200 mg/kg) tended to increase the incidence resorptions and decrease the litter size, but these changes were **not statistically significant**. There were no dead foetuses and only one dam (200 mg/kg) with total implant loss.

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Table B.6.6.3-3: Reproductive parameters, teratogenicity study in rats with difenoconazole

dose level (mg/kg)	0	2	20	100	200
Pregnancy Status					
Dams pregnant / mated (n/n)	25/25	25/25	24/25	23/25	25/25
Dams with total implant loss (n)	0	0	0	0	1
Dams with viable foetuses (n)	25	25	24	23	24
C-Section Data					
<i>Corpora lutea</i> (mean n per dam \pm SD)	16.1 \pm 3.0	17.4 \pm 3.4	15.9 \pm 2.2	17.5 \pm 2.6	15.6 \pm 3.2
Implantation sites (mean n per dam \pm SD)	14.8 \pm 3.2	14.7 \pm 2.7	14.7 \pm 1.4	15.1 \pm 2.3	14.0 \pm 3.2
Foetuses (=live foetuses) (mean n per dam \pm SD)	14.1 \pm 3.1	13.6 \pm 2.9	14.0 \pm 1.3	14.1 \pm 2.7	12.2 \pm 4.4
dead foetuses (total n)	0	0	0	0	0
Early resorptions (total n)	18	27	17	24	42
Early resorptions (mean n per dam \pm SD)	0.7 \pm 0.8	1.1 \pm 1.1	0.7 \pm 1.1	1.0 \pm 2.1	1.7 \pm 3.1
Late resorptions (total n)	0	0	1	0	2
Affected implants (mean % of implants per dam)	4.8 \pm 5.2	7.4 \pm 8.0	4.8 \pm 4.8	6.6 \pm 11.5	9.8 \pm 10.8
Foetal Data					
Sex ratio (% males of total)	52.0	49.0	51.5	46.9	46.6
Pup body weight (g, mean males \pm SD)	3.50 \pm 0.33	3.58 \pm 0.32	3.47 \pm 0.29	3.49 \pm 0.42	3.32 \pm 0.50
Pup body weight (g, mean females \pm SD)	3.28 \pm 0.32	3.39 \pm 0.24	3.34 \pm 0.30	3.28 \pm 0.41	3.20 \pm 0.44
Pup body weight (g, mean combined \pm SD)	3.39 \pm 0.30	3.48 \pm 0.27	3.41 \pm 0.30	3.38 \pm 0.41	3.25 \pm 0.46

ANOVA tests performed

There were no significant effects on litter data observations. Foetal **sex ratios** were not affected by treatment. **Foetal weights** of male and female foetuses were not significantly different among the groups, although the means of the 200 mg/kg foetuses were slightly lower than the control foetuses.

Gross pathology and histopathology

Maternal necropsy examinations at caesarean-section did not reveal any treatment-related findings attributed to CGA 169374 Technical administration.

Examinations of the caesarean-delivered foetuses did not reveal any treatment-related effects on the incidences of **external or visceral findings**.

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Skeletal alterations were seen in approximately 7% of the foetuses and 30% of the litters. Overall, more alterations were found in the control foetuses and litters than in the treated groups. Wavy, incompletely ossified ribs, incompletely ossified or non-ossified sternbra(e) and incompletely ossified pelvis (ischia or pubis) were seen in significantly more control foetuses than in the foetuses from the treated dams. **Foetal incidence of bifid and unilaterally ossified thoracic vertebral centres was significantly higher in the 200 mg/kg foetuses than in the controls, but the litter incidence was not statistically different.**

Table B.6.6.3-4: Foetal Skeletal alterations, teratogenicity study in rats with difenoconazole

dose level (mg/kg)	0				2				20				100				200			
Observation n evaluated	Foetus		Litter		Foetus		Litter		Foetus		Litter		Foetus		Litter		Foetus		Litter	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
ANY SKELETAL FINDINGS	24	13	13	52	4	2	3	12	10	6	6	25	8	5	6	26	18	11	10	42
VERTEBRAE, CERVICAL																				
C7, rib present	2	1	2	8	-	-	-	-	1	1	1	4	1	1	1	4	1	1	1	4
VERTEBRAE, THORACIC																				
T10 or T11, bifid centra	-	-	-	-	1	1	1	4	-	-	-	-	2	1	2	9	4**	3	3	13
T10+T12, bifid centra	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1**	1	1	4
T2 or T8 or T1 centra, unilateral ossification	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3**	2	3	13
T5+T8+T10 centra, unilateral ossification	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1**	1	1	4
T3 centrum not ossified	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	4
VERTEBRAE, LUMBAR																				
V5 or V6, arch(es) inc. ossified	1	1	1	4	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	4
V6+V7, arches inc. ossified	1	1	1	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RIBS																				
wavy	5**	3	3	12	-	-	-	-	1	1	1	4	-	-	-	-	-	-	-	-
inc. ossified/hypoplastic fused (3 pairs)	6**	3	3	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
STERNEBRAE																				
S1 or S2, not ossified	2**	1	2	8	-	-	-	-	2	1	2	8	2	1	2	9	5	3	3	13
S1 or S2, inc. ossified	8**	4	6	24	-	-	-	-	2	1	1	4	3	2	2	9	5	3	5	21
S1+S2, not ossified	-	-	-	-	-	-	-	-	1	1	1	4	-	-	-	-	-	-	-	-
S1+S2, inc. ossified	3**	2	3	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PELVIS/PUBES																				
not ossified	-	-	-	-	-	-	-	-	2	1	2	8	-	-	-	-	1	1	1	4
inc. ossified	8**	4	6	24	4	2	3	12	4	2	2	8	1	1	1	4	1	1	1	4
PELVIS/ISCHIA																				
inc. ossified	6**	3	4	16	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	4

** = p ≤ 0.01, binomial test

There was a significant increase in the average **number of ossification sites of ribs** in the foetuses from the 200 mg/kg dams, with a related significant increase in the **number of thoracic vertebrae** and consequently, the average **number of lumbar vertebrae was decreased** (all vertebrae with ribs were defined as thoracic). These changes are considered to be a developmental change associated with maternal toxicity. Increases in the average **number of ossified hyoid** and **caudal vertebrae** and decreases in the average **number of sternal centres** and **hindpaw phalanges** also occurred for 200 mg/kg foetuses.

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Table B.6.6.3-5: Foetal ossification sites, teratogenicity study in rats with difenoconazole

Dose level (mg/kg)	0		2		20		100		200	
n of litters evaluated	25		25		24		23		24	
n of fetuses evaluated	182		176		172		168		160	
Ossification sites / foetus / litter	mean	sd	mean	sd	mean	sd	mean	sd	n	%
hyoid	0.72	0.32	0.76	0.29	0.88	0.20	0.90	0.15	0.95*	0.12
vertebrae, cervical	7.00	0.00	7.00	0.00	7.00	0.00	7.00	0.00	7.00	0.00
vertebrae, thoracic	13.00	0.00	13.02	0.06	13.00	0.02	13.04	0.08	13.24**	0.19
vertebrae, lumbar	6.00	0.00	5.98	0.06	5.99	0.04	5.96	0.08	5.75**	0.19
vertebrae, sacral	3.00	0.00	3.00	0.00	3.00	0.00	3.00	0.00	3.00	0.00
vertebrae, caudal	4.85	0.41	4.81	0.46	4.88	0.60	4.94	0.96	4.99	0.80
ribs	13.00	0.00	13.02	0.06	13.00	0.02	13.03	0.05	13.21**	0.18
sternum, manubrium	1.00	0.00	1.00	0.00	0.99	0.04	1.00	0.00	0.98	0.10
sternum, sternal	3.73	0.25	3.73	0.28	3.63	0.34	3.53	0.33	3.40*	0.53
sternum, xyphoid	0.99	0.03	1.00	0.02	0.98	0.09	0.98	0.05	0.95	0.12
forepaws – carpals	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
forepaws – metacarpals	3.53	0.31	3.53	0.32	3.58	0.33	3.46	0.34	3.55	0.36
forepaws – phalanges	4.69	0.72	4.78	0.53	4.54	1.11	4.80	1.06	4.59	1.14
hindpaws – tarsals	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
hindpaws – metatarsals	4.00	0.02	4.00	0.02	3.99	0.04	4.03	0.22	3.97	0.11
hindpaws – phalanges	4.12	1.18	4.08	0.86	4.16	1.24	4.10	1.01	3.89	1.26

* = $p \leq 0.05$, ** = $p \leq 0.01$ ANOVA + Dunnett

Conclusion:

Slight maternal toxicity was seen in female rats treated with difenoconazole technical at doses of (100 and) 200 mg/kg/day during the period of organogenesis, as evidenced by **reduced body weight gain** and **reduced food consumption**. Increased incidences of some skeletal alterations in 200 mg/kg foetuses were considered to be indicators of delayed development, secondary to maternal toxicity. No effects were seen in dams treated with 2 or 20 mg/kg or in foetuses from dams treated with 2, 20 or 100 mg/kg.

The foetal incidence of bifid and / or unilaterally ossified thoracic vertebrae was significantly increased for the highest dose group (200 mg/kg), as compared to controls. The litter incidences of these foetal alterations were not significantly different.

The maternal NOAEL (No-Observable-Adverse-Effect Level) was 20 mg/kg and the foetal NOAEL was 100 mg/kg in this study.

Comments:

The organogenesis in rodents occurs between days 5-15 according to the OECD TG No. 414 (2001), thus the exposure began one day after the beginning of the organogenesis. Furthermore, it is recommended that the gravid uterus including cervix are weighed (OECD TG 414, 2001), which was not done in this study. Furthermore the doses did not produce significant maternal toxicity at the maximum dose tested, however a study with higher doses is not considered necessary since high doses were tested in the rabbit study (maternal deaths and abortions occurred) without significant effect on the offspring.

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B.6.6.4 Summary and conclusions on reproductive toxicity

Administration of difenoconazole technical to rats at dietary concentrations of 0, 25, 250 and 2500 ppm over two generations, with one mating in each generation, resulted in treatment related effects at 2500 ppm (approximately 178 mg/kg/day). Retarded body weight gain and reduced food consumption were noted in parental animals of both generations. Absolute pup body weights were lower than control body weights at 2500 ppm in both generations. Male and female reproductive organs, mating behaviour, conception, parturition, litter parameters, lactation and weaning were not adversely affected by the administration of difenoconazole at any dose level in either generation. The dose level of 250 ppm was considered the NOAEL for both parental animals and pups in this study.

The teratogenic potential of difenoconazole was investigated in the rabbit at 1, 25 and 75 mg/kg/day. Maternal toxicity, manifested as a reduced body weight gain and reduced food consumption, was observed during the period of organogenesis at 75 mg/kg/day. Two 75 mg/kg/day animals aborted and a third died of apparent compound related anorexia. A slight increase in resorptions was observed at 75 mg/kg/day, which may have been secondary to maternal toxicity. There were no differences in pregnancy or litter parameters among the treated and control groups. No treatment related external, visceral or skeletal effects were seen. The maternal and foetal NOAEL were both 25 mg/kg/day. There was no evidence of compound related embryotoxic, foetotoxic or teratogenic potential at doses of up to 75 mg/kg/day.

The teratogenic potential of difenoconazole was investigated in the rat at doses of 2, 20, 100 and 200 mg/kg/day. Maternal toxicity, manifested as reduced body weight gain and food consumption was seen during the period of organogenesis at 100 and 200 mg/kg/day. Slight increases in resorptions and reduction in litter size was seen at 200 mg/kg/day but did not reach statistical significance and were attributed to maternal toxicity. Increases in a number of minor skeletal abnormalities at 200 mg/kg/day were considered reversible and/or associated with maternal toxicity. No effects were seen in dams at 1 and 20 mg/kg/day or in foetuses from dams treated at 1, 2 or 20 mg/kg/day. The maternal NOAEL were 20 mg/kg bw/day and foetal NOAEL was 100 mg/kg/day. There was no evidence of compound related embryotoxic, foetotoxic or teratogenic potential at doses of up to 200 mg/kg/day.

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Table B.6.6.4-1: Summary of reproductive toxicity

Study/dose levels	NOAEL	LOAEL target organ/effect	Reference
2-generation reproduction 0, 25, 250, 2500 ppm	250 ppm \approx 17.3 mg/kg/day	2500 ppm \approx 178 mg/kg/day body weight, food consumption	Giknis, 1988
rabbit teratology 0, 1, 25, 75 mg/kg/day	25 mg/kg/day (maternal) 25 mg/kg/day (foetal)	75 mg/kg/day (maternal) body weight, food consumption, abortion 75 mg/kg/day (foetal) resorptions	Hummel et al., 1987
rat teratology 0, 2, 20, 100, 200 mg/kg	20 mg/kg/day (maternal) 100 mg/kg/day (foetal)	100 mg/kg/day (maternal) body weight 200 mg/kg/day (foetal) skeletal variations	Lochry, 1987

B.6.7 Delayed neurotoxicity (Annex IIA 5.7)

Delayed neurotoxicity studies were not performed because the structure and chemistry of difenoconazole do not resemble chemicals known to induce delayed neurotoxicity. In addition, no effects indicative of nervous system involvement were seen in any of the studies performed with difenoconazole.

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B.6.8 Further toxicological studies (Annex IIA 5.8)

B.6.8.1 Metabolites

In plants treated with difenoconazole, one difenoconazole specific metabolite and four triazole metabolites were found at levels that exceed 10% of TRR (Table B.6.10.8.1-1).

The toxicity of the plant metabolite CGA 205375 that is also formed to a large extent in the mammalian metabolism of difenoconazole is considered to be covered by the toxicity studies performed on the parent compound. The RMS suggests that the toxicity assessment of the other plant metabolites should include studies of acute oral toxicity and genotoxicity¹ as they are only detected at low levels. Toxicokinetic studies are useful to indicate if the metabolite accumulate in certain tissues. This discussion is based on the low exposure to metabolites following the representative use supported in the dossier. If the intended use within the EU is to expand the use pattern to include additional applications, this conclusion may be re-evaluated.

The major metabolites found in the mammalian metabolism of difenoconazole (CGA 205374, CGA 205375 and CGA 189138) were further investigated regarding the acute oral toxicity and the ability to induce mutations in bacteria.

¹ Considered to be represented by a combination of an Ames test, a gene mutation test on mammalian cells and a chromosome aberration test.

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B.6.8.1.1 Toxicology studies of CGA-189138

Bacterial Reverse Mutation Test

Reference:	Nakajima, M (1991a). Reverse mutation assay of CGA-189138.
Guideline:	Performed according to Japan MAFF 59 NohSan 4200 (1985), checked for compliance to OECD 417 (4 April 1984)
Deviations from TG 471	The results in this study were based on duplicate instead of triplicate platings and standard deviations were not reported. This is considered to be of low concern since the individual data showed a low degree of variation and there were no signs of genotoxicity observed. The bacterial titre was not reported but the presence of a background lawn was confirmed thus the suspension is considered to have contained a satisfactory titre of viable bacteria. The exclusion of a confirmatory experiment was not justified.
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	CGA 189138 tech (batch 910806) / 97.8%. Vehicle: Dimethylsulfoxide (DMSO)
Bacterial strains:	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535 and TA 1537 (histidine-auxotrophic) and <i>E. coli</i> WP2 <i>uvrA</i> (tryptophan-auxotrophic)
Doses:	- S9 ; TA 100 and TA 1537, + S9 ; <i>S typhimurium</i> 4 strains: 31.3, 62.5, 125, 250, 500 and 1000 µg/plate - S9 ; TA 98, TA 1535 and WP2 <i>uvrA</i> , + S9 ; WP2 <i>uvrA</i> : 62.5, 125, 250, 500, 1000 and 2000 µg/plate
Administration:	Test substance dissolved in DMSO
Exposure time:	48 h

Materials and Methods:

The mutagenicity of CGA 189138 tech was tested in four *Salmonella typhimurium* strains and in an *E. coli* WP2 *uvrA* strain in presence and in absence of metabolic activation. The highest concentration applied was determined in a preliminary toxicity study. Relevant positive controls were included in the tests and DMSO served as a negative control. After preparation, duplicate plates of all concentrations were incubated 48 hours at 37 °C in darkness and thereafter evaluated by colony counting.

Results:

In assays performed without activation, cell death was observed at 500 µg/plate in strain TA 100, at 1000 µg/plate in strains TA 1535, TA 98 and TA 1537 and at 2000 µg/plate in the *E. Coli* strain. In assays performed with activation, cell death was observed already at 500 µg/plate in strains TA 1535 and TA 1537. Precipitate of

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the test substance was observed in the *E. coli* strain at the top dose level. Treatment with difenoconazole did not lead to an increased incidence of revertant colonies compared to the negative control.

Conclusion:

CGA 189138 tech did not increase the reversion frequency in the *Salmonella typhimurium* strains and the *E. coli* WP2 *uvrA* strain tested.

Comments:

ENNG was used as a positive control of strain TA 1535 in the assay performed with metabolic activation. In TG 471, ENNG is given as an example of a strain-specific control specific for *E. coli* WP2 strains. There are no historical data reported regarding the reversion frequency of ENNG in TA 1535 but as it produced a high amount of revertant colonies in the test, it is considered acceptable as a control. In this study, 2-Aminoanthracene was used as the sole indicator of the efficiency of the S9 mix.

Summary:

The toxicological data on CGA-189138 is represented by a single bacterial reverse mutation test. However, CGA-189138 is neither found in plant residues nor found in high concentrations in groundwater thus further data is not considered to be necessary.

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B.6.8.1.2 Toxicology studies of CGA 71019 (1, 2, 4 –triazole)

B.6.8.1.2.1 Absorption, distribution, excretion (toxicokinetics)

Single dose (three dose levels) in rats

Reference:	Lai, K. and Simoneaux, B. (1986a): Balance study of ¹⁴ C-triazole in orally dosed rats.
Guideline:	Checked for compliance with OECD 417 (4 th April 1984).
Deviations from TG 417:	Test conditions regarding the acclimatisation procedure, randomisation process, temperature, humidity, lighting or composition of diet were not specified in the report. Standard deviations of the calculated means were not reported. Studies of metabolic pathways were not performed.
GLP:	No
Acceptability:	Yes
Test substance / purity:	¹⁴ C labelled triazole ([3,5- ¹⁴ C]-1,2,4-triazole) Chemical and radiochemical purity > 98% (TLC). Specific radioactivity 67.1, 0.67 and 0.034 µCi/mg in low, mid and high dose solutions. Substance dissolved in acetone.
Species / Strain:	Rat / Albino Sprague Dawley (SPF)
Doses / No. of animals:	0.4, 48.8 and 865.7 mg/kg bw 2 females and 2 males/ dose level
Administration:	Orally, by gavage Vehicle: deionised water
Exposure time / Duration:	Single dose, 168 h observation period post dose (daily sampling of urine and faeces at 24 hour intervals starting 24 hours after sampling.)

Materials and Methods:

Stock solutions of triazole were diluted with deionized water (1:10) and administered as single oral doses to groups of male and female rats. Urine and faeces were collected at 24-hr intervals starting 24 hours after dosing. All animals were sacrificed seven days after dosing and blood and a number of organs/tissues were collected for analysis. The radioactivity of the urine samples were assayed directly in a scintillation counter whereas faeces and tissue samples were homogenised and combusted prior to assay.

Results:

On average 97.8%, 97.5% and 89.8% of the low-, mid- and high dose respectively was excreted at 48 hours after treatment. The main route of excretion for all dose levels was via urine, averaging 89.3% of the dose whereas

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faeces contained an average of 10.4% of the dose. The total level of radioactivity in selected tissues was below the limit of detection in all but two animals of the high dose group. These animals showed concentrations above the limit of quantification in red blood cells/testes (male rat) and fat (female rat) respectively.

Table B.6.8.1.2.1-1: Excretion of radioactivity (in % of total radioactivity) by rats after a single oral dose of ¹⁴C-1,2,4-Triazole (mean, n = 2)

Treatment	0.4 mg/kg bw		48.8 mg/kg bw		865.7 mg/kg bw	
Sex	Male	Female	Male	Female	Male	Female
Urine, day 1	81.0	78.3	65.2	69.7	43.7	46.9
Urine, day 2	10.2	10.5	11.7	19.2	36.5	38.5
Urine, day 3	1.5	1.2	1.9	2.4	5.8	5.3
Urine, day 4	0.4	0.3	0.5	0.5	0.9	0.7
Urine, day 5	0.1	0.1	0.3	0.3	0.3	0.2
Urine, day 6	0.2	0.1	0.2	0.2	0.3	0.2
Urine, day 7	0.1	0.1	0.2	0.1	0.1	0.1
Urine, subtotal	93.5	90.6	80.0	92.4	87.6	91.9
Faeces, day 1	7.4	6.5	17.9	9.0	3.2	5.9
Faeces, day 2	1.0	0.6	1.5	0.9	2.0	2.8
Faeces, day 3	0.2	0.2	0.3	0.2	0.8	0.3
Faeces, day 4	0.1	0.0	0.1	0.1	0.2	0.1
Faeces, day 5	0.0	0.1	0.0	0.1	0.1	0.1
Faeces, day 6	0.0	0.0	0.1	0.1	0.1	0.0
Faeces, day 7	0.0	0.0	0.0	0.0	0.1	0.0
Faeces, subtotal	8.7	7.4	19.9	10.4	6.5	9.2
Tissue residues	0.8	0.6	0.8	0.9	1.6	1.3
Cage wash	0.0	0.5	0.3	0.8	1.0	1.2
Total recovery	103.0	99.1	101.0	104.5	96.7	103.6

Table B.6.8.1.2.1-2: Residual radioactivity (in ppm 1,2,4-Triazole equivalents) in selected tissues at 7 days after dosing (mean, n = 2) †

Treatment	0.4 mg/kg bw		48.8 mg/kg bw		865.7 mg/kg bw	
Sex	Male	Female	Male	Female	Male	Female
Plasma	< 0.001	< 0.001	< *0.20	< *0.18	< *3.67	< *3.77
Red Blood Cells	< *0.002	< *0.002	0.21	< *0.19	8.57	< *3.76
Fat	< *0.002	< *0.002	0.21	< *0.19	< *3.59	4.46
Brain	< 0.001	< 0.001	< *0.19	< *0.18	< *3.64	< *3.71
Muscle	< *0.002	< *0.002	< 0.06	< *0.19	< *3.60	< *3.69
Lung	< 0.001	< *0.002	< *0.19	< *0.19	< *3.56	< *3.88
Heart	< 0.001	< *0.002	< 0.06	< *0.19	< *3.73	< *3.99
Spleen	< *0.002	< *0.002	< *0.20	< *0.19	< *3.61	< *3.93
Kidneys	< *0.002	< *0.002	< *0.19	< *0.20	< *3.67	< *3.85
Liver	0.002	< *0.002	0.20	< *0.19	< *3.72	< *3.85
Gonads, female	-	< *0.002	-	< *0.19	-	< *3.81
Testes	< 0.001	-	< *0.19	-	4.79	-

† All data were calculated according to the following rules. When two numbers are less than the limit of detection (0.002, 0.20 and 4.0 ppm for low, mid and high dose respectively) signified by a (<) symbol, these two numbers are averaged. When two numbers are less than the limit of quantitation signified by a (< *) symbol, these two numbers are averaged. When one number is less than the limit of detection (<) and one number is less than the limit of quantitation (< *) only the < * number is listed. When one number is less than the limit of detection (<) or quantitation (< *) and one number is real, only the real number is listed.

Conclusion:

1, 2, 4-triazole was rapidly absorbed and eliminated, mainly via urine.

Comments:

According to OECD TG 417, the distribution pattern should be analysed by either whole-body autoradiographic techniques or by measurements of tissue concentrations at different timepoints of exposure. In this study, the

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tissue distribution was only measured seven days post administration therefore it is not known if the radioactivity initially showed a high distribution to specific tissues.

Single dose

Reference:	Weber, H., Patzschke, K. and Wegner, L. A. (1978): 1,2,4-triazole-¹⁴C: biokinetic studies on rats.
Guideline:	Checked for compliance with OECD 417 (4th April 1984).
Deviations from OECD TG 417:	Test conditions regarding the acclimatisation procedure, randomisation process, temperature, humidity and lighting were not specified in the report. Individual values were not reported and studies of metabolic pathways were not performed.
GLP:	No
Acceptability:	Yes
Test substance / purity:	¹⁴ C labelled triazole [3,5- ¹⁴ C]-1,2,4-triazole/ radiochemical purity > 97% Specific radioactivity: 470 µCi/mg Vehicle: physiological saline solution
Species / Strain:	Rat / albino Sprague Dawley (only males used in study)
Administration /doses	Elimination (renal/faecal): i.v. : 0.1, 1, 10 or 100 mg/kg bw; p.o. : 1 mg/kg bw Elimination (biliary): i.v. : 1 mg/kg bw; i.d. : 1 mg/kg bw Distribution: i.v. : 1.0 mg/kg bw
No. of animals:	5 per experiment (4 in studies of biliary excretion) (86 in total)
Exposure time / Duration:	Elimination studies: Urine and faeces analysed 48 hours post administration. Elimination studies in fistulated rats: Bile, urine and faeces analysed 24 hours post administration. Distribution studies: Relative concentration in plasma determined at 0.5, 1, 2, 3, 8, 24, 48, 72 and 144 hours post administration.

Materials and methods:

The absorption, distribution and elimination of radioactivity in air, urine, bile and faeces were studied in groups of male rats.

The renal and faecal elimination was studied in groups of five rats that received a single oral dose by stomach tube or a single intravenous dose. Urine and faeces were collected up to 48 hours post administration. Biliary elimination of activity was studied in bile collected from groups of four male rats, with or without fistulated bile ducts, up to 24 hours post administration of an intravenous or intraduodenal dose of 1.0 mg/kg bw.

The radioactivity in the urine and bile samples was assayed directly in a scintillation counter whereas faeces and tissue samples were homogenised and combusted prior to assay. The distribution of triazole in the body was studied by analysis of blood and selected tissues from groups of five male rats sacrificed at different time points during six days post administration of a single intravenous dose. The ¹⁴C activity in plasma and tissues was expressed by two different measures of concentration:

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P: the relative concentration calculated in relation to the dose (measured activity per gram serum or tissue divided by the applied activity per gram body weight at sacrifice).

C: the equivalent concentration in µg/g of the unchanged substance ($C=P \times \text{dose expressed in mg/kg}$)

Results:

All results relate to the sum of unchanged parent compound and its biotransformation products.

Excretion studies: The main route of excretion was via urine (92-94% recovery after 48 hours), irrespective of dose or route of administration (Table B.6.8.1.2.1-3). After 48 hours, 3.6-5.4% of the dose was recovered in faeces and the level of tissue residues (= body less G.I. tract¹) was low. About 0.1% of the dose was excreted in the expired air within 30 hours according to a range-finding experiment performed on two rats.

Table B.6.8.1.2.1-3: Excretion of radioactivity (in % of total radioactivity) by male rats after a single oral or intravenous dose of ¹⁴C-1, 2, 4-Triazole at 48 hours post-application (mean, n = 5).

Treatment	intravenous				oral
Dose (mg/kg bw)	0.1	1	10	100	1
Urine	93.9	92.6	92.1	93.9	91.9
Faeces	3.9	5.0	5.0	3.6	5.4
Total Elimination	97.8	97.6	97.1	97.5	97.3
Tissue residues	1.7	2.1	2.4	2.0	2.2
G.I. tract	0.51	0.44	0.51	0.47	0.47

In bile duct fistulated rats, the dose recovery at 24 hours after an intravenous or intraduodenal application was approximately 12% in bile, 60 to 65% in urine and 3.5 to 4% faeces (Table B.6.8.1.2.1-4). The 15 to 20% lower elimination via urine in fistulated compared to non-fistulated rats indicates an enterohepatic circulation.

Table B.6.8.1.2.1-4: Excretion of radioactivity (in % of total radioactivity) into urine, faeces and bile by male rats with fistulated bile ducts after a single intraduodenal or intravenous dose of 1 mg/kg bw of ¹⁴C-1,2,4-Triazole up to 24 hours post-application (mean, n = 4)

Treatment	intravenous			intraduodenal		
Time post-application	Urine	Bile	Faeces	Urine	Bile	Faeces
0 - 8 hours	29.7	6.3	-	31.8	6.2	-
8 - 24 hours	28.9	5.3	-	32.8	5.4	-
0 - 24 hours	58.6	11.6	3.5	64.6	11.6	3.7

Distribution:

Thirty minutes after an intravenous dose of 1 mg/kg bw, almost 100% of the applied activity was detectable in the body while the body less gastrointestinal tract contained about 90% of the applied dose. The amount of activity in the body less gastrointestinal tract declined to 50% of the applied dose at eight hours post administration, and to about 1.5% of the applied dose at day three post administration. The activity was uniformly distributed in the animal body (Table B.6.8.1.2.1-5). The decline of triazole concentration in plasma and in the majority of tissues was approximately mono exponential up to about day three post administration,

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with an elimination half-life of about 12 hours. The levels of radioactivity in the body less gastrointestinal tract and in selected tissues were very low at day six post administration.

Table B.6.8.1.2.1-5: Residual concentrations (in 10^{-2} μ g 1, 2, 4-Triazole equivalents / g) in selected tissues at time intervals up to 6 days after an intravenous dose of 1 mg/kg bw of 14 C-1, 2, 4-Triazole (mean, n = 5)

Time Interval after Dosing	0.5 h	1 h	2 h	3 h	8 h	1 day	2 days	3 days	6 days
Body less G.I. tract	102	83	82	78	53	20	4.2	1.7	< 0.3
Plasma	117	108	100	95	75	24	4.8	1.2	0.13
Red Blood Cells	110	100	96	91	64	22	4.4	1.1	~ 0.30
Blood	114	104	98	93	70	23	4.6	1.2	~ 0.22
Muscle	120	88	81	73	53	20	4.4	1.0	~ 0.20
Skin	100	73	66	60	45	18	3.9	1.8	~ 0.44
Brain	97	81	84	72	58	19	3.9	1.0	< 0.16
Lung	120	120	99	80	57	21	4.7	1.1	~ 0.22
Heart	100	93	97	81	64	22	4.8	1.3	~ 0.22
Liver	110	96	95	85	72	23	5.1	1.5	~ 0.36
Kidneys	98	92	110	100	71	19	4.9	1.2	~ 0.20
Renal Fat	48	33	29	33	24	3	~ 1.2	< 0.6	< 0.6
Adrenal	75	68	68	60	51	15	3.9	< 0.7	< 0.7
Testes	100	89	96	78	71	21	4.2	1.1	~ 0.22

Conclusion:

1, 2, 4-triazole was rapidly absorbed and readily eliminated, mainly via urine. Elimination was dose-proportional and independent of the route of administration.

Comments:

The whole body autoradiographs included in the study report cannot be properly evaluated due to poor resolution.

The gastrointestinal tract (GI) is in almost all assays dissected (including contents) separately from the other organs and tissues. The "body less GI tract" is used instead of the whole body to represent the sum of all organ and tissues. Using this approach the authors claim to avoid the problem of non-completely absorbed substances and recirculating absorbed substances.

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Identification of elimination product

Reference:	Ecker, W. (1980): Biotransformation of 1,2,4-[3(5)-14C] triazole in rats.
Guideline:	Checked for compliance with OECD 417 (4th April 1984).
Deviations from TG 417:	Test conditions regarding the acclimatisation procedure, randomisation process, temperature, humidity, lighting or composition of diet were not specified in the report.
GLP:	No
Acceptability:	Yes
Test substance / purity:	¹⁴ C labelled triazole [3,5-14C]-1,2,4-triazole/ Chemical and radiochemical purity ≥99% (TLC) Specific radioactivity: 103 µCi/mg
Species / Strain	Rat / Sprague Dawley (SPF)
Doses / No. of animals:	10 mg/kg bw / 10 males
Administration:	Orally, (procedure not specified). Vehicle: physiological saline solution (0.9% NaCl)
Exposure time / Duration:	Single dose, sampling of urine during 0-8 hours and 8-24 hours post administration.

Materials and methods

Urine samples were collected from male rats between 0 - 8 hours and 8 - 24 hours post administration of a single oral dose of triazole and subjected to TLC analysis. The TLC separations were visualised by extinction of fluorescence induced by UV-light (250 nm), development of radioautograms and a TLC scanner. The radioactivity of the urine samples was determined by liquid scintillation spectrometry and the distribution of radioactivity on the TLC plates was determined by evaluating the area integrals of the thin layer scans. The identification of 1, 2, 4-triazole as the major urinary elimination product was done by “reverse isotope dilution analysis”. In this method, 500 mg of unlabelled 1, 2, 4-triazole was added to a 10 ml urine sample (collected between 0-8 hours) and specific radioactivity was checked following cleanup steps (12 ethylacetate extractions and 3 recrystallizations). A stable level of specific radioactivity after different crystallization steps was used as a proof of chemical identity between the unlabelled compound and the unknown radiolabelled compound.

Results:

The major urinary zone (95-96% of urinary radioactivity) migrated on TLC with unaltered 1,2,4-triazole (Table 6.8.1.2.1-5).

Table B.6.8.1.2.1-5: Metabolite distribution after TLC in chloroform: methanol:25% ammonia:water (66+28+3+3)

R _f	% eliminated radioactivity in urine		Standard
	0 - 8 hours	8 - 24 hours	
0.18	1.8	2.8	1,2,4-triazole
0.33	1.1	1.9	
0.39	0.7	-	
0.58	96.4	95.3	

The absence of a change in specific radioactivity in the reverse isotope dilution analysis was interpreted as chemical identity between the elimination product and the standard (Table B.6.8.1.2.1-6).

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Table B.6.8.1.2.1-6: "Reverse Isotope Dilution Analysis" of 1, 2, 4-Triazole in rat urine

Step	Triazole (mg)	Radioactivity (μCi)	Specific Activity (nCi / mg)
Urine	500	70.3	141
Ethyl Acetate Extract	372	56.3	151
1st Crystallization	319	45.9	144
2nd Crystallization	271	42.0	155
3rd Crystallization	241	38.3	159

Conclusion:

1, 2, 4-triazole is rapidly eliminated via the urine, predominantly in unchanged form.

B.6.8.1.2.1.1 Summary of Administration, Distribution, Metabolism and Excretion studies:

This section was represented by three independent studies. Two of these were balance studies performed as single dose administrations of triazole via one or three administration routes (Lai, K. and Simoneaux, B. (1986a) and Weber, H., Patzschke, K. and Wegner, L. A. (1978) respectively). The third study was performed to identify urine metabolites (Ecker, W., 1980). Combined, the three studies cover all parts of the ADME of triazole.

The two balance studies showed a rapid and almost complete elimination of 1, 2, 4-triazole irrespective of the route of administration. According to the TLC and the Reverse Isotope Dilution analysis presented in the third study, it was eliminated predominately in unchanged form. The quality of the studies included do not meet the criteria of the current OECD TG 417 (see deviations in each study) and a study of toxicokinetics following repeated administration of 1, 2, 4-triazole was not performed as is customary in ADME studies. Since the exposure to 1, 2, 4-triazole via plants is low during the conditions of the representative use, the information gained in these studies is still considered sufficient.

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B.6.8.1.2.2 Acute toxicity

Oral toxicity (rat)

Range-finding study

Reference:	Procopio, K. R. and Hamilton, J. D. (1992): 1, 2, 4-triazole: acute toxicity range-finding study. Rohm & Haas Report No. 81R-057A (April 9, 1992); as reformatted version (report final) of original study by De Crescente, M. E. of July 23, 1981, amended by Chan, P. K., Fisher, P. M. and Morrison, R. D., September 25, 1987 (1,2,4-triazole: acute toxicity range-finding studies, Report Amendment No. 1, Rohm & Haas Report No. 81R-0057, Syngenta No. CGA71019/0029).
Guideline:	Checked for compliance with OECD TG 401 (24th Feb 1987).
Deviations from TG 401:	Test conditions regarding the acclimatisation procedure, randomisation process, temperature, humidity, lighting and composition of diet were not reported. Three rats/dose level were used instead of five/dose level. The maximum dose volume in TG 401, 2ml/100g, was exceeded in the high dose group that received a dose level of 2.5 ml/100g. Only two dose levels were used.
GLP:	Yes
Acceptability:	As supportive information
Test substance / purity:	1,2,4-Triazole, (batch: TD 81-112)/Purity: 92.8%
Species / Strain:	Rat/ CrI:CD@BR (males)
Doses/ No of animals:	500 mg/kg bw and 5000 mg/kg bw / 3 males/dose group.
Administration:	Orally (gavage) Vehicle: 0.5% methylcellulose
Exposure time:	Single dose, 14 days post-observation period.

Materials and methods:

The test material was suspended in 0.5% methylcellulose and administered to groups of male rats by oral gavage. Clinical signs were observed daily whereas body weights were recorded at the start and at the end of the study. All animals were subjected to necropsy.

Results:

All animals of the 5000 mg/kg bw group died within ten minutes after dosing. Gross necropsy of the decedents revealed reddened duodenums and reddened glandular portions of the stomachs. In contrast, all animals of the 500 mg/kg bw group survived treatment and no clinical signs (study log not shown) or treatment-related lesions were observed.

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Table 6.8.1.3.2-1: Acute oral toxicity of 1,2,4-Triazole in rats

Dose [mg/kg bw]	Mortality	Time of Death
5000	3/3	Within 10 minutes
500	0/3	-

Conclusion:

The oral LD₅₀ of 1, 2, 4-triazole in rats was determined to be more than 500 mg/kg and less than 5000 mg/kg bw.

Comments:

According to annex VI of the Council Directive 67/548/EEC, classification is required when the LD₅₀ is below 2000 mg/kg bw. In this study, the dose levels 500 mg/kg bw and 5000 mg/kg bw were used. In the absence of information on the toxicity between a dose of 5000 mg/kg bw that caused 100% mortality and a dose of 500 mg/kg bw which caused 0 % mortality, the precautionary approach would be to classify triazole as Xn;R22. Since there is an additional study on acute oral toxicity in this section, this study is not necessary for the assessment.

Reference:	Thyssen, J. and Kimmerle, G. (1976): 1,2,4-triazole: occupational toxicology study.
Guideline:	Checked for compliance with OECD TG 401 (24th Feb 1987).
Deviations from TG 401:	Test conditions regarding the acclimatisation procedure, randomisation process (dose levels), temperature, humidity, lighting or composition of diet were not specified in the report. The frequency of clinical examinations and the incidence of clinical signs were not reported. Body weights were not measured. Animals subjected to gross pathological examinations were not specified. The method used to determine the LD ₅₀ value was not specified.
GLP:	No
Acceptability:	Yes
Test substance / purity:	1, 2, 4-triazole / technically pure.
Species / Strain:	Rat/ Wistar II albino
Doses/ No of animals:	250, 500, 1000, 1250, 1500, 1750, 1850 (males only), 2000, 2500 mg/kg 15*/sex/dose *Exceptions: 1000 mg/kg bw (males), 2000 mg/kg bw (females) :30 animals used 2500 mg/kg bw (males): 14 animals used.
Administration:	Orally (gavage) Vehicle: distilled water and Cremophor EL
Exposure time:	Single dose, 14 days post-observation period.

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Materials and methods:

The test material was emulsified in distilled water and Cremophor EL and administered to groups of male and female rats by oral gavage.

Results:

Mortality was observed at 1250 mg/kg bw and above showing a dose-related incidence. All animals administered the highest dose died within three days (Table 6.8.1.3.2-2).

The following clinical signs were observed: sedation, breathing disorders, reduction in general well-being, lying in abdominal or side position. These symptoms appeared within an hour of administration and lasted during 13 days after administration (study log not shown).

Gross necropsy revealed no major changes (data not shown).

Table 6.8.1.3.2-2: Acute oral toxicity of 1, 2, 4-Triazole in rats

Dose [mg/kg bw]	Mortality		Time of Death	
	Males	Females	Males	Females
100	-	0/15		
250	0/15	0/15		
500	0/15	0/15		
1000	0/30	0/15		
1250	1/15	1/15	after 1 day	after 4 hrs
1500	3/15	3/15	after 1 day	after 4 hrs - 1 day
1750	10/15	9/15	after 1 day	after 1 - 12 days
1850	12/15	-	after 1 hr - 1 day	-
2000	13/15	28/30	after 2 hrs - 7 days	after 1-9 days
2500	14/14	15/15	after 1 - 6 days	after 3 hrs - 3 days

Conclusion:

The LD₅₀ reported was 1650 (1547-1744) mg/kg bw in males and 1648 (1547-1737) in females. According to annex VI of the Council Directive 67/548/EEC it should be classified as Xn, R22 (harmful if swallowed).

Comments:

This study lacks a complete description of the experimental methodology and the results obtained. It does not follow any guideline as it was performed before the guideline was adopted. Since the result is based on data from a large number of animals tested at several dose levels, it is considered reliable.

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Acute dermal toxicity**Range-finding study (rabbit)**

Reference:	Procopio, K. R. and Hamilton, J. D. (1992): 1,2,4-triazole: acute toxicity range-finding study. Rohm & Haas Report No. 81R-057A (April 9, 1992); as reformatted version (report final) of original study by De Crescente, M. E. of July 23, 1981, amended by Chan, P. K., Fisher, P. M. and Morrison, R. D., September 25, 1987 (1,2,4-triazole: acute toxicity range-finding studies, Report Amendment No. 1, Rohm & Haas Report No. 81R-057, Syngenta No. CGA71019/0029).
Guideline:	Checked for compliance with OECD TG 404 (24th April 2002).
Deviations from TG 404:	Test conditions regarding the temperature, humidity, lighting, composition of the diet, and the age of the animals were not reported. Details of patch site preparation, patch material used, patching technique, application volume, the size of the treated area and grading of the skin reactions were not specified in the report. A control area (untreated area) was not used. The exposure time was 24 hours instead of four hours as is normally used.
GLP:	Yes
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-triazole, (batch: TD 81-112), Purity: 92.8%
Species / Strain:	Rabbit/ New Zealand White
Doses/ No of animals:	200, 2000 and 5000 mg/kg / 2 males/dose group.
Administration:	Dermal application Vehicle: saline
Exposure time:	Single dose, 24 hour exposure.

Materials and methods:

The test substance was ground and moistened with saline (1:1 w/v) before application onto the closely shaved skin of two male rabbits/dose group. The application site was covered by an impervious cuff for 24 hours then the cuff was removed and the application site was wiped with paper towels. Clinical signs were observed daily and body weights were recorded at the start and at the end of the study.

Results:

All animals of the 5000 mg/kg bw group died on the first or second day after administration of test substance and all animals in the 2000 mg/kg bw group died on the third or fourth day. The clinical signs observed before death are listed in table 6.8.1.3.2-4. All animals of the 200 mg/kg bw group survived treatment and neither clinical signs nor necropsy findings were observed. Since all but the two animals of the low dose group died during the study, body weight changes between dose groups cannot be compared.

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Table 6.8.1.2.2-3: Acute dermal toxicity of 1, 2, 4-Triazole in rabbits

Dose [mg/kg bw]	Mortality	Time of Death
200	0/2	-
2000	2/2	within 4 days
5000	2/2	within 2 days

Table 6.8.1.2.2-4: Clinical signs and necropsy findings after treatment with 1, 2, 4-Triazole in rabbits

Clinical signs	2000 mg/kg bw (no positives/no observed)	5000 mg/kg bw (no positives/no observed)
passiveness	2/2	1/1
abdominal breathing	1/2	
ataxia	1/2	1/1
nasal discharge clear		1/1
nasal discharge yellow		1/1
gasping	1/2	
moribundity	2/2	
salivation	2/2	
reddescant droppings	2/2	1/2
soft faeces	1/2	
tremors		1/1
Necropsy observations		
gastric mucosa separated from serosa		1/2
yellow stained muzzle	1/2	1/2
wet yellow stained muzzle	1/2	1/2
2*2 cm gland. Mass adhering to liver and pancreas.		1/2
glandular appearance of liver		1/2
whitening of intest.tract		1/2
adjacent to stomach		
mod. skin irritation	2/2	
mod. Edema/yellow stained skin at application site		1/2

Conclusion:

In this study, the dermal LD₅₀ of 1, 2, 4-triazole in rabbits was determined to be more than 200 mg/kg bw and less than 2000 mg/kg bw.

Comments:

According to annex VI of the Council Directive 67/548/EEC, a substance with a dermal LD₅₀ between 400 and 2000 mg/kg bw should be classified as Xn, R21, and as T, R24, if the dermal LD₅₀ is between 50 and 400 mg/kg.

In the absence of information on the toxicity between a dose of 200 mg/kg bw and 400 mg/kg bw, the precautionary approach would be to classify triazole as T, R24.

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Rat

Reference:	Thyssen, J. and Kimmerle, G. (1976): 1,2,4-triazole: occupational toxicology study.
Guideline:	Checked for compliance with OECD TG 404 (24th April 2002).
Deviations from TG 404:	Test conditions regarding temperature, humidity, lighting and age of animals were not reported. Details of patch site preparation, patch material used, patching technique, application volume and grading of skin reactions were not specified in the report. An untreated area to serve as control was not used in the study. The frequency of clinical examinations and the time of onset and the duration of the symptoms was not reported. Body weights were not recorded.
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-Triazole / technically pure.
Species / Strain:	Rat/ Wistar II albino
Doses/ No of animals:	1000, 2000 (only females), 2500, 3500, 4000 (only females) or 5000 mg/kg bw 10 animals were used per dose level Exceptions: males (1000 mg/kg bw): 5 animals; females (2000 mg/kg bw): 20 animals
Administration:	Dermal application Vehicle: Cremophor EL
Exposure time:	Single dose, 14 days post-observation period.

Materials and methods:

The test article was moistened in drops of Cremophor EL and applied to the dorsal skin of rats that was shaved the day before treatment. The substance was left on for 24 hours then washed off with water and soap. The observation period lasted 14 days.

Results:

Mortality was observed in animals administered doses of 2500 mg/kg bw or more (Table 6.8.1.3.2-5). All animals showed clinical signs which were similar to those in the acute oral toxicity study (sedation, breathing disorders, reduction in general well-being, lying in abdominal or side position).

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Table 6.8.1.2.2-5 Acute dermal toxicity of 1,2,4-Triazole in rats

Dose [mg/kg bw]	Mortality		Time of Death	
	Males	Females	Males	Females
1000	0/5	0/5	-	
2000	-	0/10	-	
2500	2/10	3/10	after 3 - 4 days	after 4 - 9 days
3500	4/10	6/10	after 1 - 3 days	after 2 - 9 days
4000	-	6/10	-	after 1 - 3 days
5000	6/10	18/20	after 2 - 4 days	after 1 - 4 days

Conclusion:

The dermal LD₅₀ of 1,2,4-triazole in rats was determined to be 4200 and 3129 mg/kg bw for males and females, respectively.

Comments:

This study lacks a complete description of the experimental methodology and the results obtained. Since the size of the treated area was not reported, it is not possible to decide if the animals were sufficiently exposed to the substance. The acute dermal toxicity can therefore not be adequately determined in this study.

Acute inhalation toxicity

Reference:	Thyssen, J. and Kimmerle, G. (1976): 1,2,4-triazole: occupational toxicology study.
Guideline:	Checked for compliance with OECD TG 403 (12 th May 1981).
Deviations from TG 403:	Test conditions regarding the temperature, humidity, lighting and age of animals were not reported. Details of the experimental methodology such as the source of air, method of conditioning air, treatment of exhaust air, temperature of air, particle size, nominal and actual concentration in test breathing zone and method of housing animals in the chamber were not reported. The body weights and frequency of clinical examinations were not reported.
GLP:	No
Acceptability:	No (see comments) This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-Triazole / technically pure.
Species / Strain:	Rat/ Wistar II albino Mouse/NMRI
No of animals:	Rat: 5 males; Mouse: 10 males
Administration:	Inhalation using a 10L inhalation chamber.
Exposure time:	4/6 hours, 14 days post-observation period.

Materials and methods:

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Air was passed at 2 litres per minute through test article contained in a dust tower. The resulting air, enriched with vapour and dust, was administered to 5 rats and 10 mice in a 10 litre inhalation chamber. Exposure amounted to 4 and 6 hours.

Results:

According to the study report, the substance did not vaporize or atomize during the conditions used in the 4 and 6-hr experiments. Rats and mice tolerated the inhalation periods without clinical signs. There were no observations of any irritant effects on the mucous membrane of the eyes and noses of the animals.

Conclusion:

During the conditions used in the study, the substance did not vaporize or atomize hence it was not toxic to the animals.

Comments:

The study authors conclude that toxicity due to inhalation of triazole is of no concern as the substance does not vaporize or atomize during the conditions used in the study. However, this conclusion cannot be evaluated since neither the parameters used to determine the degree of vaporisation/atomisation nor the methodology of analysing these parameters is reported. Moreover, the substance may vaporize or atomize during other conditions than those used in this study. Since animals were not exposed to the test article, the inhalation toxicity of triazole cannot be determined.

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B.6.8.1.2.3 Skin irritation

Reference:	Procopio, K. R. and Hamilton, J. D. (1992): 1,2,4-triazole: acute toxicity range-finding study. Rohm & Haas Report No. 81R-057A (April 9, 1992); as reformatted version (report final) of original study by De Crescente, M. E. of July 23, 1981, amended by Chan, P. K., Fisher, P. M. and Morrison, R. D., September 25, 1987 (1,2,4-triazole: acute toxicity range-finding studies, Report Amendment No. 1, Rohm & Haas Report No. 81R-057, Syngenta No. CGA71019/0029).
Guideline:	Checked for compliance with OECD TG 404 (24th April 2002) .
Deviations from TG 404:	Test conditions regarding temperature, humidity, composition of diet and age of animals were not reported. Details of patch site preparation, patch material used and patching technique were not specified in the report. A control area (untreated area) was not included in the study. Two rabbits were used instead of three. Irritation scores after 48 hours were not determined. The deviations are not considered to influence the results obtained.
GLP:	Yes
Acceptability:	Yes This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-Triazole, (batch: TD 81-112), Purity: 92.8%. Vehicle: saline
Species / Strain:	Rabbit/ New Zealand White
Doses/ No of animals:	500 mg/kg / 2 males
Administration:	Dermal application
Exposure time:	Single dose, 24 hour exposure. 7 day post-observation period.

Materials and methods:

The test material was moistened in 0.9% saline (1:1 w/v) and applied under two patches to the shaved skin of two male rabbits. Each rabbit received one patch on the intact skin and a second patch on abraded skin. Both application sites were covered with an impervious cuff for a period of 24 hours then the cuff was removed and the application sites on each rabbit were wiped with paper towels. Skin irritation was evaluated at 24, 72 hrs and at 7 days after patch removal according to EEC criteria.

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Results:

In the intact skin, one application site exhibited moderate erythema at 24 hours and in abraded skin, both application sites exhibited very slight erythema (also present at 72 hours at one of the sites). No edema was observed in abraded or intact skin.

The mean erythema and edema scores of the 24 and 72 hour readings were 0.5 and 0 for intact skin and 0.75 and 0 in the abraded skin. According to annex VI of 67/548/EEC, classification is not required as the mean erythema or edema scores were less than two.

Table B.6.8.1.2.3-1: Intact rabbit skin irritation scores according to EEC criteria

	Erythema		Edema	
	81-26257	81-26258	81-26257	81-26258
Animal no.				
after 24 hours	0	2	0	0
after 72 hours	0	0	0	0
mean score 24-72 h	0.5		0	

Conclusion:

During the conditions used in this study, 1, 2, 4-triazole was not irritating to skin.

Comments:

Studies on abraded skin are not part of the test procedure described in OECD TG 404.

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Reference:	Thyssen, J. and Kimmerle, G. (1976): 1,2,4-triazole: occupational toxicology study.
Guideline:	Checked for compliance with OECD TG 404 (24th April 2002) .
Deviations from TG 404:	Test conditions regarding temperature, humidity, lighting, age of animals and grading of skin reactions were not specified in the report. A control area (untreated area) was not included in the study. Body weights and the frequency of clinical examinations were not reported. The vehicle used was not specified. Two rabbits were used instead of three. Irritation scores after 24, 48 and 72 hours were not determined. The area exposed (2.25 cm ²) was smaller than recommended in TG 404 (6 cm ²).
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-triazole / technically pure.
Species / Strain:	Rabbit/ New Zealand White
Doses/ No of animals:	Rabbit: 500 mg applied to cellulose patches/ 2 (one rabbit of each sex). Human: preliminary study: dose unknown / 1 female/exposure time main study: dose unknown / 5 males
Administration:	Dermal application Vehicle: unknown
Exposure time:	Single dose, 24 hour exposure followed by a 7 days post-observation period. Human study, preliminary study: 2 and 4 hours, main study: 8 hours, 7 days post-observation period.

Materials and methods:

Rabbit: Small cellulose patches (1.5 cm²) containing the test material was attached to the hairless skin on the ears of two rabbits using an adhesive dressing.

Human: Skin irritation in humans was first tested in a preliminary study performed on two females exposed to the test substance during two and four hours respectively and then in a main study on five men exposed during eight hours. The substance was applied to the skin of the forearm with adhesive dressings and washed off with soap and water after the exposure time. A post observation period of seven days was used in the preliminary as well as in the main study.

Results:

Rabbit: The application sites revealed no signs of skin irritation following removal of the dressing or during the 7-day post-treatment observation period.

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Human: The treated parts of the skin proved to be physiologically normal following removal of the dressing and during the post-observation period.

Conclusion:

In this study, 1, 2, 4-triazole was not irritating to skin.

Comments:

Since the size of the treated area was not reported it is not possible to decide whether the animals were sufficiently exposed to the substance thus the irritation potential of triazole cannot be adequately determined. The human study is not considered acceptable since a full description the test conditions (including the dose level used) is missing from the report.

B.6.8.1.2.4 Eye irritation (rabbit)

Range-finding study

Reference:	Procopio, K. R. and Hamilton, J. D. (1992): 1,2,4-triazole: acute toxicity range-finding study. Rohm & Haas Report No. 81R-057A (April 9, 1992); as reformatted version (report final) of original study by De Crescente, M. E. of July 23, 1981, amended by Chan, P. K., Fisher, P. M. and Morrison, R. D., September 25, 1987 (1,2,4-triazole: acute toxicity range-finding studies, Report Amendment No. 1, Rohm & Haas Report No. 81R-057, Syngenta No. CGA71019/0029).
Guideline:	Checked for compliance with OECD TG 405 (24th April 2002).
Deviations from TG 404:	Test conditions regarding the temperature, lighting, humidity, age of animals, individual weights of animals at start and end of study, composition of diet, the volume of solid material used, the condition of the eye before treatment and the time of sacrifice were not reported. Two rabbits were used instead of three. The size of the corneal opacity area was not reported.
GLP:	Yes
Acceptability:	Yes This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-triazole, (batch: TD 81-112) / Purity: 92.8%.
Species / Strain:	Rabbit/ New Zealand White
Doses/ No of animals:	100 mg/kg / 2 males
Administration:	Applied to the conjunctival sac of the left eye.
Exposure time:	Single dose, observations at 4, 24, 48, 72 and 96 hours and at 7 and 14 days post administration.

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Materials and methods:

The test substance was ground in a mortar and applied to the conjunctival sacs of the left eyes of two male rabbits. The lower eyelid was held open momentarily after treatment. The eyes were examined at 4, 24, 48, 72, 96 hours and at 7 and 14 days post administration and the grade of eye irritation was scored according to EEC criteria at 24, 48, 72 hours post administration (table 6.8.1.3-1).

Results:

Corneal, iridal and conjunctival effects were observed at four hours post treatment. Iridal effects still remained on the seventh day post administration, but were not evident on day 14.

Table 6.8.1.3-1: Eye irritation scores according to EEC criteria - unwashed eyes

Animal no.	Corneal opacity		Iris lesions		Conjunctival redness		Conjunctival chemosis	
	81-26280	81-26282	81-26280	81-26282	81-26280	81-26282	81-26280	81-26282
after 24 hours	1	1	0.5	1	2	2	2	2
after 48 hours	1	2	0.5	1	2	2	2	2
after 72 hours	2	3	0.5	1	2	2	1	2
mean score 24-72 h	1.7		0.8		2.0		1.8	

Conclusion:

Triazole caused irritating effects on the eye that were reversible within 14 days. According to the Council Directive 67/548/EEC, classification of 1, 2, 4-triazole is not required.

Reference:	Thyssen, J. and Kimmeler, G. (1976): 1, 2, 4-triazole: occupational toxicology study.
Guideline:	Checked for compliance with OECD TG 405 (24th April 2002) .
Deviations from TG 405:	Test conditions regarding the temperature, lighting, humidity, age of animals, individual weights of animals at start and end of study, the condition of the eye before treatment, volume of solid material and the time of sacrifice were not reported. Two rabbits were used instead of 3. The size of the corneal area affected was not reported. Irritation scores after 24, 48 and 72 hours were not determined.
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-Triazole / technically pure.
Species / Strain:	Rabbit/ New Zealand White
Doses/ No of animals:	50 mg / 2 (one rabbit of each sex).
Administration:	Applied to the conjunctival sac of the left eye. Vehicle: unknown (ground solid or paste)
Exposure time:	Single dose, observations reported at 24 hours, 5 days and 7 days post administration.

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Materials and methods:

The test substance was applied into the conjunctival sacs of the left eyes of two rabbits.

Results:

An intense reddening and a very intense swelling of the conjunctivae of the treated eyes developed within an hour after application and persisted up to 24 hours after treatment. A slight reddening and swelling of the conjunctivae was still observed in one animal five days post application. The conjunctivae of both animals had returned to normal seven days post application. A slight, dispersed, diffuse opacity of the cornea and a slight reddening and swelling of the iris was observed during the first and second day post application.

Conclusion:

In this study, 1, 2, 4-triazole caused irritating effects on the eye.

Comments:

Since irritation scores at 24, 48 and 72 hours post administration were not determined and since there is no information regarding the size of the affected corneal area, this study is not useful for classification according to the Council Directive 67/548/EEC.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

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B.6.8.1.2.5 Skin sensitisation

Reference:	Frosch, I. (1998): Evaluation of skin sensitisation by 1,2,4-triazole with the Guinea-Pig Maximisation Test.
Guideline:	Checked for compliance with OECD 406 (17th July 1992).
Deviations from TG 406:	The test area was not painted with 0.5 ml of 10 % to create a local effect as is recommended if the substance is not an irritant.
GLP:	Yes
Acceptability:	Yes This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-Triazole, (Cu 3994/5/1) / Purity > 98% Vehicle: Aqua pro injectione
Species / Strain:	Guinea Pig / Dunkin Hartley, CrI:HABR (SPF)
Doses/ No of animals:	Intradermal induction: see table B.6.8.1.2.5-1; dermal application: 0.5g 75:25 mixture (w/w) of 1,2,4-triazole in white Vaseline. 10 males used in treatment group, 5 males used in control group
Administration:	Intradermal induction at day 0, first dermal application at day 7 and second dermal application (challenge) at day 21.
Exposure time:	48 hour exposure during dermal application. After the second dermal application (challenge) observation of skin reactions were made at 24 and 48 hours after patch removal.

Table B.6.8.1.2.5-1

	Intradermal induction	
Treatment:	Control group	Dose group
Injection 1 (2 pairs)	50:50 (v/v) FCA and Aqua pro injectione.	50:50 (v/v) FCA and Aqua pro injectione.
Injection 2 (2 pairs)	Aqua pro injectione.	10% test article solution in Aqua pro injectione.
Injection 3 (2 pairs)	Not considered necessary in report as Aqua pro injectione is the vehicle.	10% test article solution in 50:50 (v/v) Aqua pro injectione and FCA).

Materials and methods:

The doses used in the main study were based on the results of a pilot study in which two guinea pigs were given four pairs of intradermal injections of test substance solution (20, 10, 5 and 1%) in a cranial to caudal sequence in the shoulder region. Thereafter, the dermal tolerance in the pilot study was tested by applying different formulations of test substance (75:25, 50:50, 25:75, 10:90) at the left and right side of the shoulder and flank.

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The results of the pilot study showed that the intradermal injection of a 10% solution caused slight to moderate erythema in both animals during the 72-hour observation period while dermal application of a 75:25 mixture (w/w) of the test article in vaseline caused no signs of skin irritation (data not shown). These concentrations were therefore chosen in the main study. Body weights were recorded at the start and at the end of the study. The intradermal induction in the main study was performed on day 0 (Table B.6.8.1.2.5-1) followed by epidermal induction on day 7 when a filter paper soaked with 0.5 g of the 75:25 mixture and white vaseline was placed on the skin and covered with an occlusive dressing for 48 hours. The filter-paper placed on control group animals contained 0.5 g white vaseline only. After 48 hours, the patches were removed and the test sites were washed with water. On day 21 of study, the second dermal application (challenge) was performed. Strips of gauze patch containing 0.5 g of the 75:25 mixture and white vaseline or 0.5 g white vaseline were applied to the right and left flank respectively. Both sides were covered with occlusive dressing for 24 hours then the patches were removed and the test sites were washed with water. At 24 and 48 hours after patch removal, the skin reactions were observed and graded according to OECD TG 406. In addition, the skin-fold thickness was measured.

Results:

An intradermal injection of Freund's complete adjuvant caused the same skin irritation (grade 1-2) with and without test substance. These symptoms subsided by the seventh day of the study. In some cases erosion and eschar formation occurred and these were in healing at the end of the study. Erythema (grade 1) was observed in 2 of 10 animals during the first two days after intradermal injection of 10% test substance solution. There were no skin reactions recorded after the first dermal induction and no signs of allergic skin reactions after the epidermal challenge. The treatment had no effect on bodyweight gain or on the skin-fold thickness.

Conclusion:

In this study, 1, 2, 4-triazole was not a sensitiser.

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B.6.8.1.2.5.1 Summary of acute toxicity, irritation and sensibilisation studies:

Study author	parameter	species	Dose levels	Results (incl eclassification)
Procopio, K. R. and Hamilton, J. D. (1992)	Acute oral LD ₅₀	rat	500 and 5000 mg/kg	500 < LD ₅₀ < 5000 mg/kg bw Classification: Xn; R22 (harmful if swallowed)
Thyssen, J. and Kimmerle, G. (1976)	Acute oral LD ₅₀	rat	250, 500, 1000, 1250, 1500, 1750 1850 (males only), 2000, 2500 mg/kg	LD ₅₀ (males): 1650 mg/kg bw LD ₅₀ (females): 1648 Classification: Xn; R22 (harmful if swallowed)
Procopio, K. R. and Hamilton, J. D. (1992):	Acute dermal LD ₅₀	rabbit	200, 2000 and 5000 mg/kg bw.	200 < LD ₅₀ < 2000 mg/kg bw Classification: T; R24 (toxic in contact with skin)
Thyssen, J. and Kimmerle, G. (1976)	Acute dermal LD ₅₀	rat	1000, 2000 (only females), 2500, 3500, 4000 (only females) or 5000 mg/kg bw	LD ₅₀ (males): 4200 mg/kg bw LD ₅₀ (females): 3129 Classification is not required.
Thyssen, J. and Kimmerle, G. (1976)	Acute inhalation LD ₅₀	Rat/mouse	unknown	Not applicable as animals were not exposed to the test article.
Procopio, K. R. and Hamilton, J. D. (1992)	Skin irritation	rabbit	500 mg/kg	non-irritating
Thyssen, J. and Kimmerle, G. (1976)	Skin irritation	rabbit	500 mg/kg	non-irritating
Thyssen, J. and Kimmerle, G. (1976)	Skin irritation	human	unknown	non-irritating
Procopio, K. R. and Hamilton, J. D. (1992)	Eye irritation	rabbit	100 mg/kg	Mildly irritating Classification is not required.
Thyssen, J. and Kimmerle, G. (1976)	Eye irritation	rabbit	50 mg	Irritating This study is not useful for classification due to poor study quality.
Frosch, I. (1998)	Skin sensitisation (Guinea-Pig Maximisation Test)	male Guinea pigs	Intradermal injection (x3): 10% test article solution in Aqua pro injectione. Dermal application (x2): 0.5g of 75:25 mixture and white vaseline	non-sensitising

The acute oral toxicity was determined in two independent studies. In the study by Procopio, K. R. and Hamilton, J. D., an oral LD₅₀ value could not be deduced since the gap between the high dose level that caused 100% mortality and the low dose level that caused 0 % mortality was too wide. In the other study by Thyssen, J. and Kimmerle, G. (1976), data regarding the test conditions were considered insufficient and the method used for determination of LD₅₀ was not specified. However, the result of this study was still considered reliable since it was based on data from a large number of animals tested at 8-9 dose levels. According to this study, 1, 2, 4-triazole should be classified as **Xn, R22** (harmful if swallowed). The post-mortem examinations performed did not identify any particular target organ. In the Procopio, K. R study on acute dermal toxicity, 1, 2, 4-triazole was classified as **T; R24** (toxic in contact with skin) thus it is of higher dermal toxicity than the parent compound.

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However, since 1, 2, 4-triazole is a plant residue of difenoconazole, the exposure to this substance is presumed to be far below toxic doses.

The skin and eye irritation studies contain less data than required but the Procopio, K. R. and Hamilton, J. D. studies are still considered acceptable. Since the exposure to 1, 2, 4-triazole via plants is presumed to be low during the conditions of the representative use, any irritating potential of triazole is considered to be of low concern.

In conclusion, the majority of the studies presented in this section lack a complete report of the methodology used which is necessary for an independent and adequate assessment of toxicity. However, since 1, 2, 4-triazole is a plant residue of difenoconazole, the exposure to this substance is mainly via the oral route thus only the oral toxicity of 1, 2, 4-triazole needs to be determined.

B.6.8.1.2.6 Subchronic Oral Toxicity

Reference:	Bomhard, E., Löser, E. and Schilde, B. (1979): 1,2,4-Triazole: Subchronic toxicological study with rats.
Guideline:	Checked for compliance with OECD 408 (27th July 1995).
Deviations from TG 408:	The relative humidity during housing of animals was not reported. The preparation of the test substance was not described and its stability in diet was not analysed. Blood urea nitrogen, sodium, potassium and albumin were not analysed. Epididymides, uterus and brain were not weighed and the spinal cord, parathyroid and the female mammary gland were not examined histologically. An ophthalmological examination was not performed. Histopathological examination of the high dose and control group was only performed on five animals per sex instead of all. Sensory reactivity to stimuli, assessment of grip strength and motor activity was not conducted.
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-triazole (batch: 16001/78) Purity: 99.6%.
Species / Strain:	Rat/ Wistar W.74 SPF
Doses / No. of animals:	0, 100, 500 and 2500 ppm 15/sex/dose
Administration:	Orally, via the diet.
Exposure time / Duration:	Daily dose / 3 months

Materials and methods:

The test material was administered to rats daily in diet for three months. The animals were inspected daily and a weekly record was kept of any alterations or signs occurring (study log not shown). Body weights and food

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intake were recorded weekly whereas body temperatures were measured at one and three months after the start of the study. Urine was collected during a period of approximately 17 hours. At one and three months after the start of the study, five animals of each sex and dose group were subjected to clinical examinations. At the end of the treatment period all animals were sacrificed and examined for gross changes and selected organs were weighed. A histopathological examination was performed on most organs described in OECD TG 408 (see deviations) in five animals of each sex of the high dose group and the control group. Additionally, extra liver specimens from all animals used in the study were fixed with formol calcium for fat demonstration. Arithmetic group means, standard deviations and upper and lower confidence intervals were calculated and the values of treated and control groups were compared using the significance test (U-test) of Mann, Whitney and Wilcoxon.

Results:

All animals survived to the end of treatment. Two males and two females at 2500 ppm exhibited temporary slight convulsions otherwise the appearance and behaviour was similar in control and treated groups (study log not shown). During the first two weeks of treatment, a reduced food intake was observed in animals administered 2500 ppm. This resulted in a reduced body weight gain during this time period. The bodyweight gain during the entire study period was still slightly reduced in animals administered the highest dose but as this reduction was small (12% and 8% for males and females, respectively) it was not considered adverse (Table 6.8.1.2.6-1).

Table 6.8.1.2.6-1: Body weight gains (mean values, gram)

Time interval	0 ppm		100 ppm		500 ppm		2500 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
week 0 - 1	36 (100)	25 (100)	36 (100)	24 (96)	38 (106)	25 (100)	14 (39)	16 (64)
week 0 - 4	131 (100)	70 (100)	131 (100)	70 (100)	137 (105)	64 (91)	105 (80)	58 (83)
week 0 - 8	196 (100)	100 (100)	201 (103)	101 (101)	212 (108)	94 (94)	168 (86)	92 (92)
week 0 - 13	248 (100)	113 (100)	254 (102)	117 (104)	256 (103)	108 (96)	219 (88)	104 (92)

Data in brackets express body weight gain as a percentage of that seen in controls.

Statistically significant changes in red blood cell parameters were observed in males administered 2500 ppm after 1 and 3 months of study (Table 6.8.1.2.6-2). However, the difference in haemoglobin concentration between high dose and control rats was small and the concentration of haemoglobin in the high dose males was higher after three months of treatment than after one month. The reduced level of haemoglobin is therefore not considered to be an effect of treatment. The reduced level of MCH is a consequence of the reduced level of haemoglobin as it is defined as the quotient of the haemoglobin concentration and the concentration of erythrocytes. Similar to MCH, the reduced level of MCV is a consequence of the reduced haematocrit as it is defined as the quotient of the haematocrit and the concentration of erythrocytes. Since the levels of the individual components of the haematocrit such as the concentration of erythrocytes and the leucocyte counts, are normal, the reduced haematocrit may be a false effect due to hemolysis or low blood volume. The reduced levels of haemoglobin, MCH, haematocrit and MCH are not considered to be effects of the treatment.

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Table 6.8.1.2.6-2: Red blood cell parameters (mean values) in male and female rats

Parameter	0 ppm				100 ppm				500 ppm				2500 ppm			
	Males		Females		Males		Females		Males		Females		Males		Females	
	4	13	4	13	4	13	4	13	4	13	4	13	4	13	4	13
Erythrocytes (tera/l)	7.42	8.83	7.36	7.67	7.22	8.78	7.51	7.63	7.09	8.53	7.51	7.80	7.41	8.64	7.74	7.91
Haemoglobin (g/l)	151	162	145	148	147	161	146	144	144	155	146	146	142	151	146	146
Haematocrit (l/l)	0.481	0.517	0.468	0.468	0.465	0.510	0.473	0.457	0.450	0.488	0.464	0.458	0.449	0.475	0.469	0.464
MCV (fl)	65	59	64	61	64	58	63	60	63	57	62	59	61	55	61	59
MCH (pg)	20.3	18.3	19.7	19.2	20.4	18.3	19.4	18.8	20.2	18.2	19.4	18.7	19.1	17.5	18.9	18.5

* or ** statistically significant at the 5% or 1% level, respectively.

The ↓/↑ numbers refer to the difference in per cent from the corresponding control value.

The analysis of the clinical chemistry parameters demonstrated a few changes that were statistically significant. The reduced levels of creatinine observed at the first measurement in males of all dose groups returned to normal during the following two months and they were therefore not considered to be toxicologically relevant. Moreover, no changes were observed in the histopathological examination of kidneys of these rats. Changes in blood sugar levels were observed in females administered 500 or 2500 ppm. However, the animals were not fasted prior to the clinical chemistry analysis which may increase the variability in this parameter. The decrease in GLDH observed in male rats of the 2500 ppm group was not considered to be toxicologically relevant as none of the other five parameters (ALP, ALT, AST, bilirubin and total protein) used to study liver function were affected.

Table 6.8.1.2.6-3: Clinical chemistry parameters

Parameter	0 ppm				100 ppm				500 ppm				2500 ppm			
	Males		Females		Males		Females		Males		Females		Males		Females	
	4	13	4	13	4	13	4	13	4	13	4	13	4	13	4	13
Plasma																
GLDH	n.d	7.4	n.d	2.8	n.d	11.4	n.d	4.6	n.d	4.4	n.d	7.2	n.d	2.2	n.d	6.6
Blood sugar	5.98	6.06	5.81	5.19	6.27	6.12	6.08	5.57	6.42	6.15	6.92	6.04	5.75	6.30	6.43	5.87
											**	**			**	**
											↑19%	↑16%			↑11%	↑13%
Urine																
Creatinine (μmol/l)	46	85	43	61	52	78	48	62	83	55	45	56	51	56	47	57
					*				**				**			
Protein (g/l)	0.34	0.73	0.22	0.22	0.38	0.60	0.20	0.22	0.54	0.52	0.24	0.48	0.46	0.82	0.56	0.56
					↑13%				↑80%				↑11%		*	*
															↑155%	↑155%

or ** statistically significant at the 5% or 1% level, respectively.

The ↓/↑ numbers refer to the difference in per cent from the corresponding control value.

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Changes in absolute organ weights were observed in animals administered 2500 ppm but as these changes were less than 10% (except the thymus weight that was reduced 15% in males) and not accompanied by any histopathological findings, they were not considered to be adverse.

Table 6.8.1.2.6-4: Absolute organ weights (mean values, mg) in male and female rats

Organ	0 ppm		100 ppm		500 ppm		2500 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Thymus	339	268	323	233	339	240	287* ↓15%	238
Heart	963	628	938	631	962	605	876** ↓9%	589
Lung	1217	883	1190	887	1230	847	1106* ↓9%	804** ↓9%
Spleen	603	426	597	424	578	421	534* ↓11%	382
Testes	3418	-	3308	-	3247	-	3215* ↓6%	-

* or ** statistically significant at the 5% or 1% level, respectively.

The ↓/↑ numbers refer to the difference in per cent from the corresponding control value.

The gross findings at necropsy were not considered to be associated with treatment.

The histopathological evaluation revealed slight to moderate fat accumulation in liver parenchyma cells in three of fifteen males administered 2500 ppm.

Conclusion:

The NOAEL in this study was 500 ppm (equivalent to 37.9/54.2 mg/kg bw/day in males/females) based on the temporary slight convulsions observed in both males and females and on the hepatocellular fat accumulation observed in males administered 2500 ppm (equivalent to an average test substance intake of 212 and 267 mg/kg bw/day for males and females, respectively). **The NOEL was 100 ppm** (the concentration is only reported in ppm) based on the reduced levels of creatinine observed in males administered 500 ppm.

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B.6.8.1.2.7 Genotoxicity

Reference:	Poth, A. (1989) <i>Salmonella typhimurium</i> reverse mutation assay with 1H-1, 2, 4-Triazole.
Guideline:	Performed in accordance with OECD TG 471 (26 th May 1983) and 84/449/EEC B.14. Checked for compliance with OECD TG 471 (21st July 1997).
Deviations from TG 471:	A strain detecting oxidising agents, crosslinking agents and hydrazines (i.e WP2 strains or <i>S. typhimurium</i> TA 102) was not included in the test.
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	1H-1, 2, 4-Triazole (batch: 249 950 004) / Purity 99.7%. Vehicle: water.
Bacterial strains:	<i>Salmonella typhimurium</i> TA98, TA100, TA1535 and TA 1537 (histidine-auxotrophic)
Doses:	10.0, 33.3, 100.0, 333.3, 1000 and 5000 µg/plate.
Exposure time:	72 hours at 37°C

Materials and methods:

Two sets of independent mutagenicity tests were performed in four histidine-auxotrophic strains of *Salmonella typhimurium* in presence or in absence of metabolic activation. The highest concentration applied (5000 µg /plate) was determined in a preliminary study of toxicity performed in strains TA 98 and TA 100. After preparation, the plates were incubated in darkness during 72 hours at 37 °C and thereafter evaluated by colony counting using a BIOTRAN III counter. Positive and negative controls (untreated and solvent controls) were included in the test in order to demonstrate the sensitivity of the test system. Triplicates of each concentration were tested in the original as well as in the confirmatory mutagenicity test.

Results:

The presence of a normal background lawn was confirmed in all experiments. A reduced number of revertant colonies was observed at the highest concentration tested in all strains used both in presence and absence of metabolic activation. This effect was also observed at 1000 µg/plate in strains TA 1535, TA 1537 and TA 100 (only in presence of metabolic activation). Treatment with 1H-1, 2, 4-triazole did not lead to an increased incidence of mutants compared to the negative controls tested whereas positive controls showed a marked increase of the number of revertant colonies.

Conclusion:

Based on the results of this study 1H-1, 2, 4-triazole did not induce gene mutations in the strains of *S. typhimurium* used.

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Comments:

In this study, 4-NOPD was used as a positive control of strains TA 98 and TA 1537 in assays performed without metabolic activation. In TG 471, 2- Nitrofluorene and 9- Aminoacridine serve as examples of strain-specific controls of these strains. There are no historical data reported regarding the reversion frequency of 4-NOPD in these strains but as they induce a high amount of revertant colonies in the test, they are acceptable as controls. In this study, 2-Aminoanthracene was used as the sole indicator of the efficiency of the S9 mix.

Reference:	Melly, J.G. and Lohse, K. (1982): Genetic toxicology report: 1,2,4-triazole; microbial mutagen test.
Guideline:	OECD 471 (21 st July 1997), 84/449/EEC B.14., EPA-TSCA § 798.5265, JMAFF
Deviations from TG 471:	A strain detecting oxidising agents, crosslinking agents and hydrazines (i.e. WP2 strains or <i>S. typhimurium</i> TA 102) was not included in the test. The bacterial titre was not reported and the presence of a background lawn was not confirmed. In the confirmatory experiment, only strains TA 98 and TA 100 were tested. Strain specific positive controls were not included in assays performed withpot activation.
GLP:	No
Acceptability:	As supportive information
Test substance / purity:	1, 2, 4-triazole, Batch: 113296, Purity: 92.8%. Vehicle: DMSO.
Bacterial strains:	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535 and TA 1537 (histidine-auxotrophic).
Doses:	100-7500 µg/plate (concentrations based on 100% a.i)
Exposure time:	No data

Materials and methods:

A mutagenicity test was performed in four histidine-auxotrophic strains of *Salmonella typhimurium* in presence and in absence of metabolic activation. Triplicates of each concentration were tested and the results obtained for strains TA 98 and TA 100 were confirmed in a second mutagenicity test.

Results:

Inhibition of growth was observed in several strains at doses ≥ 2000 µg/plate. 1, 2, 4- triazole did not show any mutagenic potential at the concentrations tested.

Conclusion:

Based on the results of this study 1, 2, 4-triazole did not induce gene mutations in the strains of *S. typhimurium* used.

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Comments:

There was no information regarding the experimental design or the test conditions used included in the study report.

Inhibition of growth was observed in several strains and it cannot be excluded that this may hide an increased mutation frequency. If the plates showing inhibition of growth are withdrawn from the evaluation, the evaluation of mutagenicity of 1, 2, 4-triazole will in some strains rely on the results from a few concentrations. Due to these uncertainties, the negative result obtained in this study report is only considered as data in support of the study by Poth, A. (1989).

B.6.8.1.2.7.1 Summary of genotoxicity studies:

The mutagenicity of 1, 2, 4-triazole was tested in two bacterial reversion tests (Ames test). According to the study by Poth, A (1989), 1, 2, 4-triazole did not induce gene mutations in the strains used. Due to the lack of information in the study by Melly, J.G. and Lohse, K. (1982), this study is regarded as supportive data. The RMS considers that an adequate testing of genotoxicity requires a combination of an Ames test, a gene mutation test on mammalian cells and a chromosome aberration test thus the notifier has been asked (march 2006) for a justification why these tests were excluded from the dossier submission. The notifier has replied (April 2006) that such studies are in progress.

B.6.8.1.2.8 Reproductive Toxicity

Reference:	Menegola, E., Broccia, M. L., Di Renzo, F. and Giavini, E. (2001): Antifungal triazoles induce malformations in vitro. Reproductive Toxicology 15, 421-427
Guideline:	No specific guideline (article in journal).
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1,2,4-triazole / purity: not indicated Vehicle: Ethanol
Species / Strain:	Rat / Crl:CD
Doses/ No of animals:	500, 1500, 2500 and 5000 µM; 9 embryos/dose level (10 embryos in control).
Administration:	Addition of test substance to culture medium.
Exposure time:	48 hours.

This article presents the teratogenic effects observed in a of a post-implantation whole embryo culture exposed to 1, 2, 4-triazole, flusilazole or fluconazole. Here, only the results on 1, 2, 4-triazole are presented.

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Materials and methods:

Rat embryos were explanted from pregnant rats 9.5 days after a positive vaginal smear was detected and cultured (five embryos/bottle) in sterile rat serum supplemented with antibiotics. Triazole was dissolved in 100% ethanol and added to the culture at different concentrations. After 48 h in culture, the embryos were examined with a dissecting microscope. The parameters shown in table B.6.8.1.2.8-1 were evaluated and morphologically normal and abnormal embryos from the control/ethanol group and from treated groups were processed for histological examination. The remaining embryos were evaluated for total protein content and DNA content. Statistical analysis was performed using one-way ANOVA followed by Tukey's test for continuous variables, the U test according to Mann-Whitney for ranks and the χ^2 test for frequencies. The level of significance was set at $P < 0.05$.

Results:

A significant reduction in visceral yolk sac diameter, crown-rump length, somite number, and total score was found in embryos exposed to the test substance at 5000 μM (Table B.6.8.1.2.8-1). There were no effects observed on the embryonal DNA and protein content.

The number of abnormal embryos was comparable between treated groups and the control group (Table B.6.8.1.2.8-2) and these effects were therefore not considered to be related to treatment. By contrast, 100% of embryos exposed to 2500 or 5000 μM showed a well vascularised but markedly anaemic visceral yolk sac. Although both yolk sac vessel formation and blood circulation appeared comparable to the control embryos, the erythrocytes appeared visibly paler than controls. Histopathological examination showed an increased cellular death at the level of mesenchyme of the maxillary processes and branchial arches in 1500, 2500 and 5000 groups hence the NOAEL was placed at 500 μM .

Table B.6.8.1.2.8-1: Morphometric effects on 9.5 day old rat embryos (mean values).

Parameter	Control (n = 10)	500 μM (n = 9)	1500 μM (n = 9)	2500 μM (n = 9)	5000 μM (n = 9)
Visceral yolk sac diameter (mm)	4.47	4.31	3.99 **	4.31	3.44 **
Crown-rump length (mm)	3.87	3.75	3.67	3.61	3.33 **
Head length (mm)	1.83	1.79	1.74	1.78	1.67 **
Somite number	25.1	23.8	24.6	24.3	22.3 **
Total score ¹ (median)	39.0	39.0	39.0	38.5	36.0 **

** statistically significant at the 1% level.

^a control + ethanol

¹ The developmental degree evaluated according to the scoring method by Brown and Fabro, *Teratology* 1981;24:65-78.

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Table B.6.8.1.2.8-2: Effects on 9.5 day old rat embryos: abnormalities and developmental delays.

Parameter	Control (n = 10)	500 μ M (n = 9)	1500 μ M (n = 9)	2500 μ M (n = 10)	5000 μ M (n = 9)
Abnormal embryos	2	2	2	0	3
General aspect	1	2	0	0	3
Rotation anomalies	1	2	0	0	1
Hook-shaped tail	0	0	0	0	2
Heart	1	0	2	0	0
Heart malrotation	1	0	2	0	0
Abnormal yolk sac	0	0	0	10 **	9 **
Yolk sac anaemic	0	0	0	10 **	9 **
Developmental delays	0	0	0	1	1
Neuropore	0	0	0	1	0
Optic vesicle	0	0	0	0	1

** statistically significant at the 1% level.

Conclusion:

The NOAEL of this *in vitro* study was 500 μ M due to the well vascularised and markedly anaemic visceral yolk sac observed at 1500 μ M.

Reference:	Wickramaratne, G A de S. (1987). The Chernoff-Kavlock Assay: its validation and application in rats. Teratogen, Carcinogen. and Mutagen. 7, 73-83
Guideline:	No specific guideline (article in journal). Checked for compliance with OECD TG 414 (22nd January 2001).
Deviations from TG 414:	Test conditions regarding the acclimatisation procedure, randomisation process, temperature, humidity, lighting, age of animals, food consumption and data regarding the mating procedure were not reported. Body weights were not reported at the third day of study and clinical observations were not reported at all. Ten females were used instead of 20. The sex and body weight of each foetus was not determined and a post-mortem examination of dams or foetuses was not performed. The uterus weight, the number of corpora lutea, number of implantations/resorptions, number and percent of pre- and post-implantation losses was not reported.
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-Triazole / purity: not indicated. Vehicle: not indicated.
Species / Strain:	Rat / Alpk:AP (Wistar-derived)
Doses/ No of animals:	0, 25 and 100 mg/kg bw
Administration:	Orally (gavage).
Exposure time:	Daily dose from day 7 to 17 of pregnancy (sperm-positive vaginal smear = day 1 of gestation).

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Triazole was assayed in a teratogenicity study as one of 26 chemical substances forming the basis for the Chernoff-Kavlock Assay (a model used for detection of teratogens based on mouse data) applied to Alpk: AP₀ (Wistar-derived) rats. In transposing the model to rat, the number of animals used was reduced.

Material and methods:

The test article was administered by oral gavage to groups of 10 successfully mated and presumably pregnant rats at daily dose levels of 25 or 100 mg/kg bw. Maternal observations were restricted to body weight determinations on days 1, 7-17, and 22 and the offspring was observed for litter weights of live pups on days 1 and 5 post-partum and the number of live and dead pups on these days.

Results:

The effects of triazole were reported as the group mean weight change during pregnancy (day 1-22), group mean weight change during dosing (day 7-16), number of pregnant rats, mean litter size (dead and alive) the number of viable litters at day 1 and 5, the number of live and dead pups at day 1 and 5 (total and mean), per cent survival, mean pup weight at day 1 and 5 and mean % weight gain per litter (days 1-5).

In this assay, 1, 2, 4-triazole had no effect on maternal weight gain, the number of viable litters, litter size, survival or post-natal weight gain.

Conclusion:

During the conditions used in this assay, the NOAEL for maternal and developmental toxicity was ≥ 100 mg/kg bw.

Comments:

Only two dose levels and a limited spectrum of parameters were tested in this study.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

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Reference:	Renhof, M. (1988a): 1,2,4-triazole: investigations into embryotoxic effects on rats after oral administration.
Guideline:	Checked for compliance with OECD TG 414 (22nd January 2001) .
Deviations from TG 414:	Body weight measurements at the third day of study were not performed and food consumption was not measured. Dams were not subjected to a post-mortem examination. The uterus weight as well as the number of corpora lutea and number/ per cent of pre- and post-implantation losses was not reported.
GLP:	The study is not reported to have been performed in compliance with GLP. However, an unsigned declaration that the study has been tested by a QA unit is included in the report.
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1,2,4-Triazole (batch: 270/84) / Purity: 95.3% Vehicle: 0.5% aqueous Cremophor-EL emulsion.
Species / Strain:	Rat / Bor: WISW (SPF Cpb)
Doses/ No of animals:	0, 10, 30 and 100 mg/kg bw; 25 females
Administration:	Orally (administration route is not specified but is supposed to be by gavage).
Exposure time:	Daily dose from day 6 to 15 of pregnancy (sperm-positive vaginal smear = day 0 of gestation).

Materials and methods:

The test article or vehicle mixture was administered daily to groups of 25 successfully mated and presumably pregnant rats during day 6 to 15 of pregnancy. The dose levels chosen were based on the results of a preliminary study during which doses of 1000 and 3000 mg/kg bw caused a high maternal toxicity.

The dams were observed daily regarding appearance and symptoms and body weights were determined on days 0, 6-15, and 20 of study. On day 20 of pregnancy the dams underwent caesarian section and the following parameters were determined: number of implantations, number of foetuses/embryos (live and dead), sex of surviving foetuses, weight of each foetus, average foetal weight per litter, runts per litter, total and average placental weight per litter, examination of all foetuses for external malformations, investigation of a number of foetuses (approximately 30% of total) for visceral malformations (modified Wilson technique), remaining foetuses assigned to skeletal and soft tissue evaluations. Weight gain, number of implantations, number of foetuses and resorptions were statistically evaluated using the WILCOXON-MANN-WHITNEY test, the number of runts was determined by Chi-square test (correction after YATES) and the number of fertilised and pregnant animals was evaluated by Chi-square test (correction after YATES or Fischer's exact test depending on frequency expected).

Results:

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All animals survived treatment and the few clinical signs observed were comparable between treated groups and the control group. A significantly reduced mean body weight gain was observed in dams administered the highest dose (Table 6.8.1.2.8-3) and reduced mean weights were observed in their foetuses (Table 6.8.1.2.8-4). Among these foetuses, the number of runts (defined as the arithmetic mean - 2S_x of control) was significantly increased.

Table 6.8.1.2.8-3: Body weight gains of dams (mean values, gram)

Time period	0 mg/kg bw	10 mg/kg bw	30 mg/kg bw	100 mg/kg bw
Dosing	28.2 (100)	25.4 (90.1)	26.8 (95.0)	21.8 * (77.3)
Pregnancy	92.9 (100)	86.6 (93.2)	90.0 (96.9)	79.8 * (85.9)

* Statistically significantly different from control group mean ($P < 0.05$). Data in brackets express body weight gain as a percentage of that seen in controls.

Table 6.8.1.2.8-4: Effects on foetuses (mean values)

Parameter	0 mg/kg bw	10 mg/kg bw	30 mg/kg bw	100 mg/kg bw
No. of implantations per dam	11.6	10.5	11.4	10.6
No. of male foetuses per dam	6.5	5.1 *	6.0	5.0 *
No. of female foetuses per dam	4.5	5.0	4.6	4.5
No. of male and female foetuses per dam	11.0	10.1	10.6	9.5
No. of losses per dam	0.6	0.4	0.8	1.1
Mean weight of foetuses (g)	3.58	3.59	3.53	3.25**
Mean weight of placenta (g)	0.56	0.56	0.57	0.56
No. of foetuses with minor skeletal deviations	2.00	2.41	2.84	2.42
No. of foetuses with malformations	0.05	0.05	0.05	0.17
No. of runts	0.33	0.23	0.53	2.21**

* or ** statistically significant at the 5% or 1% level, respectively.

The malformations observed were predominantly eye deformities (Table 6.8.1.2.8-5). According to the historical data included in the study report, the normal frequency of Microphthalmia in this strain is 6/1344 which correlates well with the result obtained in the control group of this study. The frequency at the highest dose level was slightly elevated.

Table 6.8.1.2.8-5: Malformations observed in foetuses (of total number of foetuses)

Parameter	0 mg/kg bw	10 mg/kg bw	30 mg/kg bw	100 mg/kg bw
Microphthalmia, bilateral	1/231			
Microphthalmia, right side		1/222		1/228
Microphthalmia, left side				1/228
Anophthalmia				1/228
False posture of right hind leg			1/202	
Dysplasia and asymmetry of body of vertebrae and vertebral arches of thoracic spine and abnormal position of one rib				1/228

Conclusion:

The maternal NOAEL was 30 mg/kg bw based on the reduced body weight gain observed at 100 mg/kg bw.

The developmental NOAEL was 30 mg/kg bw based on the lower foetal weights and the increased incidence of runts observed at 100 mg/kg bw. The maternal and developmental NOEL was 30 mg/kg bw based on the absence of any treatment-related effects at this level.

Comments:

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Since the dams used in this study were not subjected to a post-mortem examination, the maternal NOAEL was only based on clinical signs and body weight measurements. Since food consumption was not recorded it is possible that the reduced body weight gain observed at 100 mg/kg was due to palatability and that the NOAEL in that case was overrated. On the other hand, it is also possible that the NOAEL was underestimated if a post-mortem examination of the dams had been performed and pathological changes were observed in the 30 mg/bw dose group. The developmental NOAEL cannot be determined with certainty either since the effects observed may be secondary to the maternal toxicity.

The anophthalmia observed in 1/228 fetuses of the 100 mg/kg bw group may indicate a teratogenic effect of triazole but in the absence of other effects it is considered to be a random finding. If higher doses had been used, this might have been clarified.

Reference:	Renhof, M. (1988b): 1,2,4-triazole: investigations into embryotoxic effects on rats after oral administration.
Guideline:	Performed in accordance with the "Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals", EPA, 83-3, "Teratogenicity study", Revised Edition, November 1984. Checked for compliance with OECD TG 414 (22nd January 2001) .
Deviations from TG 414:	Body weight measurements at the third day of study were not performed. Autopsy findings in dams were not reported.
GLP:	Yes (self-certification of the laboratory)
Acceptability:	Yes (only for a determination of the maternal NOAEL) This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1,2,4-Triazole (batch: 270/84) / Purity: 94.0% Vehicle: 0.5% aqueous Cremophor-EL emulsion.
Species / Strain:	Rat / Bor: WISW (SPF Cpb)
Doses/ No of animals:	0, 100 and 200 mg/kg bw; 25 females
Administration:	Orally (gavage)
Exposure time:	Daily dose from day 6 to 15 of pregnancy (sperm-positive vaginal smear = day 0 of gestation).

This study report is a supplement to the previous study and was performed to elucidate if 1, 2, 4-triazole had a teratogenic potential.

Materials and methods:

The test article or vehicle mixture was administered daily to groups of 25 successfully mated and pregnant rats during day 6 to 15 of pregnancy. The dose levels chosen were based on the results of the previous study (Renhof, M. (1988a) in which the highest dose level, 100 mg/kg bw, was insufficient to determine any teratogenic effects of 1, 2, 4-triazole. In the present study 200 mg/kg bw was used as the highest dose level.

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The dams were observed daily regarding appearance and symptoms and body weights were determined on days 0, 6-15, and 20 of study. On day 20 of pregnancy the dams underwent caesarian section and the following parameters were determined: number of implantations, number of corpora lutea, weight of uterus, number of foetuses/embryos (live and dead), sex of surviving foetuses, weight of each foetus, weight of each placenta, average foetal weight per litter, number of malformations, length (crown to rump) of each foetus, runs per litter, examination of all foetuses for external malformations, investigation of a number of foetuses (approximately 50% of total) for visceral malformations (modified Wilson technique), remaining foetuses assigned to skeletal and soft tissue evaluations. In the foetal skeletal evaluation, changes were categorised as biological changes, deviations or malformations and the last two categories were recorded (biological changes were described in the study report). Weight gain, number of implantations, number of foetuses and resorptions were statistically evaluated using the WILCOXON-MANN-WHITNEY test, the number of runs was determined by Chi-square test (correction after YATES) and the number of fertilised and pregnant animals was evaluated by Chi-square test (correction after YATES or Fischer's exact test depending on frequency expected).

Results:

Analyses of each dosing suspension confirmed that all animals received the intended dose within experimental limits of $\pm 10\%$ except for the first dosing suspension of the high dose which was 87% of the nominal value. All animals survived the treatment and the few clinical signs observed were comparable between treated groups and control. There were no autopsy findings. Food consumption was comparable between groups but a significantly reduced mean body weight gain was observed in dams administered the highest dose level (Table 6.8.1.2.8-6). Foetal and placental weights were reduced at 100 and 200 mg/kg bw and the the number of runs (defined as the arithmetic mean - $2S_x$ of control) was significantly increased at these dose levels (Table 6.8.1.2.8-7). In addition, the foetal lengths were reduced in the highest dose group compared to controls but this parameter was not statistically evaluated.

Table 6.8.1.2.8-6: Body weight gains of dams (mean values, gram)

Time period	0 mg/kg bw	100 mg/kg bw	200 mg/kg bw
Dosing	29.3 (100)	27.4 (93.5)	21.5 * (73.4)
Pregnancy	96.9 (100)	91.9 (94.8)	60.4 ** (62.3)

* or ** statistically significant at the 5% or 1% level, respectively. Data in brackets express body weight gain as a percentage of that seen in controls.

The number of surviving foetuses per dam was significantly reduced at 200 mg/kg bw and the effect was most pronounced in female foetuses (Table 6.8.1.2.8-7).

The incidence of foetuses with minor skeletal deviations was highest in the low dose group but the incidence of foetuses with bone changes (retardation) was comparable between the treated groups (74% in 200 mg/kg bw group and 70% in the 100 mg/kg bw group. The total number of foetuses with malformations increased with dose (Table 6.8.1.2.8-7). The distribution of the malformations observed is summarized in Table 6.8.1.2.8-8.

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Table 6.8.1.2.8-7: Effects on foetuses (mean values)

Parameter	0 mg/kg bw	100 mg/kg bw	200 mg/kg bw
No. of corpora lutea per dam	13.6	13.9	14.2 *
No. of implantations per dam	12.5	12.2	11.8
No. of males per dam	5.9	6.0	3.1 **
No. of females per dam	6.1	5.9	2.4 **
No. of males and females per dam	12.0	11.9	5.5 **
No. of losses per dam	0.5	0.3	6.3 **
Mean weight of foetuses (g)	3.55	3.06 **	2.35 **
Mean weight of placenta (g)	0.59	0.52 *	0.49**
Foetuses with minor skeletal deviations	2.67	4.32 *	2.24
Foetuses with malformations	0.29	0.63	0.80 *
No. of runts	0.24	2.84 **	4.96 **

* or ** statistically significant at the 5% or 1% level, respectively.

Table 6.8.1.2.8-8: Rat teratology study – Malformations

Type of malformation	Number of affected foetuses		
	0 mg/kg bw (253)	100 mg/kg bw (226)	200 mg/kg bw (138)
Microphthalmia, left side	2	0	0
False posture of hind legs	0	0	1
Undescended testicle	2	1	6
Hydronephrosis	1	1	7
Multiple malformation	1	0	0
Cleft palate	0	0	4
Humeral dysplasia	0	0	1
General oedema	0	0	1
Long bone dysplasia	0	0	2
Diaphragmatic hernia	0	0	1

The total number of foetuses is given within brackets.

Conclusion:

The maternal NOAEL was 100 mg/kg bw based on retarded weight gain at 200 mg/kg bw. The developmental **NOAEL is less than 100 mg/kg bw** based on an increased incidence of runts, lower foetal and placental weights, and a higher incidence of minor skeletal deviations at 100 mg/kg bw.

Comments:

In this study, a developmental NOAEL could not be determined since adverse effects were observed at all dose levels used. The reduced foetal/placental weights and the reduced number of surviving foetuses may be due to maternal toxicity and not due to a reproductive toxicity of 1, 2, 4-triazole. However, the incidence of cleft palate (4/138) observed in the high dose group points at 1, 2, 4-triazole as being a teratogen substance.

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Reference:	Wickings, E. J., Middleton, M. C. and Hillier, S. G. (1987): Non-steroidal inhibition of granulosa cell aromatase activity in vitro. J. Steroid. Biochem. 26 (6), 641-646.
Guideline:	No specific guideline (article in journal).
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	1, 2, 4-Triazole / purity: not indicated
Strain:	Granulosa cells isolated from female Wistar rats.
Doses/ No of cells:	10^{-5} M; $0.25-0.63 \times 10^5$ cells/0.25ml medium.
Administration:	Addition of test substance to granulosa cell culture culture.
Exposure time:	48 hours.

In this article 1, 2, 4-triazole was included in a serie of structurally related azoles that were tested to identify the moiety/moieties of the chemical structure of R151885 (1,1-di (4-fluoro-phenyl)-2-(1, 2, 4-triazol-1-yl)-ethanol that is responsible for its inhibitory effect on sterol biosynthesis in fungi and for the delayed ovulation observed when administered to cyclic rats.

Materials and methods:

Immature female rats were implanted subcutaneously with a capsule containing diethylstilboestrol to stimulate granulosa cell proliferation. Six days later, the animals were killed and granulosa cells were harvested from the ovaries. The cells were grown in cultures in the presence of human FSH (100 ng/ml), testosterone (10^{-7} M) and the test substance (10^{-5} M) for 48 hours at 37°C in a humidified tissue culture incubator gassed with 95% air / 5% carbon dioxide. The medium was collected at the end of this period and stored at -20°C until analysis for oestradiol and progesterone content by radioimmunoassays.

Results:

The levels of oestradiol and progesterone in rat granulosa cell cultures were unaffected by treatment with 1, 2, 4-triazole.

Conclusion:

1, 2, 4-triazole does not modulate ovarian oestrogen biosynthesis *in vitro*.

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B.6.8.1.2.8.1 Summary of reproductive toxicity

This section was represented by three literature studies and two developmental toxicity studies performed in rats. The first literature study presented in this section was performed on a post-implantation rat embryo culture and the results showed that treatment with triazole resulted in reductions of visceral yolk sac diameter, crown-rump length, somite number and total score. In the other two literature studies there were no toxic effects of triazole observed. Since none of the literature studies meet the criteria of the current OECD guideline, conclusions therein should be interpreted with caution.

Developmental toxicity: The two developmental studies presented in this section were performed by the same lab and the second study served as a complement to the first by adding a higher dose level and additional parameters. In the first study, decreased foetal weights and an increased incidence of runts were observed in the offspring of dams administered 100 mg/kg. However, the developmental NOAEL could not be determined with certainty since the effects observed could be secondary to the maternal toxicity. The developmental NOAEL was therefore estimated to 30 mg/kg bw. In the second study, adverse effects were observed at both dose levels used (100 and 200 mg/kg bw) thus the RMS considers that it is not possible to determine a developmental NOAEL based on this study. Cleft palate was observed in 4/138 fetuses of dams administered 100 mg/kg and triazole was therefore considered to be a teratogen substance. However, although triazole is currently classified (ATP24) as R63 "Possible risk of harm to the unborn child", the teratogenic effects of triazole are not considered to be of concern considering that the exposure to this substance is low during the conditions of the representative use of difenoconazole.

Maternal toxicity: Since the dams used in the first study were not subjected to a post-mortem examination, the maternal NOAEL was only based on clinical signs and body weight measurements in this study. The reduced body weight gain observed at 100 mg/kg resulted in a maternal NOAEL of 30 mg/kg bw. However, since food consumption was not recorded, palatability could not be excluded as a plausible explanation of the reduced body weight gain and the NOAEL would in that case be overrated. On the other hand, it was also possible that the NOAEL was underestimated since pathological changes might have been discovered in the 30 mg/bw dose group if a post-mortem examination of the dams had been performed. Due to these uncertainties, a maternal NOAEL could not be determined in this study.

A reduced body weight gain in dams was observed also in the second study. In contrast to the first study, this effect was observed only in the 200 mg/kg bw group and not in the 100 mg/kg bw group. In this study, the food consumption was estimated and the results showed that the reduced body weight gain was not due to palatability. A maternal NOAEL of 100 mg/kg bw was determined.

Although a reproductive study performed in accordance with the current guideline would be desirable to establish a clear picture of the toxicity of triazole on the reproductive system, it is not justified in this context. Triazole is a plant residue of difenoconazole and the exposure to this substance is presumed to be far below toxic doses during the conditions of the representative use considered in this DAR. Further studies are not required.

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B.6.8.1.3 Toxicology studies of CGA 131013 (Triazole alanine)

[Synonyms: Triazole alanine = Triazolylalanine = TA = THS 2212 = CGA 131013 = R152056 = ICI 156,342]

B.6.8.1.3.1 Absorption, distribution, excretion (toxicokinetics)

Single dose (two dose levels) in rat

Reference:	Hamboeck, H. (1983a): Distribution, degradation and excretion of D,L-2-amino-3-(1H-1,2,4-triazol-yl)-propanoic acid (D,L-triazolylalanine) in the rat.
Guideline:	Checked for compliance with OECD TG 417 (April 4 th 1984).
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	[3, 5- ¹⁴ C]-randomly labelled D,L-2-amino-3-(1H-1,2,4-triazol-yl)-propanoic acid (D,L-triazolylalanine) Chemical and radiochemical purity > 99% (TLC). Specific radioactivity 80.3 µCi/mg and 26.77 µCi/mg (diluted with unlabelled authentic reference compound) for low and high dose respectively.
Species / Strain:	Rat / Tif:RAI f (SPF)
Doses / No. of animals:	0.5 mg/kg bw and 50 mg/kg bw. 4 male and 4 female rats/dose level. Two rats of each sex served as controls.
Administration:	Orally, by gavage Vehicle: tap water
Exposure time / Duration:	Single dose, 168 h observation period post dose (sampling of urine, faeces, acidic and basic volatiles at 24 hour intervals starting 24 hours prior to dosing).

Materials and methods:

The absorption, distribution, and elimination of D,L-triazolylalanine was studied in male and female rats that received either a high or a low single oral dose of the test substance. Urine, faeces, acidic and basic volatiles were collected at 24-hr intervals starting 24 hours prior to dosing. Seven days post administration, all animals were sacrificed by cervical dislocation and blood and a number of organs and tissues were collected for analysis. Radioactivity was measured in a scintillation counter using appropriate scintillation mixtures. The urinary metabolite pattern was analysed using thin layer chromatography (TLC), high voltage electrophoresis (HVE) and high performance liquid chromatography (HPLC).

Results:

Absorption and elimination: D,L-triazolylalanine was rapidly eliminated mainly via urine in animals irrespective of the dose administered (B.6.8.1.3-1). Within 24 hours, approximately 92-99% and 3-5% of the administered doses were recovered in urine and faeces respectively and less than 0.5% was excreted in the expired air.

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Table B.6.8.1.3-1: Excretion of radioactivity (in % of the dose) by rats after a single oral dose of ^{14}C -triazolylalanine (mean \pm SD, n = 4)

Treatment	0.5 mg/kg		50 mg/kg	
Sex	Male	Female	Male	Female
Urine, 0 - 24 h	96.06 \pm 7.28	92.01 \pm 3.96	97.67 \pm 3.08	98.96 \pm 3.34
Urine, 24 - 48 h	1.71 \pm 0.86	2.05 \pm 0.80	6.34 \pm 1.36	1.56 \pm 0.46
Urine, 48 - 72 h	0.37 \pm 0.22	0.15 \pm 0.11	0.37 \pm 0.19	0.27 \pm 0.12
Urine, 72 - 168h	0.11 \pm 0.05	0.10 \pm 0.07	0.23 \pm 0.13	0.15 \pm 0.09
Urine, subtotal	98.25 \pm 7.00	94.31 \pm 3.81	104.62 \pm 4.30	100.94 \pm 3.54
Faeces, 0 - 24 h	3.64 \pm 4.61	4.93 \pm 3.76	2.64 \pm 3.16	3.02 \pm 2.60
Faeces, 24 - 48 h	0.38 \pm 0.04	1.12 \pm 0.60	0.44 \pm 0.12	2.10 \pm 2.20
Faeces, 48 - 72 h	0.14 \pm 0.05	0.52 \pm 0.53	0.09 \pm 0.04	0.19 \pm 0.22
Faeces, 72 - 168 h	0.09 \pm 0.04	0.16 \pm 0.11	0.10 \pm 0.06	0.16 \pm 0.18
Faeces, subtotal	4.26 \pm 4.59	6.70 \pm 4.02	3.28 \pm 3.36	5.47 \pm 3.16
Expired air ¹ , 0-24 h	0.01 \pm 0.00	0.01 \pm 0.005	0.01 \pm 0.00	0.01 \pm 0.00
Expired air, 24 -48 h	n.d. ²	n.d.	n.d.	n.d.
Expired air, 48-168 h	-	-	-	-
Expired air, subtotal	0.01 \pm 0.00	0.01 \pm 0.005	0.02 \pm 0.00	0.02 \pm 0.01
Total excretion	102.52 \pm 2.55	101.02 \pm 0.63	107.92 \pm 1.26	106.43 \pm 0.40
Cage wash	0.06 \pm 0.05	0.63 \pm 0.76	0.25 \pm 0.16	0.19 \pm 0.16
Tissue residues ³	0.00	0.00	0.01 \pm 0.01	0.00 \pm 0.00
Total recovery	102.58 \pm 2.58	101.65 \pm 1.33	108.17 \pm 1.14	106.62 \pm 0.26

¹ Sum of acidic and basic volatiles in the expired air

² n.d. = not detected

³ Calculated from table B.6.8.1.3-3 assuming that fat, blood and muscle represent 11%, 5.9% and 45% of the body weight, respectively, but without carcass values.

Identification of metabolites:

TLC analysis of the urinary metabolite pattern in the 0-24 hour urine revealed a major (U1) and a minor metabolite (U2), accounting for 72-86% and 8-19% of recovered radioactivity, respectively (B.6.8.1.3-2). The major fraction co-chromatographed with D, L-triazolylalanine on HVE at pH 1.9, HPLC and in TLC with different solvent systems (data not shown).

Table B.6.8.1.3-2: TLC analysis of urinary metabolites (in % of recovered radioactivity) in 0 - 24 hour rat urine after a single oral dose of ^{14}C -triazolylalanine (mean \pm SD, n = 4)

Treatment	0.5 mg/kg		50 mg/kg	
Urine fraction	Male	Female	Male	Female
U1	72.0 \pm 7.4	80.3 \pm 2.2	83.4 \pm 2.0	86.4 \pm 1.7
U2	19.4 \pm 6.4	12.7 \pm 3.1	10.6 \pm 1.9	7.7 \pm 1.8
BG1	8.6 \pm 2.0	7.0 \pm 1.2	6.0 \pm 0.6	6.0 \pm 0.6
Total	100.0	100.0	100.0	100.0

¹ Background = radioactivity not attributed to fractions U₁/ U₂

Distribution:

The tissue levels of D,L-triazolylalanine equivalents in animals were low and were primarily found in organs involved in compound elimination (liver, kidneys, blood) (B.6.8.1.3-3). All tissue residues were below the limit of detection after seven days in animals administered the low dose. There was no difference between genders with respect to excretion, the pattern of tissue residues, or urinary metabolite pattern.

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Table B.6.8.1.3-3: Residual radioactivity (in ppm D,L-triazolylalanine equivalents) in selected tissues at seven days post administration (mean \pm SD, n = 4)

Sex		Male		Female	
Tissue	LQ 1 [ppm]	0.5 mg/kg	50 mg/kg	0.5 mg/kg	50 mg/kg
Spleen	0.006	< LD	< LQ 2	< LD	~ LD
Liver	0.001	< LD	0.010 \pm 0.001	< LD	0.008 \pm 0.001
Fat	0.006	< LD	< LD	< LD	< LD
Kidneys	0.003	< LD	0.011 \pm 0.007	< LD	0.007 \pm 0.001
Muscle	0.002	< LD	0.002 \pm 0.000	< LD	0.002 \pm 0.000
Blood	0.002	< LD	0.003 \pm 0.000	< LD	0.003 \pm 0.000
Brain	0.004	< LD	< LD	< LD	< LD
Heart	0.005	< LD	< LQ	< LD	< LQ
Lungs	0.003	< LD	< LQ	< LD	< LQ
Pancreas	0.005	< LD	< LQ	< LD	< LQ
Stomach	0.003	< LD	0.004 \pm 0.001	< LD	0.004 \pm 0.001
Small intestine	0.002	< LD	< LQ	< LD	< LQ
Testes	0.003	< LD	< LQ	< LD	-
Ovaries	0.010	< LD	-	< LD	< LQ
Carcass	0.002	< LD	0.007 \pm 0.001	< LD	0.008 \pm 0.005

L_Q = limit of determination, L_D = limit of detection; $L_D = 0.33 L_Q$

< L_Q = value below L_Q but higher than L_D

Conclusion:

The compound was rapidly absorbed and was eliminated mainly via urine, predominantly in unchanged form.

Comments:

According to OECD TG 417, the correct way to analyse distribution pattern is to use either whole-body autoradiographic techniques or to measure tissue concentrations at different timepoints of exposure. In this study, the tissue distribution was only measured seven days post administration therefore it is not known if the radioactivity initially showed a high distribution to specific tissues.

The results of the TLC, HVE and HPLC experiments are not supported by any data showing that the sample and the reference compound (D,L-triazolylalanine) were identical. In order to prove the identity of the sample, the migration rates, the retention times and the gel pictures from the HVE of both the sample and the reference compound should have been included in the study report. Despite this, the results are considered to be reliable since the study report has been evaluated by a quality assurance unit.

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Single dose (three dose levels) in rat

Reference:	Lai, K. and Simoneaux, B. (1986b): Balance study of ¹⁴C- triazole alanine in orally dosed rats.
Guideline:	Checked for compliance with OECD TG 417 (April 4 th 1984).
Deviations from TG 417:	Test conditions regarding acclimatisation procedure, randomisation process, temperature, humidity, lighting or composition of diet were not specified in the report. Standard deviations of the mean values were not presented.
GLP:	No
Acceptability:	Yes
Test substance / purity:	¹⁴ C labelled triazole alanine (α -amino-[3,5- ¹⁴ C]-1,2,4-triazole-1-propionic acid) (D,L-triazolylalanine) Chemical and radiochemical purity > 99% (TLC). Specific radioactivity: 29.4 (low), 0.3 (mid) and 0.015 (high) μ Ci/mg
Species / Strain:	Rat / Albino Sprague Dawley
Doses / No. of animals:	0.56 (low), 54.4 (mid) and 993.71 (high) mg/kg bw 2 male and 2 female rats/dose level
Administration:	Orally, by gavage Vehicle: PEG
Exposure time / Duration:	Single dose, 168 h observation period post dose (sampling of urine and faeces at 24 hour intervals starting 24 hours after dosing).

Materials and methods:

The absorption, distribution, and elimination of labelled triazole alanine were studied in three groups of male and female rats that received a single oral dose of triazole alanine. Urine and faeces were collected at 24-hr intervals starting 24 hours after dosing. After seven days, all animals were sacrificed and blood and selected organs and tissues were collected for analysis. Urine samples were measured directly in a scintillation counter whereas faeces and tissue samples were homogenised and combusted prior to radioassay.

Results:

Elimination: The main route of excretion was via urine (B.6.8.1.3-4). Within 24 hours, an average of approximately 66–80% and 4–16% of the administered doses were recovered in urine and faeces respectively. On average 97.4%, 87.3% and 88.2% of the total dose was excreted within 48 hours post administration in the low-, mid- and high dose, respectively.

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Table B.6.8.1.3-4: Excretion of radioactivity (in % of total radioactivity) by rats after a single oral dose of ¹⁴C-triazolylalanine (mean, n = 2)

Treatment	0.56 mg/kg bw		54.4 mg/kg bw		993.7 mg/kg bw	
Sex	Male	Female	Male	Female	Male	Female
Urine, day 1	66.1	78.1	71.9	79.7	76.3	63.0
Urine, day 2	13.2	6.1	7.8	3.4	3.8	18.9
Urine, day 3	0.9	0.7	1.1	0.5	0.8	1.4
Urine, day 4	0.3	0.2	0.3	0.2	0.2	0.4
Urine, day 5	0.2	0.1	0.2	0.2	0.2	0.2
Urine, day 6	0.2	0.0	0.2	0.1	0.2	0.2
Urine, day 7	0.2	0.0	0.1	0.1	0.1	0.1
Urine, subtotal	81.1	85.2	81.6	84.2	81.6	84.2
Faeces, day 1	16.2	12.4	4.3	4.7	4.4	7.7
Faeces, day 2	1.2	1.3	1.7	1.0	0.8	1.5
Faeces, day 3	0.6	0.2	0.2	0.1	0.1	0.3
Faeces, day 4	0.1	0.0	0.1	0.1	0.1	0.1
Faeces, day 5	0.1	0.0	0.0	0.1	0.1	0.0
Faeces, day 6	0.1	0.0	0.1	0.1	0.1	0.1
Faeces, day 7	0.0	0.0	0.0	0.0	0.1	0.0
Faeces, subtotal	18.3	13.9	6.4	6.1	5.7	9.7
Tissue residues	1.0	0.4	0.1	1.4	0.7	0.1
Cage wash	1.4	1.0	1.0	0.6	1.6	0.5
Total recovery	101.8	100.5	89.1	92.3	89.6	94.5

Distribution: The levels of radioactivity were at or below the limits of quantitation in almost all of the tissues analysed seven days post administration irrespective of the dose administered (Table B.6.8.1.3-5).

Elevated levels of radioactivity were seen in the spleen (65.49 ppm) and fat (11.12 ppm) of one female rat administered a high dose, in the plasma (0.97 ppm) of one male rat administered a mid dose and in fat (0.026 ppm) of one male rat administered a low dose. However, since these values were obtained in single animals and there was no accumulation observed in these tissues in the previous study (Hamboeck, H. 1983a), these findings are not considered to be toxicologically relevant.

Table B.6.8.1.3-5: Residual radioactivity (in ppm D,L-triazolylalanine equivalents) in selected tissues at 7 days after dosing (mean, n = 2) †

Treatment	0.56 mg/kg bw		54.4 mg/kg bw		993.7 mg/kg bw	
Sex	male	female	male	female	male	female
Plasma	< 0.001	< 0.001	0.97	< 0.13	< 2.86	< 2.97
Red blood cells	< 0.001	< 0.001	< * 0.43	< 0.14	< 2.87	< 2.93
Fat	0.016	< * 0.004	< * 0.44	< * 0.40	< * 8.50	11.12
Brain	< 0.001	< 0.001	< 0.14	< 0.14	< 2.82	< 2.91
Muscle	< 0.001	< * 0.004	< 0.15	< * 0.40	< 2.87	< 2.97
Lung	0.005	< 0.001	< * 0.46	< * 0.43	< 2.84	< * 9.00
Heart	0.006	< * 0.004	< * 0.45	< * 0.41	< 2.88	< 2.94
Spleen	0.006	0.006	< 0.14	< 0.13	< 2.89	65.49
Kidneys	< 0.001	< 0.001	< * 0.41	< * 0.42	< 2.84	< * 8.87
Liver	< 0.001	< 0.001	< * 0.14	< 0.14	< 2.82	< * 8.69
Gonads, female		< 0.002		< * 0.40		< 3.12
Testes	< * 0.004		< 0.15		< * 8.78	

† All data were calculated according to the following rules:

When two numbers are less than the limit of detection (0.004 ppm, 0.40 ppm and 8.5 ppm in the low-, mid- and high dose, respectively) signified by a (<) symbol, these two numbers are averaged.

When two numbers are less than the limit of quantitation signified by a (< *) symbol, these two numbers are averaged.

When one number is less than the limit of detection (<) and one number is less than the limit of quantitation (< *) only the < * number is listed.

When one number is less than the limit of detection (<) or quantitation (< *) and one number is real, only the real number is listed.

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Conclusion:

The compound was rapidly absorbed and readily eliminated, mainly via urine.

Identification of the major metabolic pathways of (D, L-triazolylalanine) in rats.

Reference:	Hamboeck, H. (1983b): The metabolism of D,L-2-amino-3-(1H-1,2,4-triazol-yl)-propanoic acid (D,L-triazolylalanine) in the rat.
Guideline:	Checked for compliance with OECD TG 417 (April 4th 1984).
GLP:	No
Acceptability:	Yes
Test substance / purity:	¹⁴ C labelled D,L-2-amino-3-(1H-1,2,4-triazol-yl)-propanoic acid (D,L-triazolylalanine)/ Chemical and radiochemical purity > 99% (TLC). Specific radioactivity 80.3 µCi/mg and 26.77 µCi/mg (diluted with unlabelled authentic reference compound) in low and high dose respectively.
Species / Strain:	Rat / Tif:RAI f (SPF)
Doses / No. of animals:	0.5 mg/kg bw and 50 mg/kg bw. 4 male and 4 female rats/dose level. Two rats of each sex served as controls.
Administration:	Orally, by gavage Vehicle: tap water
Exposure time / Duration:	Single dose Sampling of urine and faeces 0-24 hours post dose. <i>Analysis of urine by TLC and HVE:</i> pooled urine containing 85-103% of the dose <i>Analysis of metabolites by TLC and HVE (faeces):</i> separately pooled faeces from male and female rats administered the high dose. <i>Analysis of metabolites by IEC/HPLC (urine):</i> pooled urine from three male rats administered the high dose.
Expression of results:	Metabolite levels are reported as % of total radioactivity in urine/faeces pools. Identification of metabolites: Mass spectrometry data shown for metabolite 1a/b and metabolite 2 and reference compounds. NMR spectra of metabolite 2 +/- reference compound.

Materials and methods:

This report is a complement to the the rat balance experiment (Hamboeck, H. (1983a) and adds the major metabolic pathways of D,L-triazolylalanine. All samples analysed in this study originate from the (1983a) study. Urine samples were frozen until analysis whereas faeces was lyophilized and kept at room temperature. The radioactivity in urine was measured directly in a scintillation counter whereas the radioactivity present in faeces was determined after combustion. The major urinary fractions were characterised and scanned for radioactivity using thin layer chromatography (TLC) and high voltage electrophoresis (HVE).

Metabolites were isolated from pooled (0-24 hours) urine samples from three male rats administered the high dose in a purification process involving steps of ion-exchange chromatography, HPLC, and size-exclusion

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chromatography. Column eluates were monitored for radioactivity and the purified compounds were submitted to mass spectroscopy (MS) and nuclear magnetic resonance analysis (NMR). The 0-24 hour faeces samples of male and female rats were separately pooled, extracted with water, and characterised on TLC and HVE (data not shown).

Results:

Two major urinary fractions denoted U-1 and U-2 accounting for 69-86% and 8-19% of the dose respectively were detected in the TLC analysis. In HVE analysis, the major urine metabolite showed an ionic behaviour that resembled that of D, L-triazolylalanine (data not shown). Minor metabolites present in the genuine urine were non-identical to CGA 142852 (2-(1H-1,2,4-triazol-1-yl)-acetic acid) and CGA 71019 (1H-1,2,4-triazole) according to the TLC analysis but were not further investigated.

In TLC analysis of faeces, two to three minor faecal metabolites were identified, none of which exceeded 3% of the dose. The two major fractions co-migrated with D, L-triazolylalanine and its acetylated derivative in both TLC and HVE (data not shown).

Isolation of metabolites:

Metabolites M1a and M1b were isolated in the purification process of fraction U1 and subjected to FD-MS. Both metabolites yielded the spectra of the sodium salt of D,L-triazolylalanine.

Metabolite M2 was isolated in the purification of fraction U-2. This metabolite co-migrated with the reference compound N-acetyl- D,L-triazolylalanine (CGA 143548) on TLC in different solvent systems (data not shown) and in the MS and NMR analyses, these compounds showed identical spectra, both per se and after esterification using 2-propanol/HCl.

Fraction FE-1, representing approximately 50% of the radioactivity in faeces, co-migrated on TLC and HVE (pH 2) with D,L-triazolylalanine (data not shown) and the minor fraction in faeces, FE-2, representing approximately (16%), behaved identically to N-acetyl- D,L-triazolylalanine on TLV and HVE (pH 2). A third fraction, that was not present in urine, accounting for approximately 30% of the radioactivity in faeces, remained unidentified.

Conclusion:

D,L-triazolylalanine was excreted predominantly in unchanged form and to a minor extent as N-acetyl-D,L-triazolylalanine in both urine and in faeces. In this study, D,L-triazolylalanine represented 69-86% of the dose in urine and 1-2% of the dose in faeces whereas N-acetyl- D,L-triazolylalanine represented 8-19% of the dose in urine and < 1% of the dose in faeces.

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Reference:	Lai, K. and Simoneaux, B. (1986c): The metabolism of triazole alanine in the rat.
Guideline:	Checked for compliance with OECD TG 417 (April 4 th 1984).
Deviations from TG 417:	See Lai, K. and Simoneaux, B. (1986a).
GLP:	No
Acceptability:	As supportive data
Test substance / purity:	¹⁴ C labelled triazole alanine (α -amino-[3,5- ¹⁴ C]-1,2,4-triazole-4-propionic acid) (D,L-triazolylalanine) Chemical and radiochemical purity > 99% (TLC). Specific radioactivity 29.4, 0.3 and 0.015 μ Ci/mg in low, mid and high dose respectively.
Species / Strain:	Rat / Albino Sprague Dawley
Doses / No. of animals:	0.56, 54.4 and 993.71 mg/kg bw in low, mid and high dose. 2 male and 2 female rats/dose level.
Administration:	Orally, by gavage (1ml). Vehicle: PEG
Exposure time / Duration:	Single dose, sampling of the 0-24 hour post dose urine containing the greatest percentage of recovered radioactivity regardless of sex at each dose level.
Expression of results:	Metabolites and reference compounds are considered to be identical when showing similar migration rates in TLC experiments.

This report is based on the rat balance experiment by Lai, K. and Simoneaux, B. (1986a) and the urine samples analysed herein originates from this study.

Materials and methods:

The 0-24 hour urine samples containing the greatest percentage of recovered radioactivity at each dose level, regardless of sex, was selected for TLC analysis with five different solvent systems. Radioactivity was measured in a scintillation counter and the TLC separations were visualised either by development of radioautograms or with a spark chamber.

Results

In the TLC analysis, two urinary fractions accounting for 82-93% and 13-30% of radioactivity, respectively, were identified in all dose groups. The major urinary zone co-migrated in all five systems with unaltered D,L-triazolylalanine. The second zone cochromatographed with standard N-acetyl- D,L-triazolylalanine.

Conclusion:

Absorbed D,L-triazolylalanine is rapidly eliminated via the urine, predominantly in unchanged form. A minor proportion of the dose is excreted as N-acetyl- D,L-triazolylalanine.

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Comments: Data presented in this study cannot be judged properly due to poor resolution of autoradiogram and UV visualisation.

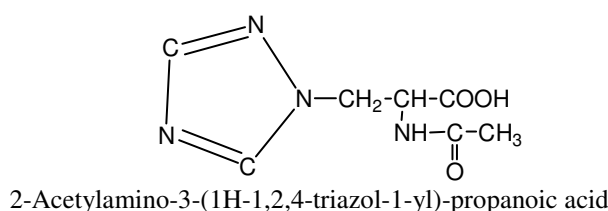
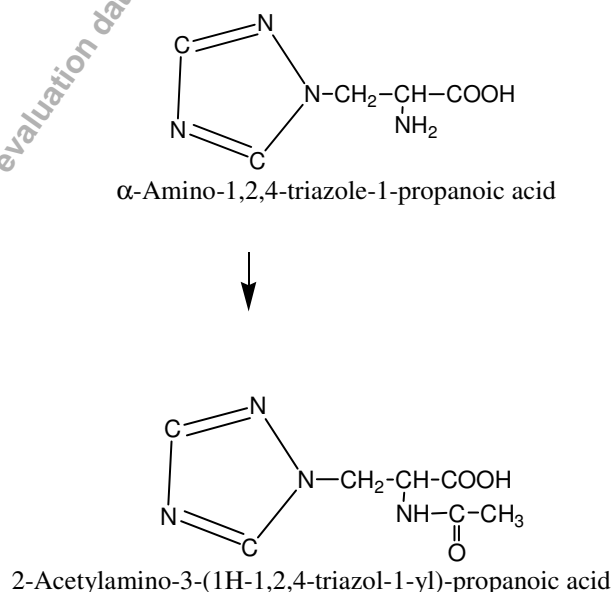
B.6.8.1.3.1.1 Summary of Administration, Distribution, Metabolism and Excretion studies:

This section was represented by two balance studies and accompanying metabolism studies performed by two different labs. The metabolite study by Lai, K. and Simoneaux, B. (1986b) was not considered to be of acceptable quality but the ADME of triazole alanine was still sufficiently covered by the two Hamboeck, H. studies in combination. In general, the results of the two labs correlated well with each other and the slightly lower renal elimination observed in the Lai, K. and Simoneaux, B. study compared to the Hamboeck, H. study may be due to the use of PEG as vehicle instead of water.

Absorbed D, L-triazolylalanine was shown to be rapidly eliminated mainly via urine. It was excreted predominantly in unchanged form and to a minor extent as N-acetyl- D,L-triazolylalanine. There was no difference between genders with respect to excretion, distribution or the qualitative urinary metabolite pattern. The Hamboeck study showed that almost all radioactivity was recovered in urine and faeces within 24 hours and there was no indication of any accumulation of the test substance. However, since the tissue distribution was measured only at seven days post administration, it is not known if the radioactivity initially showed a high distribution to specific tissues.

It is customary in ADME studies to investigate the properties of the test compound also after repeated administration. This was not performed in any of the studies of this section. Considering the efficient elimination of triazolylalanine and that the exposure to this plant residue of difenoconazole is presumed to be low during the conditions of the representative use, such study is not considered necessary.

Figure B.6.8.1.3-1: Metabolic pathway of triazolylalanine in rats



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B.6.8.1.3.2 Acute Oral Toxicity

Reference:	Mihail, F. (1982): Triazolylalanine (THS 2212), Acute Toxicity Studies.
Guideline:	Checked for compliance with OECD TG 401 (Feb 24th 1987) .
GLP:	No
Acceptability:	Yes
Test substance / purity:	Triazolylalanine, Batch 238099 / analytically pure (not given in percentage).
Species / Strain:	Rat / Wistar Bor:WISW (SPF-Cpb) Mouse/ NMRI (SPF-Hah)
Doses / No. of animals:	Rat/(fasted): 500, 1000, 2500 and 5000 mg/kg bw (male), 5000 mg/kg bw (female)/ 10/sex/dose. Rat (fed): 2500 and 5000 mg/kg bw (male), 5000 mg/kg bw (female) /10/sex/dose. Rat (i.p): 250, 400, 630, 1000, 2500 or 5000 mg/ml (male) 630, 1000, 2500 or 5000mg/ml (female) / 10/sex/dose. Mouse (fasted): 5000mg/ml (10 ml/kg bw) /10/sex/dose.
Administration:	Rat: Orally (by gavage)/ intraperitoneally Vehicle: distilled water (using Cremophor KL: 0.2 ml to 10 ml water) / physiological saline solution (using Cremophor KL: 0.2 ml to 10 ml 0.9% NaCl) Mouse: Orally by gavage Vehicle: distilled water (using Cremophor KL: 0.2 ml to 10 ml water)
Exposure time / Duration:	Single dose / Observation period of 14 days post dose.

Materials and methods:

Triazolylalanine was administered to mice by gavage and to rats by either gavage or an intraperitoneal injection. The body weights were determined prior to administration and after the first and second week of observation. The behaviour and appearance of the animals were observed and recorded on the day of administration and thereafter at least once per day. The median lethal dose (LD₅₀) was determined by the method of Litchfield and Wilcoxon.

Results:

Acute oral toxicity in mouse (fasted): All animals survived treatment and no clinical signs were observed during the study. At gross necropsy, minor effects on lungs were observed in one of ten female and male rats probably as an effect of the diethyl ether treatment used at sacrifice.

Acute oral toxicity in rat: All animals survived treatment. All male (fasted) rats administered 5000 mg/kg bw exhibited polyuria on the day after treatment. One male rat in the 5000 mg/kg bw group (fed) exhibited symptoms such as piloerection, accelerated breathing, stiff/stastic gait and staggering that started on the sixth day of study and lasted for two (first two symptoms) or five days. These symptoms which were graded as barely

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perceptible were not considered treatment-related as none of the other rats of the dose group showed a similar behaviour. A reduced body weight gain was observed in fasted as well as fed female rats administered 5000 mg/kg bw. At gross necropsy, moderate effects on lungs (mottled or distended appearance, dark discoloration) were observed in several animals of both sexes (fasted as well as fed) at all dose levels probably as an effect of the diethyl ether treatment used at sacrifice. Effects observed in single rats were not regarded as related to treatment.

Acute intraperitoneal toxicity in rat: All animals survived treatment. The animals exhibited behavioural disturbances, impaired activity and reduced muscle tone. Phonation during application interpreted as pain sensation was observed in all animals administered 2500 mg/kg bw and above (males administered 1000 mg/kg bw and above). Piloerection was observed in animals administered 2500 mg/kg bw and above and diarrhoea was observed approximately 3 hrs after administration in 3 males and 1 female administered 5000 mg/kg bw. In females administered 630 mg/kg bw and above, a dose-related reduction in body weight gain was noted. At gross necropsy, the formation of a connective tissue-like capsule surrounding the liver and, in some cases also the spleen, was observed in animals administered 2500 mg/kg bw and above. This was interpreted as a local effect of the test compound when administered intraperitoneally. At gross necropsy, small testes were observed in one male of the 400 mg/kg bw group and a mottled appearance of the lungs was observed in both sexes of animals administered doses from 400 mg/kg bw and above. This was probably an effect of the diethyl ether treatment used at sacrifice.

Conclusion:

The oral LD₅₀ and intraperitoneal LD₅₀ of triazolylalanine in rats is > 5000 mg/kg. The oral LD₅₀ of triazolylalanine in mice is > 5000 mg/kg

WARNING: This document forms part of an EC evaluation data package and should not be relied upon for regulatory purposes. Registration must not be granted on the basis of this document.

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Reference:	Henderson, C. and Parkinson, G.R. (1980): R152056: Acute Oral Toxicity to Rats.
Guideline:	Checked for compliance with OECD TG 401 (24th February 1987).
Deviations from TG 401:	The bodyweights were not reported and a pathological examination was not performed. Rats used in this study are younger than recommended in TG 401 (between 5 and 7 weeks instead of 8 and 12 weeks).
GLP:	Yes, although the study was performed before the OECD GLP guidelines were finalised (self-certification of the laboratory).
Acceptability:	Yes (see comments) This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	R152056, Batch P4; purity not indicated.
Species / Strain:	Alderley Park (SPF) rats
Doses / No. of animals:	2000 mg/kg bw (application volume 10 ml/kg) 10/sex/dose.
Administration:	Orally, by gavage. Vehicle: Distilled water (20 % w/v suspension).
Exposure time / Duration:	Single dose / Observation period of 14 days post dose.

Materials and methods:

Triazole alanine was administered to fasted rats by oral gavage. The animals were observed for 2 weeks.

Results:

All animals survived treatment and no clinical signs were observed throughout the observation period (study log not shown).

Conclusion:

The oral LD₅₀ of triazolealanine in rats was > 2000 mg/kg.

Comments:

In this study, bodyweights were not recorded and a pathological examination was not performed. These endpoints are important as they may indicate toxic effects to focus on in subsequent toxicity studies. However, it can be concluded from this study that the oral toxicity in rats is greater than 2000 mg/kg as no animals died at this dose level. The use of animals younger than recommended is acceptable since no signs of toxicity or mortality were observed.

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B.6.8.1.3.2.1 Summary of acute toxicity studies:

Study	Parameter	Species	Route of Administration	Dose level	Results
Mihail, (1982):	F. Acute LD ₅₀	Rat (fasted)	Oral (gavage)	M:500, 1000 2500, 5000 F:5000 mg kg ⁻¹ bw	LD ₅₀ > 5000 mg/kg bw Classification is not required.
Mihail, (1982):	F. Acute LD ₅₀	Rat (fed)	Oral (gavage)	M:2500, 5000 F:5000 mg kg ⁻¹ bw	LD ₅₀ > 5000 mg/kg bw Classification is not required.
Mihail, (1982):	F. Acute LD ₅₀	Rat	Intraperitoneal	M:250, 400, 630, 1000, 2500,5000 F: 630, 1000, 2500, 5000 mg kg ⁻¹ bw	LD ₅₀ > 5000 mg/kg bw Classification is not required.
Mihail, (1982):	F. Acute LD ₅₀	Mouse	Oral (gavage)	M/F:5000 mg kg ⁻¹ bw	LD ₅₀ > 5000 mg/kg bw Classification is not required.
Henderson, C. and Parkinson, G.R. (1980)	Acute LD ₅₀	Rat	Oral (gavage)	M/F:2000 mg kg ⁻¹ bw	LD ₅₀ > 2000 mg/kg bw Classification is not required.

This section was represented by studies of acute oral toxicity only. However, since triazole alanine is a plant residue of difenoconazole, the exposure to this substance is presumed to occur mainly via the oral route thus data on the acute dermal/ inhalation toxicity and the irritation/sensitisation potential of triazole alanine are not considered necessary. The acute oral toxicity following repeated administration was not investigated as is customary in toxicological studies but as the exposure to triazole alanine via plants is presumed to be low during the conditions of the representative use, such study is not required.

Triazole alanine is of low acute oral toxicity and classification is not required according to annex VI of the Council Directive 67/548/EEC.

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B.6.8.1.3.2 Subacute Oral toxicity**Dose-range finding study**

Reference:	Bomhard, E. (1982). THS 2212, Preliminary Subacute Toxicity Study on Male Rats, Administration in the Drinking Water
Guideline:	Checked for compliance with OECD TG 407 27th July 1995 .
Deviations from TG 407:	Only males were tested in this study. Two dose levels were used instead of at least three. The clinical signs investigated were not specified. Analyses of haematology and clinical chemistry were not performed. Testes, epididymides and the heart were not weighed. A histopathological examination was not performed.
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	Triazolylalanine (batch E 238099) / Purity: approximately 100%.
Species / Strain:	Rat/ Bor:WISW (SPF-Cpb) Wistar
Doses / No. of animals:	0, 3000 and 10000 ppm (448 and 1491 mg/kg bw/day) 10 males/ dose level.
Administration:	Orally, in drinking water
Exposure time / Duration:	Daily dose / Observation period of 14 days post dose.

Materials and methods:

Traizolylalanine was administered to groups of male rats (5 to 6 weeks old) during two weeks. Drinking water was used as the route of administration due to the analytical problems encountered with doses below 2000 ppm in food. The test compound was shown to be stable in water over a period of eight days (results not documented). The bodyweights were recorded at the beginning of the study then once a week. All animals were observed daily (once on week-ends and holidays) for symptoms (study log not shown) and the food and water consumption was recorded weekly. After two weeks all animals were sacrificed and grossly examined. The weights of thyroid, tymus, spleen, liver, kidneys, adrenals and brain were determined. Treated and untreated animals were compared using the Mann, Whitney and Wilcoxon (U-test) at significance levels of 5% and 1%.

Results:

All animals survived treatment and there were no clinical signs of toxicity observed throughout the study. Food and water intake was not affected by treatment and the body weight development was similar in all groups. The incidence and type of necropsy findings did not differ between treated and control animals and the weight analysis of selected organs did not show any differences that were statistically significant.

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Conclusion:

NOEL > 1491 mg/kg bw/day based on the absence of treatment-related findings at this dose level (highest dose tested).

Subacute oral study in rat

Reference:	Mihail, F and Vogel, O. (1983): Triazolylalanine (THS 2212), Subacute Oral Toxicity Study on Rats.
Guideline:	Checked for compliance with OECD TG 407 27th July 1995 .
Deviations from TG 407:	The blood clotting time/potential and the levels of total protein/albumin in blood, blood urea nitrogen, sodium and potassium were not analysed. Thymus, epididymides and uterus were not weighed and a histopathological examination of the spinal cord, thymus, trachea, accessory sex organs, urinary bladder or the peripheral nerve was not performed. Additionally, a test of the sensory reactivity to stimuli was not performed.
GLP:	No
Acceptability:	This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	Triazolylalanine (2-amino-3-(1, 2, 4-triazol-1-yl)-propionic acid) (batch E 238099) Purity: analytically pure.
Vehicle:	Distilled water (using 5 drops Cremophor EL to 10 ml water)
Species / Strain:	Rat/ Bor:WISW (SPF-Cpb) Wistar
Doses / No. of animals:	0, 25, 100 or 400 mg/kg bw / 20/sex/dose
Administration:	Orally, by gavage
Exposure time / Duration:	Daily dose / 28 days followed by a 28-day treatment-free recovery period.

Materials and methods:

Groups of male and female rats were daily administered triazolylalanine by oral gavage during 28 days followed by a 28-day treatment-free recovery period. The animals were weighed at the start of each study week and before autopsy and the appearance and behaviour were observed and recorded daily. At the end of the treatment period, ten animals were sacrificed and the remaining ten animals/group/sex were sacrificed at the end of the recovery period. Half of the sacrificed animals (five animals/group/sex) were subjected to a histopathological examination and the other half were subjected to laboratory examinations of blood and urine.

Brain, heart, testicles, liver, lung, spleen, adrenals, kidneys, thyroid and ovaries were weighed and the following organs from five males and five females were fixed in 10% aqueous formaldehyde solution and subjected to histopathological examination: intestine (three localisations), brain, heart, testicles, bones with bone marrow (femur) and musculature, liver, lymph nodes, stomach, spleen, epididymis, adrenals, kidneys, oesophagus, ovaries, thyroid and uterus. Analyses of the test substance preparations were performed at the start of the study and then once during the study (results not documented). The control group values were compared to the corresponding test group values using the Mann, Whitney and Wilcoxon's U-test.

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Results:*General:*

All animals survived treatment and there were no clinical signs of toxicity observed throughout the study. The food and water intake was unaffected by the treatment and the body weight gain was similar in all groups.

Haematology, clinical chemistry, urine analysis and liver tissue:

A statistically significant decrease in the thrombocyte level was observed in females at all dose levels.

According to the study author, all values were within the physiological range of variation but this was not supported by any historical data. However, a variation of thrombocyte levels is observed in control animals sacrificed after four weeks of treatment and at the end of the post-observation period respectively (Table B.6.8.1.3.2-2). Since there were no statistically significant differences in thrombocyte levels between the control and the treated groups that were sacrificed at the end of the post-observation period, the reduced level of thrombocytes observed in the treated groups is not considered to be an adverse effect. A slightly increased MCHC value (5%) was observed in females administered 25 and 400 mg/kg triazolylalanine. However, erythrocytes are normally saturated with haemoglobin thus increased levels of MCHC may indicate technical errors at analysis or the biological variation. Moreover, since MCHC is defined as the quotient of the concentration of haemoglobin and the haematocrit and these parameters are normal, this is not considered to be an effect. A statistically significant reduced level of creatinine was observed in both males and females administered 400 mg/kg bw and a reduced level of urea was also recorded in the males of this group. Since these findings were not accompanied by any histopathological indications on kidney damage, they were not considered to be adverse effects.

The examinations of urine and urine sediments revealed no dose-related differences between control and dose group animals. The analysis of microsomal enzyme systems and triglyceride content performed at the end of the treatment period did not indicate any effects on the liver. These parameters were not examined in the restitution group.

Organ weight and gross pathology:

Small but statistically significant changes of lung and liver weights were observed in female rats administered the high dose. However, since these differences are small and not accompanied by any histopathological findings, they are not considered to be adverse effects.

Since there were no indications of treatment related organ changes in the histopathological examinations performed at the end of the study, the restitution group was not examined.

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Table B.6.8.1.3.2-1: Clinical chemistry parameters (mean values) in male rats

Parameter	0 mg/kg bw/day		25 mg/kg bw/day		100 mg/kg bw/day		400 mg/kg bw/day	
	Treatment	Recovery	Treatment	Recovery	Treatment	Recovery	Treatment	Recovery
Urea (mmol/l)	6.65	7.00	6.39	7.25	6.64	5.95	5.35 * (↓ 20%)	5.78 * (↓ 17%)
Creatinine (μmol/l)	61	47	66	48	60	49	49 ** (↓ 20%)	52

* or ** statistically significant at the 5% or 1% level, respectively.

The ↓/↑ numbers refer to the difference in percent from the corresponding control value.

Table B.6.8.1.3.2-2: Haematological and Clinical chemistry parameters (mean values) in female rats

Parameter	0 mg/kg bw/day		25 mg/kg bw/day		100 mg/kg bw/day		400 mg/kg bw/day	
	Treatment	Recovery	Treatment	Recovery	Treatment	Recovery	Treatment	Recovery
Thrombocytes	1063	810	824** (↓ 23%)	864	905* (↓ 15%)	862	794** (↓ 25%)	872
Creatinine (μmol/l)	59	43	56	43	53	58* (↑ 35%)	50** (↓ 15%)	48

* or ** statistically significant at the 5% or 1% level, respectively.

The ↓/↑ numbers refer to the difference in percent from the corresponding control value.

Table B.6.8.1.3.2-3: Deviations in liver and lung weights (mean values) in female rats at the end of the treatment phase

	0 mg/kg bw/day	25 mg/kg bw/day	100 mg/kg bw/day	400 mg/kg bw/day
Liver weight				
Absolute (gram)	6.79	6.80	6.52	5.88 (↓ 13%) **
Relative (% of body weight)	3.98	3.88	3.80	3.59 (↓ 10%) *
Lung weight				
Absolute (gram)	746	786	748	751
Relative (% of body weight)	437	448	436	457* (↑ 4.6%)

* or ** statistically significant at the 5% or 1% level, respectively.

The ↓/↑ numbers refer to the difference in percent from the corresponding control value.

Conclusion:

The **NOAEL** was **≥ 400 mg/kg bw** based on the absence of adverse effects at the highest dose level tested. The LOELs in males and females was 400 mg and 25 mg/kg bw based on changes in clinical chemistry parameters and changes in blood parameters respectively.

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B.6.8.1.3.3 Subchronic Oral Toxicity

Subchronic oral toxicity in rat

Reference:	Maruhn, D. and Bomhard, E. (1984): Triazolylalanine (THS 2212), Study for Subchronic Toxicity to Rats (Three-month feeding study).
Guideline:	Checked for compliance with OECD 408 (21 st september 1998).
Deviations from TG 408:	Thymus, epididymides, uterus and ovaries were not weighed. Tests of the sensory reactivity to stimuli, an assessment of the grip strength and the motor activity were not conducted.
GLP:	No
Acceptability:	Yes (see comments) This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	Triazolylalanine (2-amino-3-(1, 2, 4-triazol-1-yl)-propionic acid)(Batch TLB1207) Purity: 97.5
Species / Strain:	Bor: WISW (SPF-Cpb) Wistar (5-6 weeks old)
Doses / No. of animals:	0, 1250, 5000 and 20000 ppm (equivalent to 90, 370, 1510 mg/kg bw (male) and 100, 400, 1680 mg/kg bw (female)) added to powdered feed 20/sex/dose
Administration:	Orally, via the diet.
Exposure time / Duration:	Daily dose / 97 days (96-99)

Materials and methods:

Groups of male and female rats were daily administered triazolylalanine in feed during approximately 90 days. The animals were inspected once or twice daily and body weights, food consumption and water intake was recorded weekly. Ophthalmological examinations (ten animals/sex/dose) were performed before and at the end of the study and clinical chemistry/urine analysis (ten animals/sex/dose) were performed after one month and at the end of the study. All animals were subjected to autopsy and a histopathological examination was performed on rats administered the highest dose, on the control group and on all grossly altered organs or possible target organs from animals administered lower doses. The brain, heart, testicles, liver, lung, spleen, adrenals and kidneys were weighed and all organs requested for histopathology in OECD guideline 408 were fixed in Bouin's solution. The MANN, WHITNEY and WILCOXON test was applied to detect significant deviations from control group parameters.

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Results:*General observations:*

The homogeneity/stability of the test compound in food was established before the study and the concentration in food was then checked and confirmed during the study (results not documented).

All animals showed a similar appearance and general behaviour (study log not shown) and the food/water consumption was comparable between treated and untreated rats. A reduced body weight gain was recorded in males administered 20000 ppm but this reduction was only statistically significant (at least 5%) during week 3-8 and week 12-13 of study. During these weeks, the difference in bodyweights between high dose and control animals were less than 10% and this effect was therefore not considered adverse. Two females administered 1250 ppm and two females administered 5000 ppm died during blood sampling procedures. These deaths were considered to be caused by an overdose of ether and/or hypovolaemia and not related to treatment. The ophthalmoscopy examinations revealed no indications of substance-induced damage to the eye.

Haematological and clinical laboratory examinations:

The haematological analysis showed differences in leucocyte counts that were statistically significant in males administered 5000 or 20000 ppm after one month as well as after three months of study. This was also observed in females after three months of study (Table B.6.8.1.3.3-2). In males, statistically significant changes in lymphocyte and polymorphonuclear neutrophil (segm) levels were also observed. All changes were reported to be within normal variation according to the study report but this was not supported by any historical data. In the clinical chemistry analysis performed one month after dosing, decreased levels of triglyceride, creatinine, calcium and urea were observed in females administered 5000 or 20000 ppm. After three months, only the reduced level of triglycerides was still statistically significant. The clinical chemistry analysis of males performed one month after administration of 5000 or 20000 ppm demonstrated decreased levels of bilirubin and glucose that were statistically significant. After three months of dosing, only the reduced level of bilirubin was still statistically significant. However, in this analysis, reduced levels of triglycerides, urea and protein P were also observed. The slightly reduced level of protein P was not dose-related and not considered to be related to treatment. Analysis of urine and sediment did not show any effects apart from minor individual variations without dose correlation. The altered clinical chemistry parameters were not accompanied by any histopathological findings thus these changes were not considered adverse.

Pathological and histopathological examinations:

Although a few variations in absolute and relative organ weights were observed, they were not considered to be of concern since the variations were small (<10% difference).

The histopathological changes observed were generally found in both untreated and treated rats and therefore they were considered to be common spontaneous findings and not associated with treatment. Atrophy of testes (slight and marked respectively) was observed in 2/20 male rats administered the highest dose.

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Table B.6.8.1.3.3-1 Body weights (mean values) in male rats at the end of the treatment phase

Body weight (gram)	0 ppm	1250 ppm	5000 ppm	20000 ppm
week 0	71	72	71	71
week 13	315 ^a	312	309	288 ^b ** (↓9%)

** statistically significant at the 1% level.

The ↓/↑ numbers refer to the difference in per cent from the corresponding control value.

Table B.6.8.1.3.3-2: Haematological and clinical chemistry parameters (mean values) in male and female rats

Parameter	0 ppm				1250 ppm				5000 ppm				20000 ppm			
	Males		Females		Males		Females		Males		Females		Males		Females	
	wk 4	wk 13	wk 4	wk 13	wk 4	wk 13	wk 4	wk 13	wk 4	wk 13	wk 4	wk 13	wk 4	wk 13	wk 4	wk 13
Leucocytes (giga/L)	8.6	10.1	8.4	9.6	7.5	9.6	7.8	8.2	7.0**	8.4**	7.8	8.3	7.1**	8.3*	7.7	7.2**
									↓19%	↓17%			↓17%	↓18%		↓25%
Lymphocytes	88.6	92.6	90.8	89.3	89.8	89.2	95.3**	89.8	91.3*	87.7*	92.8	86.1	91.8*	86**	92.4	83.9
									↑3%	↓5%			↑3.6%	↓7%		
Polymorpho nuclear neutrophils (segm)	10	6.8	8.4	9.7	9.5	10	4.1**	9.0	8.0	11.8*	6.2	12.6	7.8*	12.6*	7.1	15.0
										↑74%			↓12%	↑85%		
Bilirubin (μmol/l)	2.7	4.4	1.7	3.2	2.5	4.2	1.5	3.2	2.5*	4.1	1.4	2.7	2.0**	3.8**	1.6	3.1
									↓7%				↓26%	↓14%		
Urea (mmol/l)	8.0	7.6	8.5	7.9	7.6	7.2	8.1	7.6	7.7	6.9	7.3**	7.6	7.7	6.8*	7.5*	7.9
											↓14%			↓11%	↓12%	
Creatinine (μmol/l)	48	61	48	53	45	59	44	52	48	57	40**	67	46	66	41**	50
											↓17%				↓15%	
Triglycerides (μmol/l)	1.00	1.22	0.90	1.30	0.98	1.03	0.87	1.24	1.05	1.11	0.61*	0.77**	0.82	0.72**	0.58**	0.85**
											↓33%	↓41%		↓11%	↓36%	↓35%

* or ** statistically significant at the 5% or 1% level, respectively.

The ↓/↑ numbers refer to the difference in per cent from the corresponding control value.

Conclusion:

The NOAEL was $\geq 20\ 000$ ppm (equivalent to 1510/1680 mg/kg bw in males/females) based on the absence of adverse effects in the high dose.

NOEL in males and females was 1250 ppm (equivalent to 90 mg/kg bw) and 5000 ppm (equivalent to 400 mg/kg bw) respectively based on the changes in haematological (M) and clinical chemistry parameters (M/F) observed in animals of the next dose levels.

Comments:

The retarded body weight gain development observed in males administered the high dose is not considered adverse since the reduction was below 10% and it was not present during the entire study period. The atrophy of testes observed in this study was also observed in the Two-generation study by Milburn, G. M (1986) included in this DAR (section B.6.8.1.3.6). However, in the Milburn, G. M study, this effect was observed both in F0 males administered the high dose (severe atrophy in 1/15) and in untreated F1 males (slight and severe degeneration in 2/15). The atrophy of testes observed is thus considered to be a random finding and not an effect of treatment.

Weighing of thymus, epididymides, uterus and ovaries and assessment of grip strength, motor activity and sensory reactivity to stimuli were not required in the guideline existing at the time of the study.

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Sub chronic toxicity study in dog

Reference:	von Keutz, E. and Gröning, P. (1984): THS 2212 (Triazolylalanine), Subchronic Toxicity Study to Dogs on Oral Administration.
Guideline:	Checked for compliance with OECD 409 (21 st September 1998).
Deviations from OECD 409:	The gall bladder, epididymides, uterus and thymus were not weighed. A histopathological examination of the trachea and the parathyroid was not performed. The animals were not fasted prior to the clinical chemistry analysis as recommended in the guideline. A statistical evaluation of bodyweight gain and food consumption was only performed on the sexes combined.
GLP:	No
Acceptability:	Yes This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	Triazolylalanine; (batch TLB 1207) Purity: 97.5%.
Species / Strain:	Dog/Beagle
Doses / No. of animals:	0, 3200, 8000, or 20000 ppm /4/dose/sex
Administration:	Orally, via the diet.
Exposure time / Duration:	Daily dose / 91-92 days

Materials and methods:

Groups of male and female dogs were daily administered the test article in the diet during approximately 90 days. The animals were inspected several times daily and any abnormalities were recorded in the study log. Food consumption was recorded daily whereas body weights were recorded weekly. Prior to the start of the study and during treatment weeks 2, 4, 7 and 13, reflex tests, body temperature measurements, pulse rates and haematological, clinical chemistry and urine analyses were performed. Differences between treated and untreated animals were checked for statistical significance using Wilcoxon's non-parametric rank sum test. All animals were subjected to an ophthalmological examination prior to the start of the study and during treatment weeks 7 and 13. At the end of the study all animals were autopsied and selected organs from animals in the control and in the high dose groups were examined histopathologically.

Results:

General observations:

The diet analysis showed that the test compound was stable for at least ten days in dry food and that it was homogeneously distributed in the mixtures. The test substance content was analysed at monthly intervals and showed an actual content within 12% of the nominal values in all concentrations.

All animals survived treatment and there were no differences observed between treated and untreated animals regarding body temperatures, pulse rates, appearance or behaviour. Isolated incidences such as vomiting or altered condition of faeces were reported to be distributed between dose groups (study log not shown) and they were therefore considered to be related to treatment.

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The food intake was reduced in females receiving the highest dose (not analysed for statistical significance) and this was accompanied by a reduced body weight gain (Table B.6.8.1.3.3-3).

Ophthalmoscopic and clinical laboratory examinations:

There were no treatment-induced effects found in the eye and there were no differences in haematological parameters, clinical chemistry parameters, in urine or in liver enzymes observed.

Pathological and histopathological examinations:

A reduced relative weight of testes was observed in two male rats administered the highest dose. However, the differences in absolute and relative organ weights between treated and untreated animals were not analysed for statistical significance and the standard deviations of the means indicated a large variation in the control group (control: mean 1.915, S.D 0.416; 20 000 ppm: mean: 1.52, S.D 0.071). Since there were no histopathological findings in testes, the reduced relative weight was not considered an adverse effect. Gross pathological alterations were detected in the spleen, liver, lung and tonsils. However, these alterations did not show any dose-relation and were not correlated with any histopathological findings hence they were considered to be random findings and not associated with triazolylalanine.

Table B.6.8.1.3.3-3: Body weight development (mean values) in female and male dogs

Body weight (kg)	0 ppm female / male	3250 ppm female / male	8000 ppm female / male	20000 ppm female / male
week -1	7.4 / 8.2	7.4 / 7.9	7.4 / 8.5	7.2 / 8.2
week 13	8.4 / 8.9 (+1/0.7 kg)	8.6 / 8.9 (+1.2/1 kg)	8.1 / 9.8 (+0.7/1.3 kg)	7.4 / 9.0 (+0.2/0.8 kg)

Conclusion:

The NOAEL in female dogs was rats 8000 ppm (equivalent to ca 200 mg/kg bw) based on the reduced body weight gain observed in females administered 20 000 ppm. **The NOAEL in males is \geq 20 000 ppm (equivalent to ca 500 mg/kg bw)** based on the absence of adverse effects at this dose level.

Comments:

The body weight gain observed in females administered the high dose was 80% less compared to the body weight gain observed in control animals (Table B.6.8.1.3.3-3). Since the food intake was normal in males and the reduced food intake in females was of approximately similar magnitude over the entire study period, this effect can probably not be explained by palatability. The reduced body weight gain is thus considered to be an adverse effect of treatment.

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B.6.8.1.3.3.1 Summary of subacute and subchronic toxicity studies

Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
Subacute oral toxicity in rat (28 day)					
Mihail, F and Vogel, O 1983	0,25,100, 400 mg kg ⁻¹ day ⁻¹	Orally (gavage)	NOAEL: M/F:400 mg/ kg bw	N/A	No adverse effects observed
			NOEL: M: 100 mg/ kg bw F: N/A	LOEL: M: 400 mg/kg bw F: 25 mg/ kg bw	400 mg/kg/day: ↓ Liver weight (F) Altered clinical parameters: ↓ Creatinine (M/F) ↓ Urea (M) 25 mg/kg/day: ↓ Thrombocytes (F)
Subchronic oral toxicity in rat					
Maruhn, D., Bomhard E 1984	M/F: 0, 1250, 5000, 20 000 ppm ≈ 90/100, 370/400, 1510/1680 mg/kg bw	Orally via the diet	NOAEL: M/F: 1510/1680 mg/ kg bw 20 000 ppm	LOAEL: Not applicable	No adverse effects observed
			NOEL: M/F: 90/100 mg/ kg bw 1250 ppm	LOEL: M/F: 370/400 mg/ kg bw 5000 ppm	5000 ppm: Altered haematological (M) and clinical chemistry parameters
Subchronic oral toxicity in dog					
Von Keutz, E., Gröning, P. 1984	M/F: 0, 3200, 8000, 20 000 ppm	Orally via the diet	NOAEL: M/F: 500/200 mg/ kg bw M/F: 20 000/8000 ppm	LOAEL: M/F: N/A./20 000 ppm	20 000 ppm ↓ Body weight gain (F)
			NOEL: M/F: 20 000/3250 ppm	LOEL: M/F: N/A./8000 ppm	8000 ppm ↓ Body weight gain (F)

This section was represented by a dose-finding study in rat, a subacute study in rat and two subchronic studies performed in rats and dogs respectively. There were no adverse effects observed in the rat studies performed but a reduced body weight gain was observed in the female dogs administered the highest dose. According to TG 408 and 409, a substance should be tested in doses until toxicity is apparent or at a limit dose of 1000 mg/kg bw. In the studies performed on rats, triazole alanine was thus not adequately tested. However, triazole alanine is a plant residue of difenoconazole and the exposure is presumed to be low during the conditions of the representative use considered in this DAR. Further studies are not considered to be required.

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B.6.8.1.3.4 Genotoxicity**Bacterial Reverse Mutation Test (1)**

Reference:	Deparade, E. (1986): CGA 131 013 tech - Salmonella / mammalian-microsome mutagenicity test.
Guideline:	Checked for compliance with OECD 471 (21 st July 1997).
Deviations from TG 471:	Information regarding strain characteristics or the titer was not reported. Since there is no information on the background lawn it is difficult to assess if this had any influence on the results. However, since the positive controls used induced a high number of revertants this is considered to be of less concern.
GLP:	Yes
Acceptability:	As supportive information (see comments)
Test substance / purity:	CGA 131013 (batch: TLB 1207 6.Lief) / Purity 97.4% Dissolved in DMSO
Bacterial strains:	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 and TA 102.
Doses:	20, 78, 313, 1250 and 5000 µg/0.1 ml (+/- microsomal activation).
Exposure time:	48 hours at 37 °C

Materials and methods:

Two sets of independent mutagenicity tests were performed, in the presence and in the absence of metabolic activation, in the histidine-auxotrophic *Salmonella typhimurium* strains: TA 98, TA 100, TA 102, TA 1535 and TA 1537. The highest concentration applied was determined in a preliminary toxicity test. After preparation, the plates were incubated 48 hours at 37 °C in darkness and then evaluated by colony counting. Positive controls were included in the test in order to demonstrate the sensitivity of the test system and DMSO served as a negative control.

Results:

The test substance precipitated in agar at concentrations of 78 µg/plate and above.

Treatment with CGA 131 013 did not lead to an increased incidence of mutants in comparison with the negative controls in the interval tested. Positive controls showed a marked increase of the number of revertant colonies thus indicating the sensitivity of the test system.

Conclusion:

CGA 131 013 tech. did not induce gene mutations in the strains of *S. typhimurium* used.

Comments:

The solubility of the test substance was not reported but as it precipitated on agar plates it is possible that it did not dissolve completely in DMSO and it is therefore unclear if bacteria were exposed to the concentration

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intended. However, since there are two additional bacterial reverse mutation tests included in this section, this study is not necessary for the assessment.

In this assay, 4-Nitroquinoline-N-oxide and Daunorubicin-HCl were used as positive controls of strains TA100 and TA 98 in assays performed without metabolic activation. These are not the examples of strain specific controls given in TG 471. There are no historical data regarding the reversion frequency of 4-Nitroquinoline-N-oxide and Daunorubicin-HCl in strains TA100 and TA 98 included but as they induce a high amount of revertant colonies in the test, they are considered acceptable as controls.

Bacterial Reverse Mutation Test (2)

Reference:	Hertner, Th. (1993): CGA 131 013 tech - Salmonella and Escherichia/liver-microsome test.
Guideline:	Checked for compliance with OECD 471 (21st July 1997) .
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	CGA 131013 tech. (batch: RV-1469/8 Fr.1) / Purity: 96% Dissolved in DMSO
Bacterial strains:	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 (histidine auxotrophs) <i>Escherichia coli</i> WP2 uvrA (tryptophan auxotrophs).
Doses:	312.5, 625, 1250, 2500 and 5000 µg/plate (+/- microsomal activation).
Exposure time:	48 hours at 37 °C

Materials and methods:

Two sets of independent mutagenicity test were performed, in presence or in absence of metabolic activation, on the histidine-auxotrophic *Salmonella typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 and on the tryptophan-auxotrophic *Escherichia coli* strain WP2 uvrA. The highest concentration applied was determined in a preliminary toxicity test. After preparation, the plates were incubated 48 hours at 37 °C in darkness and thereafter evaluated by colony counting and determination of the background lawn. Positive controls were included in the test in order to demonstrate the sensitivity of the test system and DMSO served as negative control. The test concentration was confirmed by HPLC with UV detection.

Results:

The analysis of the test substance, performed with the lowest concentration used, showed that the concentrations used were in agreement with the concentrations intended thus demonstrating a sufficient stability of the test substance in the vehicle.

A normal background growth was observed in all strains at all concentrations. Treatment with CGA 131 013 tech, in presence or in absence of metabolic activation, did not increase the incidence of revertant colonies compared to the negative controls at any of the five concentrations tested in the original and the confirmatory mutagenicity experiments. The positive controls used showed a marked increase of the number of revertant colonies indicating the sensitivity of the test system.

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Conclusion:

CGA 131 013 tech. did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used.

Bacterial Reverse Mutation Test (3)

Reference:	Herbold, B. (1983a): THS 2212 Triazolylalanine. Salmonella/ microsome test for point mutagenic effect.
Guideline:	Checked for compliance with OECD 471 (21st July 1997).
Deviations from TG 471:	No strain detecting cross-linking mutagens, oxidising agents and hydrazines (<i>E. coli</i> WP2 <u>uvrA</u> , <i>E. coli</i> WP2 <u>uvrA(pKM101)</u> or <i>S. typhimurium</i> TA 102) was included in the test. Cyclophosphamide (in the form of the drug endoxan), 2-Aminoanthracene and tryptaflavine were used as positive controls but as all of these require metabolic activation for mutagenicity, there was no positive control included showing mutagenicity in assays without metabolic activation. There was no justification why a confirmatory experiment was excluded.
GLP:	No
Acceptability:	Yes (see comments)
Test substance / purity:	Triazolylalanine, (2-amino-3-8I, 2, 4-triazol-1-yl)-propionic acid) (batch E238099) Purity: not indicated. Vehicle: dimethylsulphoxide (DMSO).
Bacterial strains:	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 and TA 1538 (histidine auxotrophs)
Doses:	20, 100, 500, 2500 and 12500 µg/plate (+/- microsomal activation).
Exposure time:	48 hours at 37 °C

Materials and methods:

The reversion frequency in the histidine-auxotrophic *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 was tested upon exposure to triazolylalanine at five concentrations spaced by a factor of five. Quadruplicates of each concentration were tested in presence and in absence of metabolic activation. After preparation, the plates were incubated during 48 hours at 37°C and thereafter evaluated by colony counting and a determination of the background lawn. Positive controls were included in the test in order to demonstrate the sensitivity of the test system and DMSO served as negative control.

Results:

Treatment with triazolylalanine did not lead to an increased incidence of mutants compared to the negative controls. The positive controls increased the number of revertant colonies considerably, indicating the sensitivity of the test system. A normal background growth was observed in all strains at all concentrations.

Conclusion:

In this study **triazolylalanine did not induce gene mutations** in the strains of *S. typhimurium* used.

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Comments:

In this assay, a confirmatory experiment was not performed. However, since the results from the four plates/concentration used showed a low spread, the result is considered to be clearly negative and a confirmatory experiment is not considered necessary.

***In vitro* Mammalian Cell Gene Mutation Test**

Reference:	Dollenmeier, P. (1986b): CGA 131 013 tech – Point mutation test with Chinese Hamster Cells V79.
Guideline:	Checked for compliance with OECD 476 (21st July 1997) .
Deviations from TG 476:	The phenotypic expression time was 5 days instead of 6-8 as recommended in guideline.
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	CGA 131013 (batch: TLB 1207 6.Lieferung) / Purity 97.4%. Dissolved in Ham's F10 culture medium and 3% foetal calf serum (plus 10% activation mixture).
Cell line:	V 79 Chinese hamster cells (clone 65/3)
Doses:	Cytotoxicity test: 305 ng/ml-10 mg/ml (+/- microsomal activation). Mutagenicity test: 500, 1000, 2000, 4000, 6000, 8000 and 10000 µg/ml (+/- microsomal activation). Dissolved in water
Exposure time:	5 hours with and 21 hours without microsomal activation at 37 °C. phenotypic expression time: 5 days Incubation with 6-thioguanine: 7-8 days

Materials and methods:

V79 Chinese hamster cells were exposed to triazole alanine, in presence or in absence of metabolic activation, in two sets of independent mutagenicity studies (original and confirmatory study). The highest concentration applied was determined in a preliminary cytotoxicity test. Two cultures per dose level were exposed to the test substance for 5 or 21 hours in tests performed in the presence and in the absence of metabolic activation respectively. The cultures were extensively washed with phosphate-buffered saline to terminate treatment and then reincubated in fresh growth medium for five days for phenotypic expression. At the end of the expression time, the cells were trypsinized and plated into dishes for mutant selection using 8 µg/ml 6-thioguanine. After 7-8 days, the cultures were fixed with methanol and stained with Giemsa stain and mutant colonies were counted. Positive controls were included in the test in order to demonstrate the sensitivity of the test system and water was used as negative control.

Cell viability was determined as relative cloning efficiency and the mutant frequencies were normalised to a virtual cloning efficiency of 100% if the true cloning efficiency was above 15%.

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Results:

Treatment with CGA 131 013 tech. in the absence of metabolic activation did not lead to an increased incidence of 6-thioguanine resistant mutants compared to the negative controls in the original or the confirmatory mutagenicity experiments. Treatment with CGA 131 013 tech in the presence of metabolic activation led to a mutant factor of 2.2 and 1.4 in original and confirmatory experiments respectively. With the positive controls a marked increase in the number of colonies was observed mutant factors (169/267 without activation and 76/20 with activation), thus indicating the sensitivity of the test system.

Conclusion:

In this assay CGA 131 013 tech. did not induce gene mutations in V79 Chinese hamster cells.

Comments:

The phenotypic expression time was one day shorter than required in TG 476, but this is not considered to influence the results since the positive controls induced an increased number of colonies.

Pol A1- test on *E. coli*

Reference:	Herbold, B. (1983b): THS 2212 Triazolylalanine. Pol A1- test on <i>E. coli</i> during testing for effects harmful to DNA.
Guideline:	In-house method (no specific guidelines)
Comments:	The methodology used was not described. The results obtained in assays performed without metabolic activation were not shown.
GLP:	No
Acceptability:	As supportive information
Test substance / purity:	Triazolylalanine, (batch E238099)/ Purity not indicated Dissolved in dimethylsulphoxide (DMSO).
Cell line:	<i>E. coli</i> (K 12) p 3478 (pol A ₁ ⁻) and <i>E. coli</i> W 3110 (pol A ⁺).
Doses:	62.5, 125, 250, 500 and 1000 µg/plate µg/plate +/- metabolic activation.
Exposure time:	Not specified.

Materials and methods:

The potential to induce DNA damage was studied in two *Escherichia coli* mutants, either deficient or proficient in DNA repair. In this spot-test, bacteria are exposed to the test substance in agar plates and the sizes of the growth inhibition zones (the diameters) are compared between the two strains. A cytotoxic compound causes growth inhibition zones of similar sizes in both strains whereas a mutagenic substance causes a larger zone of inhibition in the strain with a deficient DNA repair system. In this assay, five concentrations were tested in presence and in absence of metabolic activation and the diameters of the zones of inhibition were compared between the two *E. coli* strains. Methyl methane sulphonate and Chloramphenicol were included in the test as a positive control of mutagenicity and a negative control causing cytotoxicity respectively.

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Results:

Treatment with triazolyalanine did not cause growth inhibition in any of the two strains. The mutagen MMS showed a marked difference in the inhibition zone diameters between the two strains indicating the sensitivity of the test system. The growth inhibition caused by Chloramphenicol was larger in the DNA repair deficient strain compared to the DNA repair proficient strain.

Conclusion:

During the conditions of this assay, triazolyalanine did not induce DNA damage in the strains of *E. coli* used.

Comments:

This method is considered to be insensitive, in particular towards substances that require metabolic activation.

DNA repair test (rec assay)

Reference:	Watanabe, M. (1993): CGA 131 013 – DNA repair test (rec-assay).
Guideline:	No specific guideline.
GLP:	Yes
Acceptability:	As supportive information
Test substance / purity:	CGA 131 013 tech. (batch RV –1469/8 Fr. 1) / Purity > 96%. Dissolved in sterile water.
Bacteria/strain:	<i>Bacillus subtilis</i> / H17 (rec+) and M45 (rec ⁻)
Doses:	20, 50, 100, 200, 500 and 1000 µg/plate +/- metabolic activation.
Exposure time:	24 hours at 37°C

Materials and methods:

Possible DNA damaging effects of triazolyalanine were investigated in a recombination-deficient and a recombination-proficient strain of *B. subtilis*. Filter papers soaked with test substance were spotted onto agar dishes coated with solutions of spores of the two *B. subtilis* strains. Duplicates of each concentration were tested in presence and in absence of metabolic activation. The plates were incubated during 24 hours at 37°C and were then evaluated by comparing the diameters of growth inhibition between the two *B. subtilis* strains. Trp-P-1 (3-Amino-1,4-dimethyl-5H-pyrido[4,3b]indole) and Mitomycin C were included in the test as a positive control of mutagenicity in assays with and without metabolic activation respectively. Kanamycin served as a negative control.

Results:

The highest concentration applied induced growth inhibitory zones of 2-4 mm and 0-1 mm in the recombination-deficient (rec-) strain M45 and the recombination-proficient (rec+) strain H17 respectively both in presence and in absence of metabolic activation. The negative control kanamycin induced growth inhibition zones in strains M45 and H17 of similar size (2 mm difference in diameter) as expected for a cytotoxic substance. The positive

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controls mitomycin C and Trp-P-1 caused larger growth inhibitory zones in strain M45 than in strain H17 as expected (20-22 mm difference in diameter for mitomycin C and 8 mm difference in diameter for Trp-P-1) for mutagens.

Conclusion:

In this study, CGA 131 013 did not induce DNA damage in the strains of *B. subtilis* used.

Comments:

This method is considered to be insensitive, in particular towards substances that require metabolic activation.

Unscheduled DNA Synthesis in rat hepatocytes

Reference:	Puri, E. (1986): CGA 131 013 tech – Autoradiographic DNA repair test on rat hepatocytes.
Guideline:	Checked for compliance with OECD 482 (23rd October 1986).
Deviations from TG 482:	4-BPD was used as a positive control instead of 7, 12-DMBA and 2-AAF which serve as examples of positive controls in the guideline.
GLP:	Yes
Acceptability:	Yes (see comments)
Test substance / purity:	CGA 131 013 tech., (batch TLB 1207) / Purity 97.4%.
Cell line:	Primary hepatocytes isolated from a male Tif RAIf (SPF) rat.
Doses:	Cytotoxicity test: 10 to 10 000 µg/ml (increasing by a factor of 2) Main test: 80, 400, 2000 10000 µg /ml (4 cultures per dose). Dissolved in culture medium.
Exposure time:	5 hours

Materials and methods:

Primary hepatocytes isolated from a male rat (induced with Aroclor 1254) by in situ collagenase perfusion were used to detect possible DNA damaging effects of triazolyalanine. The highest concentration applied was determined in a preliminary cytotoxicity test. Medium served as a negative control and 4-aminobiphenyl (50 µM) was used as positive control. Cells (4×10^5) were seeded onto cover slips (in a 2 hour attachment period followed by an overnight adhesion period) and incubated for five hours in fresh medium supplemented by ³H-thymidine. At the end of the incubation period the cells were washed twice and fixed with ethanol: acetic acid (3:1, v/v). The cover slips were mounted on microscope slides and prepared for autoradiography. After six days of exposure, the mean gross and net number of silver grains per nucleus was determined. Three slides (50 cells/slide) from each concentration were counted (see comments at end). Cells showing more than 120 silver grains/nucleus (0.2%) were considered to be in S-phase and were therefore excluded from counts.

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Results:

Treatment with CGA 131 013 tech. did not lead to an increased number of silver grains per nucleus when compared to the vehicle control whereas the positive control produced a high number of silver grains per nucleus indicating the sensitivity of the test system.

Conclusion:

In this study CGA 131 013 tech. did not induce DNA damage in primary rat hepatocytes.

Comments:

The historical positive control data included in the study report refers to DMN and not 4-BPD. However, since 4-BPD produced a high number of silver grains per nucleus, the sensitivity of the test system is considered to have been adequately tested.

It is stated in the general data of the report that four cultures per group were used. However, it is unclear to the RMS if the three slides/dose level analysed originate from one or three different cultures. According to OECD TG 482, at least two cultures per experimental point should be used. If three different cultures were used, the exclusion of the fourth culture should have been motivated. Since the quality of the study has been confirmed by a QA unit, the RMS assumes that the study was adequately performed.

Transformation/liver microsome test

Reference:	Beilstein, P. (1984): CGA 131 013 tech – Transformation/liver-microsome test.
Guideline:	In-house method (no specific guidelines)
GLP:	The report has been checked by a quality assurance unit but there is no information regarding any compliance with GLP.
Acceptability:	As supportive information
Test substance / purity:	CGA 131 013 tech. (batch TLB 1207) / Purity 97.4%. Vehicle and solvent control: bidistilled water.
Cell line:	(Balb / 3T3) mouse fibroblasts.
Doses:	62.5, 125, 500 and 1000 µg /ml +/- microsomal activation (15 plates per dose group).
Exposure time:	24/72 hours with/without microsomal activation.

Materials and methods:

The ability of CGA 131 013 to induce cell transformation was tested at five concentrations in Balb/3T3 mouse fibroblasts. The highest concentration applied was determined in a preliminary cytotoxicity test.

Balb/3T3 cells multiply in culture until a monolayer is formed, after which no further division and growth occurs. If the treatment with the test substance induces cell transformation, visible colonies superimposed on the monolayer of normal parent cells appear.

In this study, fifteen cultures per dose level were treated for 24 hours in presence and 72 hours in absence of a microsomal activation mixture respectively. After removal of the test substance, the cells were washed and

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incubated four weeks in the growth medium which was replenished twice weekly. The experiment was terminated by fixation of the cell monolayers with methanol and Giemsa staining. The transformed colonies were counted in a microscope. Methylcholanthrene and 2-Acetylaminofluorene were used as positive controls in absence and presence of metabolic activation respectively and the solvent and an untreated sample were used as negative controls. In parallel, a cell-viability experiment was performed and the values obtained were used to normalise the results of the transformation test. The transformation frequency value, defined as the number of foci of transformed cells per 10 000 surviving cells, was determined.

Results:

A second experiment in presence of metabolic activation had to be performed since the first could not be evaluated due to the high background of transformed colonies obtained in all dishes, including the negative control. The results from the second experiment with metabolic activation and the experiment without metabolic activation did not show any differences in transformation frequency between treated and untreated dishes that were statistically significant¹ (table 6.8.1.3.4-1).

Table 6.8.1.3.4-1 Mean values

Experiment without metabolic activation:		
Concentration (µg/ml)	Transformation frequency	Viability control (%)
Solvent control	0	21.3
Untreated control	0	20.7
CGA 131 013: 62.5	0.67	20.0
CGA 131 013: 125	0	16.7
CGA 131 013: 250	0.96	13.8
CGA 131 013: 500	0	25.5
CGA 131 013: 1000	0	17.8
Methylcholanthrene 1.5	90.7	2.5
Methylcholanthrene 3.0	528	0.8
Experiment with metabolic activation:		
Solvent control	1.15	23.1
Untreated control	1.09	26.1
CGA 131 013: 62.5	0.49	27.3
CGA 131 013: 125	1.71	23.3
CGA 131 013: 250	1.83	21.9
CGA 131 013: 500	2.34	17.1
CGA 131 013: 1000	4.1	13.0
2-Acetylaminofluorene 50	19.4	6.9
2-Acetylaminofluorene 100	33.5	4.8

Conclusion:

Based on the results of this study, CGA 131 013 tech. did not induce transformation of Balb / 3T3 mouse fibroblasts.

¹ tested by calculation of the fiducial limits for p according to the binomial-distribution model.

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Comments:

The report lacks data on the individual transformation frequency for each of the fifteen cultures per dose level and the standard deviations of the calculated means are not reported.

Reference:	Richold, M., Allen, J.A., Williams, A., Ransome, S.J. (1981): Cell transformation test for potential carcinogenicity of R152056.
Guideline:	No specific guideline.
Comment:	A description of the methodology used was not included in the study report. The results of this assay were not statistically evaluated.
GLP:	Yes
Acceptability:	As additional information (see comments)
Test substance / purity:	R152056 (batch P2 B2427 – 153) / Purity: no impurities identified. Solvent: MEMS.
Cell line:	Baby Hamster Kidney (BHK 21 C13) cells
Doses:	500, 1000, 2000, 4000 and 8000 µg /ml without microsomal activation. 1000, 2000, 4000, 8000 and 16000 µg /ml with microsomal activation.
Exposure time:	Cell survival assay: 5 days Transformation assays: 19/20 days with/without microsomal activation.

Materials and methods:

The assay was based on the observation that cells that have undergone malignant transformation *in vitro*, will, unlike normal cells, continue to grow in soft agar medium to produce macroscopically visible colonies. The concentrations used in this study were based on the results of a preliminary cytotoxicity test. MEMS or DMSO treated cells were seeded into agar plates at 5×10^5 , 2.5×10^5 , 1.25×10^5 , and 0.625×10^5 cells/plate to provide negative control cultures with simulated 100%, 50%, 25% and 12.5% survival. The purpose was to facilitate comparisons between treated cultures and negative control cultures with similar viable cell densities since cell density was known to affect the transformation frequency. Colonies that were greater than 450 µm were counted and the transformation frequency value (the number of foci per 100,000 surviving cells) was determined. Different concentrations of 4-Nitroquinoline-N-oxide and p-Dimethylaminoazobenzene were used as positive controls in absence and presence of metabolic activation respectively.

Results:

The transformation frequencies obtained in treated cultures were compared to the transformation frequencies obtained in solvent controls at the corresponding viability level. The results showed that treatment increased the transformation frequency both in the absence and in the presence of metabolic activation.

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Conclusion:

Based on the results of this study, the authors concluded that R152056 induced transformation of Baby Hamster Kidney (BHK 21 C13) cells.

Comments:

The notifier argues that there is ample evidence that the methodology used is inadequate as some laboratories have encountered sudden and dramatic increases in the spontaneous transformation frequency in these cell cultures whereas some laboratories have been unable to observe any growth of carcinogen-treated BHK cells in semi-solid media. The notifier also comments that it has been observed, also in the current study, that untreated cells seeded at progressively lower densities in agar display concomitant increases in apparent spontaneous transformation frequencies, relative to the surviving cell fraction, which raises the possibility of generating false positive results with cytotoxic doses of non-genotoxic compounds. The notifier concludes that BHK cells seem sensitive to environmental perturbations that render them unsuitable for assay purposes and refers to an article by Meyer A.L. (In vitro transformation assays for chemical carcinogens, *Mutation Research* 115, pp. 323-338, 1983).

Micronucleus test (Chinese Hamster)

Reference:	Strasser, F. (1986): CGA 131 013 tech - Micronucleus test (Chinese Hamster).
Guideline:	Checked for compliance with OECD TG 474 (21st July 1997).
Deviations from TG 474:	The choice of using Chinese hamsters which is not the conventional animal for this type of assay was not justified. Individual body weight data, food/water quality and data from the range-finding study were not reported. The mean and standard deviations of micronucleated immature erythrocytes per group were not reported. 1000 polychromatic erythrocytes were scored instead of 2000.
GLP:	Yes
Acceptability:	Yes (see comments)
Test substance / purity:	CGA 131 013 tech. (batch: TB 1207 (6. Lief)) / Purity: 97.4%.
Animal/strain:	Chinese hamster (<i>Cricetulus griseus</i>) / random outbred strain.
Administration:	Oral, by gavage Vehicle: 0.5% sodium carboxymethylcellulose (CMC).
Doses/no of animals:	5000 mg/kg / 8/dose/sex/ timepoint; negative control / 8/dose/sex/ timepoint; positive control 8/sex.
Sampling time:	16, 24, 48 hours after treatment.

Materials and Methods:

Groups of Chinese hamsters were administered a single oral dose of either 5000 mg/kg test substance (selected after a tolerability test), the negative control CMC (0.5%) or the positive control cyclophosphamide by gavage. The animals were sacrificed by cervical dislocation and bone marrow was harvested from the shafts of both

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femurs. Samples were taken from animals in treated groups and in the negative control group, sacrificed at 16, 24 and 48 hours after treatment, whereas samples from the positive control group animals were taken only at 24 hours post treatment (Table B.6.8.1.3.4-2). Erythrocyte smears were prepared on slides which were coded prior to analysis. Slides from five animals/sex/dose that showed a good differentiation between mature and polychromatic erythrocytes (PCEs) were scored. In order to detect any disturbance of erythropoiesis, the ratio of polychromatic to normochromatic erythrocytes was determined and 1000 polychromatic erythrocytes from each animal were scored for micronuclei. The χ^2 -test was used to compare the number of polychromatic erythrocytes with micronuclei in treatment and control groups.

Table B.6.8.1.3.4-2: Treatment schedule

Treatment	Preparation of Bone Marrow after Treatment		
	16 hours	24 hours	48 hours
5000 mg/kg	8m + 8f	8m + 8f	8m + 8f
negative control (0.5% CMC)	8m + 8f	8m + 8f	8m + 8f
positive control (cyclophosphamide, 64 mg/kg)		8m + 8f	

Results:

There was no statistically significant increase in the number of micronucleated polychromatic erythrocytes (PCEs) compared to the negative control animals. In the positive control sample, the percentage of micronucleated PCEs was significantly increased at 24 hours.

Conclusion:

In this study, a dose of 5000 mg/kg did not cause any evidence of clastogenic or aneugenic effects in Chinese hamsters.

Comments:

Despite the lack of data (see deviations from TG 474) this study is considered to be acceptable since the data was statistically evaluated and the study report was checked by a QA unit.

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Micronucleus test (mouse)

Reference:	Watkins, P.A. (1982): R 152056: 3-(1,2,4-triazol-1-yl) alanine (ICI 156,342). Micronucleus test in CBC F1 mice.
Guideline:	Checked for compliance with OECD TG 474 (21st July 1997).
Deviations from TG 474:	Individual body weight data and the food/water quality was not reported. The mean and standard deviations of micronucleated immature erythrocytes per group were not reported. Historical negative/positive control data were not included. 1000 polychromatic erythrocytes were scored instead of 2000.
GLP:	Yes
Acceptability:	Yes (see comments)
Test substance / purity:	R 152056: 3-(1,2,4-triazol-1-yl) alanine (ICI 156,342) (batch : 02199/49) / Purity: 97.4%.
Animal/strain:	Mouse / CBC F1
Administration:	Intraperitoneal Vehicle: 0.5% aqueous solution of Tween 80.
Doses/no of animals:	2500 and 5000 mg/kg, positive and negative control / 5 males/dose/timepoint
Sampling time:	24, 48, 72 hours after treatment

Materials and Methods:

Groups of mice were administered either 2500 or 5000 mg/kg of test substance, 0.5% aqueous solution of Tween 80 (negative control) or Cyclophosphamide (positive control) as a single intraperitoneal dose. The animals were killed by cervical dislocation after 24, 48 and 72 hours respectively. A small paint brush, lightly moistened in saline, was pushed into the exposed bone marrow of the femur of one leg, and three or four smears were prepared on slides which were coded and assessed blind. The number of micronuclei per 1000 polychromatic erythrocytes (PCEs) were counted and recorded. In order to get an indication of any disruption of bone marrow function, the number of normocytes in the same field as the PCEs being counted was recorded whilst counting the first 200 PCEs of every 1000. The results were statistically evaluated using Students t-test (one-sided) and Fisher's exact test.

Results:

There was no statistically significant increase in the number of micronucleated polychromatic erythrocytes (PCEs) at 2500 or 5000 mg/kg bw compared to the negative control animals at the 3 sampling times. In the positive control, the percentage of micronucleated PCEs was significantly increased at 24 and 48 hours, but not at 72 hours.

Conclusion:

In this study, 3-(1, 2, 4-triazol-1-yl)alanine did not cause any evidence for clastogenic or aneugenic effects.

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Comments:

Despite the lack of data (see deviations from TG 474) this study is considered to be acceptable since the data was statistically evaluated and this evaluation was checked by a Quality Assurance unit.

Micronucleus test (mouse)

Reference:	Herbold, B. (1983c): THS 2212 (Triazolylalanine), Micronucleus test for mutagenic effect on mice.
Guideline:	Checked for compliance with OECD TG 474 (21st July 1997) .
Deviations from TG 474:	Data from the preliminary test was not included in the study report.
GLP:	No
Acceptability:	Yes
Test substance / purity:	THS 2212 (Triazolylalanine), (batch: E238099) / Purity: not indicated
Animal/strain:	Bor:NMRI (SPF)
Administration:	Orally Vehicle: 0.5% Cremophor emulsion.
Doses/no of animals:	8000 mg/kg bw / 5dose/sex/ timepoint; positive/negative control 5/sex
Sampling time:	24, 48, 72 hours after treatment

Materials and Methods:

Groups of mice were administered a single oral dose of test substance, vehicle (negative control) or Cyclophosphamide (positive control). Animals were sacrificed at 24, 48 and 72 hours after treatment and the femur marrow was processed. The number of micronuclei per 1000 polychromatic erythrocytes (PCEs) were counted and recorded. In order to get an indication of any disruption of bone marrow function, the number of normocytes per 1000 PCEs was also recorded. The highest values of the treatment group and the positive controls were statistically evaluated by Wilcoxon's distribution-free signed rank test.

Table B.6.8.1.3.4-2

Treatment		Preparation of Bone Marrow after Treatment		
		24 hours	48 hours	72 hours
8000	mg/kg	5m + 5f	5m + 5f	5m + 5f
negative control (0.5% Cremophor)		5m + 5f		
positive control (cyclophosphamide, 60 mg/kg)		5m + 5f		

Results:

A statistically significant increase in the number of micronucleated polychromatic erythrocytes (PCEs) compared to the negative control animals at 24 hours after dosing was observed. However, this increase was almost entirely due to one male and one female mouse. Therefore, 1000 additional polychromatic erythrocytes (PCEs) were counted in all animals (reported as first and second series). In the second serie no statistically significant difference between triazole alanine treated animals and control animals was found. In the positive

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control the percentage of micronucleated PCEs was significantly increased at 24 hours. A statistically significant increase of the number of normochromatic erythrocytes per 1000 polychromatic erythrocytes was observed in treated animals after 72 hours but this number was within the biological range of variation according to the historical data included in the study report.

Conclusion:

In this study, triazolylalanine did not cause any evidence of clastogenic or aneugenic effects.

B.6.8.1.3.5 In vivo studies in germ cells

In absence of any mutagenic findings, no *in vivo* tests in germ cells were performed with triazolylalanine.

B.6.8.1.3.5.1 Summary of genotoxicity

Table 6.8.1.3.5.1-1: Survey of mutagenicity studies of acceptable quality

Study	Concentrations/ Dose levels	Results	Reference
Salmonella/E. coli <i>in vitro</i>	312.5-5000 µg/plate +/-activation	negative	Hertner, Th. 1993
Salmonella/ microsome test	20-12500 µg/plate +/-activation	negative	Herbold, B. 1983
Cytogenetic test on Chinese hamster cells <i>in vitro</i>	500 -10 000 µg/ml +/-activation	negative	Dollenmeier, P. 1986
Unscheduled DNA synthesis	80, 400, 2000 10000 µg /ml	negative	Puri, E. 1986
Micronucleus test <i>in vivo</i> Chinese hamster	M/F: 5000 mg/kg bw	negative	Strasser, F. 1986
Micronucleus test <i>in vivo</i> mouse	M: 2500, 5000 mg/kg bw	negative	Watkins, P.A. 1982
Micronucleus test <i>in vivo</i> mouse	M/F: 8000 mg/kg bw	negative	Herbold, B. 1983c

This section was represented by several *in vitro* and *in vivo* tests performed to detect any genotoxic potential of triazolylalanine. The quality of these studies varied and only those considered to be of acceptable quality are listed in table 6.8.1.3.5.1-1, the rest are regarded as supportive or additional information. Triazole alanine was shown to induce transformation of Baby Hamster Kidney (BHK 21 C13) cells. However, the use of this strain seems to be an inappropriate choice for this type of assay since it was previously known and also shown in this study that the incidence of spontaneous transformation frequencies is dependent on the cell density in the agar. In the transformation/liver microsome study performed in mouse fibroblasts, triazole alanine did not induce transformation of cells.

Triazole alanine did not induce mutations in any of the bacterial reversion tests, in the gene mutation test on mammalian cells, in the UDS assay or in the three micronucleus tests performed. In combination, these studies suggest that triazole alanine is not a genotoxic substance.

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B.6.8.1.3.6 Reproductive Toxicity

Dose-range finding study

Reference:	Birtley, R.D.N. (1983). Triazole Alanine : Preliminary Reproduction Study in the Rat.
Guideline:	No specific guidelines
GLP:	No
Acceptability:	As a dose-range finding study to determine dose levels for the main study.
Test substance / purity:	Triazole alanine (3- [1, 2, 4 triazol-1-yl] alanine) / Purity: 48 % (see comments).
Species / Strain:	Rat / Alpk:AP (Wistar-derived)
Doses / No. of animals:	0, 150, 625, 2500 and 10000 ppm (nominal values after correction for impurities) / 12 females and 6 males/dose
Administration:	Orally, via the diet.
Exposure time /	Daily dose / 6 weeks prior to mating (females/males), treatment continued in
Duration:	females throughout pregnancy, lactation and weaning of offspring.

Materials and Methods:

Groups composed of twelve female and six male rats were fed diets containing triazole alanine. The body weight gain, food consumption and food utilisation during the pre-mating period and the body weight gains in females during pregnancy were recorded during the study. Additional parameters including male/ female fertility indices, gestation length, pre-coital interval, live born index, survival index, litter size, sex distribution and body weight gain of litters from birth to weaning were also recorded. Male rats were sacrificed after mating, while treatment was continued in females throughout pregnancy, lactation and weaning of offspring. Thereafter all females and offspring were sacrificed. All parents and selected offspring were subjected to a post-mortem examination and a histopathological examination of certain tissues was performed in some animals. The mean values of the bodyweight gain, food consumption, food utilisation, reproductive parameters, litter size and litter bodyweights in males and females were statistically analysed using the Student's t-test (two-sided) in all treatment groups.

Results:

Diet analysis: Triazole alanine was demonstrated to be stable and homogeneously distributed in diet up to 11 weeks in the 150 and 10000 ppm samples. Analyses of the concentration achieved, measured on three occasions during the study, showed that the mean values in all dose levels were within 14% of the nominal value in all but one case; on the second occasion when analysis was done the diets from the 10000 ppm nominal concentration were found to contain 79% of this value.

The food consumption was similar in all dose groups except during the first week of the study when a statistically significant increase was observed in animals administered the highest dose. However this increase was small (6%) and it is not considered to be toxicologically relevant. The food utilisation was reduced in males

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and females of the high dose group during the first and second week respectively. This was not considered to be toxicologically relevant as the values returned to normal during the following week.

Parental Animals: One male administered the highest dose was found dead during the fifth week of the pre-mating period. This animal had deposits in its bladder and kidney. The pathology report concluded that death was caused by obstructive urolithiasis and chronic inflammation of the urinary tract. The study author claims that this is a condition that occurs sporadically in this strain and therefore this death was not considered to be related to treatment. However, this statement was not supported by any historical data on Alderly Park rats. There were no treatment-related clinical signs observed in any of the animals. Treatment did not affect the food consumption or the body weight gain during the pre-mating period.

A change in the pre-coital interval was observed in rats administered the highest dose (Table B.6.8.1.3.6-1). This effect could be due to hormonal changes and would in that case also affect the length of the oestrus cycle (not reported). In the absence of any data to explain the increased pre-coital interval, it is considered to be an adverse effect. There were no macroscopic or microscopic findings that were considered to be related to treatment.

Table B.6.8.1.3.6-1: Precoital interval in rats (mean \pm standard deviation)

Reproductive Index	0 ppm	150 ppm	625 ppm	2500 ppm	10000 ppm
Precoital interval (days)	2.1 \pm 1.1	3.0 \pm 0.9	1.8 \pm 0.8	1.9 \pm 1.2	4.3 \pm 2.4 *

* Statistically significantly different from control group mean $P < 0.05$ (Student's t-test 2 sided and Mann-Whitney U-test)

Offspring: A statistically significant higher proportion of male pups was observed both on day 1 and on day 29 in the litters from parents administered the highest dose. However, the control values were considered to be unusually low by the study author (no historical data presented) therefore this observation was considered to be incidental and not related to treatment. In male and female pups from parents administered the highest dose, reduced mean initial weights that were statistically significant were observed in both males and females on day 1 ($\leq 10\%$). However, the subsequent growth rates of these pups were similar to the control values (Table B.6.8.1.3.6-1). Individual data on pup weights were not included in the study report but the standard deviations indicate that there is a variation in this parameter and thus the reduction is not considered to be an adverse effect.

Table B.6.8.1.3.6-2: Offspring data (mean \pm standard deviation)

Offspring parameters	0 ppm	150 ppm	625 ppm	2500 ppm	10000 ppm
Sex distribution (day 1) (% male)	41.6 \pm 13.4	50.1 \pm 10.4	42.1 \pm 11.8	49.3 \pm 13.3	57.3 \pm 12.9**
Sex distribution (day 29) (% male)	41.9 \pm 11.3	53.0 \pm 8.6	41.0 \pm 12.0	44.5 \pm 7.9	59.2 \pm 14.2**
Initial weight of males (gram)	6.08 \pm 0.44	5.87 \pm 0.48	5.89 \pm 0.64	5.97 \pm 0.59	5.67 \pm 0.84 *
Initial weight of females (gram)	5.71 \pm 0.70	5.53 \pm 0.69	5.67 \pm 0.76	5.68 \pm 0.54	5.14 \pm 0.85 *
Weight of males on day 29 (gram)	79.7 \pm 7.0	76.4 \pm 14.2	79.1 \pm 11.7	74.3 \pm 6.5	76.0 \pm 13.8
Weight of females on day 29 (gram)	73.9 \pm 7.6	71.4 \pm 13.8	73.1 \pm 12.0	69.8 \pm 6.3	69.3 \pm 11.3

* Statistically significantly different from control group mean (Student's t-test 2 sided based on pooled error estimate $P < 0.05$)

** Statistically significantly different from control group mean $P < 0.05$ (Fisher's exact test 2 sided)

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Conclusion:

Reproductive NOAEL: 2500 ppm based on the increased length of pre-coital interval observed at 10000 ppm.

Offspring NOAEL: ≥ 10000 ppm based on the absence of adverse findings in the offspring of dams administered the highest dose.

Offspring NOEL: 2500 ppm based on the slight reductions in neonatal weights observed in the offspring of dams administered the highest dose.

Comments:

The purity of the first triazole alanine batch was 48% and not > 90% as was originally specified. Thus the diets prepared from this first batch contained about half of the target concentration of triazole alanine. Diets prepared from a second, purer batch of triazole alanine were formulated to contain similar actual concentrations of triazole alanine as those prepared from the first batch of triazole alanine.

However, the adverse effect observed cannot with certainty be assigned to triazolealanine since the purity of one of the batches was very low.

The dose levels used should also be reported in mg/kg bw and the diet used should be specified in the report since the same diet should be used in the main study.

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Main study (Two Generation Reproduction Study in Rat)

Reference:	Milburn, G.M., Birtley, R.D.N., Pate, I., Hollis, K., Moreland, S. (1986): Triazole Alanine: Two Generation Reproduction Study in the Rat. The report was supplemented and amended on 02.03.1988.
Guideline:	Checked for compliance to OECD 416 (22nd January 2001) .
Deviations from experimental methodology:	Power failures of 3.5 hours and 10 minutes on 29th July 1984 and 8th August 1984, respectively which affected the light cycle in the animal room when F1 parental females were littering to produce F2b litters. Elevated animal room temperatures for a period of 2 weeks during the F0 premating period (in July 1983), when the maximum recorded temperature was 25-32 °C
Deviations from TG 416:	The following parameters were not reported: the number of F0 and F1 females cycling normally, cycling length, total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, percent of sperm with each identified abnormality, the number of implantations and corpora lutea and the age of vaginal opening. Individual data on body weight and clinical signs was not reported. Organs (uterus, ovaries, testes, epididymides, prostate, seminal vesicles, brain, liver, kidneys, pituitary, thyroid and adrenal glands) were not weighed. Individual body weights of F1 and F2 pups selected for necropsy was not reported and organs (brain, spleen and thymus) from one randomly selected pup/sex/litter was not weighed. The age of vaginal opening and preputial separation in F1 weanlings selected for mating was not reported. The 500 ppm dose level was not reported in mg/kg bw.
GLP:	Yes
Acceptability:	Yes (see comments)
Test substance / purity:	Triazole alanine [2-amino-3-(1, 2, 4 triazol-1-yl) propionic acid] (batch no TLB 1207/018-24 / Purity: 97.8 % w/w)
Species / Strain:	Rat / Alpk:AP (Wistar-derived)
Doses / No. of animals:	0, 500, 2000 and 10000 ppm / F0:30 females/15males/dose Additionally, 4 females/2 males in control and high dose group respectively were used as microbiological sentinels.
Administration:	Orally, in diet.
Exposure time /	Daily dose / F0/F1: during pre-mating period, treatment continued in females
Duration:	throughout pregnancy, lactation and weaning of offspring (table).

Materials and methods:

Four groups of rats (approximately 28 days old) were daily fed triazolalanine in diet during the pre-mating period. The treatment was continued in females throughout pregnancy, lactation and weaning of offspring. Four

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additional females and two males were included in the control and high dose groups as microbiological sentinels. The bodyweights of all rats were recorded at weekly intervals during the premating period. Females were weighed on selected days during pregnancy whereas males were weighed at four week intervals. During the premating period the rats were observed daily for abnormal clinical conditions and behaviour and the food consumption was recorded at weekly intervals. After the pre-mating period, the females were first mated with males from the same group and allowed to produce a litter (A). Six days after weaning the females were remated with a different male to produce a second litter (B). Pups were randomly selected as F1 parents from F1B litters on day 36 post-partum and the mating procedure was performed as in the F0 generation. The offspring was investigated regarding litter size, total litter weight, mean pup weight gain, pup survival to day 22 and the clinical condition was observed daily. The reproductive parameters examined included fertility (females/males), length of gestation, precoital interval and maternal neglect.

Gross post mortem examinations were performed on two pups per sex of the F1/F2 A litter and of at least two pups/B litter of the F1 (not selected as F1 parents) and F2 generation. A full post mortem examination was performed on designated* F1/F2 litter B pups and on F0/F1 parents.

*F1: 5 pups/dose/sex (10000 ppm group: 4 males and 6 females); F2: 10 pups/dose/sex (2000 ppm group: 11 males and 9 females).

	1st Generation (F0)	2nd Generation (F1)
Pre-mating period	12 weeks	11 weeks
Mating trials	Two females housed with one male from the same group, vaginal smears examined daily, for up to 10 days. If there was no evidence of mating, first male was removed, and second male, which had successfully produced a positive smear in at least one female, was introduced after a 3-day rest period. No further pairing if second pairing was unsuccessful.	
Gestation period	3 weeks from mating	3 weeks from mating
Weaning	Day 28/29 post partum	Day 28/29 post partum
Progeny	F1a & F1b	F2a & F2b

Results:

Diet analysis: Samples taken at approximately monthly intervals throughout the study confirmed that the mean test substance concentrations were within 10% of the nominal concentration except for one diet prepared for the 10000 ppm level which was 13% lower than target. The test substance was evenly distributed and chemically stable in diet for approximately 3 months at the 500 ppm level and approximately 2 months at the 10000 ppm level.

Microbiology: The bacterial pathogen *Staphylococcus aureus* was isolated (the cage(s) affected was not specified) and a species of *Trichomonas* was observed in the pooled faeces of some of the F1 rats. In addition, a low antibody titre of 1/20 to Mouse Pneumonia Virus (PVM) was recorded. None of these findings were considered to affect the outcome of the experiment as the overall clinical conditions of the rats were good.

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Parental animals: Two F0 females (administered 500 ppm and 2000 ppm respectively) with imperforate vagina were sacrificed at the end of the pre-mating period, one F0 female in the control group died/were sacrificed due to poor clinical condition and three F0 females (two in the control group and one administered 500 ppm) and three F1 females (one in the control group and two administered 10000 ppm) died/ were sacrificed as a result of parturition difficulties. Since mortality was evenly distributed over all groups it was considered to be unrelated to treatment. Four F1 males (two administered 500 ppm and two administered 2000 ppm) died or were killed due to poor clinical conditions. Since all animals administered the highest dose survived treatment, these deaths were not considered to be related to treatment.

Hair loss at one or more areas was observed in six females at 10000 ppm during the pre-mating period and in ten females at 10000 ppm during the mating, gestation and lactation period, otherwise no treatment-related clinical signs were observed. There were no effects observed on body weight gains of the F0 animals but a minor reduction in body weight gain in F1 males was observed at 500 ppm and above. However, since this reduction was low (2.5-6%) and since it was not dose related it is not considered to be an adverse effect. The food consumption during the premating period was not affected by treatment in the F0 and F1 generation except for a slight decrease in food consumption in all F0 parents including the controls at weeks 6-7 of the premating period which coincided with a brief period of elevated temperatures in the animal room.

In the F0 parents administered 10 000 ppm, the female fertility index was reduced in the production of both the F1A and the F1B litters (Table B.6.8.1.3.6-3). The histopathological examination of females showing an abnormal breeding pattern revealed senile ovarian changes (recent corpora lutea absent, follicular cysts and luteal cyst) and endometrial stromal cell hyperplasia. Female fertility was also reduced in F0 parents at 500 ppm on mating for the F1A litter but was not affected at the higher dose levels or in subsequent generations and was therefore not considered to be related to treatment. Senile ovarian changes were also observed in the untreated F1 females producing the F2B litter.

In untreated dams and dams administered 10 000 ppm, a reduced number of live born F2B pups was observed. This coincided with power failures that affected the light cycle in the animal room. A slightly prolonged gestation period was also apparent in all groups on production of the F2B litter. A marginal increase in the severity of the nephrocalcinosis was detected in the kidneys of the F0 females administered 10 000 ppm. The study author claims that this is a very common lesion in rats of this strain and age and that this observation therefore is without toxicological relevance. However, this statement was not supported by any historical data on Alderly Park rats. In kidneys of F0 males administered 10000 ppm, a slight increase in the incidence of hyaline casts and focal tubular dilatation were observed but since they were minor changes and absent in the F1 parent males, they were considered to be of low concern.

Offspring (Table 6.8.1.3.6-4): There were no treatment-related effects apparent in the percentage of pups born live, the percentage of pups surviving to day 22 or in the litter size. Reduced initial weights were observed in male F1B and male/female F2A offspring of dams administered 10000 ppm. The total F2b litter weights were reduced both on day 1 and on day 29 when compared to control values. In table B.6.8.1.3.6-4, a variation in foetal weights is observed between A and B litters and differences that are statistically significant arise when the treated B litters are compared to control B litters but may not arise if compared to control A litters. Moreover, on

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Table B.6.8.1.3.6-4: Offspring data

Offspring parameters	0 ppm				500 ppm				2000 ppm				10000 ppm			
	F ₁ A	F ₁ B	F ₂ A	F ₂ B	F ₁ A	F ₁ B	F ₂ A	F ₂ B	F ₁ A	F ₁ B	F ₂ A	F ₂ B	F ₁ A	F ₁ B	F ₂ A	F ₂ B
Litter size on day 1	12	11	12	11	10	11	11	10	10	11	10	11	10	11	12	9
Total litter weight on day 1 (g)	64	65	67	64	58	67	65	60	56	63	59	65	55	62	61	52*
Initial weight of males (g)	5.7	6.5	6.0	6.2	6.2**	6.3	6.3	6.6	5.9	6.3	6.3	6.4	5.8	6.0**	5.6	6.0
Initial weight of females (g)	5.3	6.1	5.7	5.8	5.8*	5.9	5.8	6.3*	5.6	5.9	5.9	6.0	5.4	5.6**	5.3*	5.6
Litter size on day 29	10	10	11	10	10	11	11	9	9	10	9	11	9	11	11	9
Total litter weight on day 29 (g)	710	803	813	818	712	806	790	782	659	770	690	839	649	808	770	672*
Weight of males on day 29 (g)	69	75	73	81	71	71	70	81	70	75	72	79	72	76	76	78
Weight of females on day 29 (g)	65	69	69	76	65	67	66	74	63	70	67	72	66	70	71	74

* or ** statistically significant at the 5% or 1% level, respectively.

Litter size, total litter size, and mean pup weight at day 1 was considered by analysis of variance, separately for each litter. Mean pup weight gain to days 5, 11, 22 and 29 was considered by analysis of covariance on initial mean pup weight. Analyses were carried out separately for male and female pups and for A and B litters.

Conclusion:

The parental NOAEL in this study was $\geq 10\ 000$ ppm (equivalent to an average test substance intake of 500 mg/kg bw/day) based on the absence of adverse effects in rats administered the high dose.

The reproductive/offspring NOAEL in this study was 2000 ppm (equivalent to an average test substance intake of 100 mg/kg bw/day) based on the reduced female fertility index observed at 10 000 ppm and the incidence of kinked ureters affecting 10/ pups in one litter at 10 000 ppm.

Comments:

Since the study was made before the current OECD guideline 416 was adopted, many important parameters of reproductive and developmental toxicity are missing and the information is therefore limited. Despite this, the study is considered to be acceptable. The reduced female fertility index observed in dams administered 10 000 is not considered to be sufficient for a classification of triazole alanine as being toxic to reproduction. The use of higher dose levels could have been more useful in this study.

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Reference:	Clapp, M.J.L., Killick, M.E., Hollis, K.J., Godley, M.J. (1983): Triazole Alanine: Teratogenicity Study in the Rat.
Guideline:	Checked for compliance with OECD 414
Deviations from TG 414:	The detection of spermatozoa in vaginal smear was defined as day 1 of study. Treatment started one day later than recommended in guideline. Test relative humidity was lower (33-41%) than recommended in guideline.
GLP:	Yes
Acceptability:	Yes (see comments)
Test substance / purity:	Triazole alanine [2-amino-3-(1, 2, 4 triazol-1-yl) propionic acid] (batch no TLB 12072/018-24 / Purity: 94.8 %)
Species / Strain:	Rat / Alpk:AP (Wistar-derived)
Doses / No. of animals:	0, 100, 300 and 1000 mg/kg bw/day / 24 females/dose level
Administration:	Orally, by gavage. Vehicle: distilled water
Exposure time /	Daily dose / day 7 until day 16 of pregnancy (day 1 defined as the day of detection
Duration:	of spermatozoa in vaginal smear).

Materials and methods:

Mixtures of test article or vehicle were administered daily by oral gavage to groups of 24 successfully mated and presumably pregnant rats during day 7 to 16 of pregnancy. Any clinical signs were recorded daily and body weights were determined on days 1, 4, 7-16, 19 and 22. The food consumption was determined on days 1, 4, 7, 10, 13, 16 and 19. On day 22, all dams were sacrificed by halothane BP vapour inhalation and subjected to a macroscopical examination.

The uterine weights were determined and the uterus and ovaries were further examined to determine the number of corpora lutea in each ovary and the number and position of implantations. The location of live and dead (early/late intra-uterine deaths) foetuses in the uterine horns were investigated.

Foetuses were removed, numbered, sexed, externally examined, weighed and killed by intracardiac injection of pentobarbital sodium solution (200 mg/ml). The foetuses in each litter were assigned to either visceral or skeletal and soft tissue evaluation, approximately at a ratio of 2:1.

Results:

Analyses of dosing suspensions from each dose level confirmed that all animals received the intended dose within experimental limits of $\pm 10\%$.

All dams survived treatment and there were no treatment-related clinical signs were observed. The mean food consumption and body weight gain were similar in all groups. The postmortem examination did not reveal any treatment related maternal findings.

There were no adverse effects observed among litter except for one foetus of dams administered 1000 mg/kg bw/day that was severely malformed. This foetus had both external/visceral and skeletal defects. Since only one

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foetus of the 293 examined for visceral defects was affected this was considered to be a spontaneous abnormality that was unrelated to treatment. There were no effects on the incidence of skeletal variants that were considered to be related to treatment.

In the offspring of dams administered 1000 mg/kg bw, the incidence of minor skeletal defects was increased, indicating an effect on ossification (Table B.6.8.1.3.6-5). In general, a delay in the ossification process combined with low foetal weights can indicate a retardation of development. However, since there were no effects on foetal weights in this study, these effects were not considered adverse.

Table B.6.8.1.3.6-5: Incidence of statistically significant skeletal defects

Skeletal defects	0 mg/kg bw/day	100 mg/kg bw/day	300 mg/kg bw/day	1000 mg/kg bw/day
No. of foetuses examined	185	201	178	195
No. of foetuses showing minor skeletal defects only	95	95	92	122*
Skull: odontoid process not ossified	12	10	24*	29**
Vertebrae: partial ossification transverse process of 7th cervical vertebra (bilateral)	1	3	2	12**
Vertebrae: partial ossification of the 13th thoracic centrum	1	4	4	7*
Sternebrae: 5th sternebra not ossified	0	0	1	7**

* or ** statistically significantly greater than the control group at the 5% or 1% level, respectively (Fisher's exact test, one-sided)

Conclusion:

The maternal and developmental NOAEL was ≥ 1000 mg/kg bw/day based on the absence of adverse findings at this dose level. The developmental NOEL was 100 mg/kg bw/day based on an increased incidence of non-ossification of the odontoid process at 300 mg/kg bw/day.

Comments :

The deviations from TG 414 listed are not considered to affect the result of this study.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registrants must not be granted on the basis of this document.

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B.6.8.1.3.6.1 Summary of Reproductive toxicity in rat

Two-generation reproduction study in rats					
Milburn, G.M. <i>et al</i> 1986	F: 0, 500, 2000, 10000 ppm	Orally via the diet	NOAEL: Maternal: 500 mg/kg bw (10 000 ppm)	LOAEL: N/A	No adverse effects observed
			Reproductive/offspring: 100 mg/kg bw/day (2000 ppm)	500 mg/kg bw	<u>500 mg/kg bw (10 000 ppm):</u> ↓ female fertility index of F0 rats. ↑ incidence of kinked ureters in F2B litter
			NOEL: M: 100 mg/kg bw (2000 ppm)	LOEL: 500 mg/kg bw (10 000 ppm)	<u>10 000 ppm</u> ↑ incidence of hyaline casts and focal tubular dilatation in kidneys of F0 males
Teratology study in rats					
Clapp <i>et al.</i> , 1983	F: 0, 100, 300, 1000 mg/kg	Orally via the diet	NOAEL: Maternal: ≥1000 mg/kg bw	LOAEL: N/A	No adverse effects observed
			Developmental: 1000 mg/kg bw		No adverse effects observed
			NOEL: 100 mg/kg bw	LOEL: 300 mg/kg bw	<u>300 mg/kg bw</u> ↑ incidence of non-ossification of the odontoid process

This section was represented by a dose-range finding study, a two generation study and a teratogenicity study performed on rats. In the dose-range finding study, an increased length of the pre-coital interval was observed. However, these effects cannot with certainty be assigned to triazole alanine since the purity of one of the batches was very low. According to TG 416, a substance should be tested in doses until toxicity is apparent or to the limit dose of 1000 mg/kg bw. Since only few effects were observed in the preliminary study, the test substance should have been tested at higher dose levels in the subsequent main study but this was not done. The main study was made before the current OECD guideline 416 was adopted and many important parameters of reproductive and developmental toxicity are missing. The NOAEL determined was based on the reduced **female fertility index** observed in females administered 10 000 ppm. This effect is however not considered sufficient for a classification of triazole alanine as being toxic to reproduction. In the teratology study, no teratogenic effects were observed at the limit dose of 1000 mg/kg bw. Although a reproductive study performed in accordance with the current guideline would be desirable it is not justified in this context. Triazole alanine is a plant residue of difenoconazole and the exposure to this substance is presumed to be far below toxic doses during the conditions of the representative use considered in this DAR. Further studies are not required.

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B.6.8.1.4 Toxicology studies of CGA 142856 (triazole acetic acid)

B.6.8.1.4.1 Absorption, distribution, excretion (toxicokinetics)

Single dose (three dose levels) in rat

Reference:	K. Lai, B. Simoneaux, L. 1986d. Balance study of ¹⁴ C-triazole acetic acid in orally dosed rats.
Guideline:	Checked for compliance to OECD TG 417 (4 th April 1984).
Deviations from TG 417:	Test conditions regarding the acclimatisation procedure, randomisation process, temperature, humidity, lighting or composition of diet were not described in the study report. Standard deviations of the mean values were not presented.
GLP:	No
Acceptability:	Yes
Test substance / purity:	[3, 5- ¹⁴ C] ¹⁴ C-1, 2, 4-Triazole Acetic Acid (CL-V-92) / Purity > 99% (TLC)
Species / Strain:	Rat/Albino Sprague-Dawley
Doses / No. of animals:	Low: 0.58 mg/kg bw (22.6 uCi/mg) Mid: 58.63 mg/kg bw (0.227 uCi/mg) High: 1034.7 mg/kg bw (0.027 uCi/mg) Stock solutions of TAA (HCl salt) were prepared for each dose level 2 rats/sex/dose
Administration:	Orally, by gavage Vehicle: water
Exposure time / Duration:	Single dose, 168 h observation period post dose (daily sampling of urine and faeces).

Materials and methods:

The absorption and rate and route of excretion of ¹⁴C-ring labeled triazole acetic acid (TAA) was investigated in rats administered the test substance as a single oral dose. The rate and route of the excretion of radioactivity in urine and faeces was monitored at 24 hour intervals and the uptake of radioactivity in blood, heart, lungs, spleen, kidney, liver, brain, muscle, fat and gonads was analysed seven days post administration. The radioactivity of the urine samples was measured directly in a scintillation counter whereas faeces and tissue samples were homogenized and combusted prior to analysis. Aliquots of 1 mL of the dosing solution were diluted to 100 ml with acetone for the radioassay.

Results:

The main route of excretion was via urine, averaging 95.4% (90.6%, 102.5%, and 93.0% in animals administered a low, medium or high dose) of the radioactivity recovered during seven days post administration (Table B.6.8.1.4.1-1). An average of 3.7% of the dose (3.1%, 4.3%, and 3.8% in animals administered a low,

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medium, and high dose respectively) was recovered in faeces during the same time period. The excretion pattern was approximately similar in all animals irrespective of the dose administered. Within 48 hours, an average of 91.1%, 105.8%, and 94.9% of the total dose was excreted in animals administered a low, medium or high dose respectively.

Table 6.8.1.4.1-1: Excretion of radioactivity (in % of total radioactivity) by rats after a single oral dose of ^{14}C -triazole-acetic acid (mean, n=2)

Treatment	0.58 mg/kg bw		58.6 mg/kg bw		1034.7 mg/kg bw	
Sex	Male	Female	Male	Female	Male	Female
Urine, day 1	88.4	80.6	98.8	101.2	83.8	93.5
Urine, day 2	1.6	5.6	1.6	1.6	2.2	3.0
Urine, day 3	0.5	1.3	0.5	0.4	0.4	0.9
Urine, day 4	0.2	1.5	0.1	0.2	0.2	0.5
Urine, day 5	0.1	0.7	0.3	0.1	0.3	0.4
Urine, day 6	0.1	0.4	0.2	0.1	0.2	0.1
Urine, day 7	0.1	0.2	0.2	0.1	0.2	0.2
Urine, subtotal	91.0	90.3	101.7	103.7	87.3	98.6
Faeces, day 1	4.7	0.6	1.1	6.4	4.1	2.3
Faeces, day 2	0.3	0.3	0.1	0.8	0.4	0.3
Faeces, day 3	0.1	0.1	0.0	0.1	0.1	0.1
Faeces, day 4	0.0	0.1	0.0	0.1	0.0	0.1
Faeces, day 5	0.0	0.0	0.0	0.0	0.0	0.1
Faeces, day 6	0.0	0.0	0.0	0.0	0.0	0.0
Faeces, day 7	0.1	0.1	0.0	0.0	0.0	0.1
Faeces, subtotal	5.2	1.2	1.2	7.4	4.6	3.0
Tissue residues	0.8	0.1	0.1	0.1	3.1	0.2
Cage washes	0.9	12.3	0.4	0.4	1.3	2.1
Total Recovery	97.9	103.9	103.4	111.6	96.3	103.9

The levels of radioactivity in the tissues analysed were mostly at or below the limits of quantitation in all animals irrespective of the dose administered (Table 6.8.1.4.1-2). Elevated levels of TAA equivalents were observed in fat tissue in one male rat administered the low dose, in the spleen of one female rat administered the mid dose and in the testes of one rat administered the high dose. Since the elevated level observed in the testes was only found in one rat (the other rat had levels below the limit of detection) and since the levels were below the limit of detection in the other dose groups, this was considered to be of marginal toxicological relevance.

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Table 6.8.1.4.1-2: Residual radioactivity (in ppm triazole-acetic acid equivalents) in selected tissues of rats at 7 days after a single oral dose (mean, n=2).

Treatment	0.58 mg/kg bw		58.6 mg/kg bw		1034.7 mg/kg bw	
Sex	Male	Female	Male	Female	Male	Female
Plasma	<*0.006	<*0.006	0.68	<*0.59	<*12.06	<*11.57
Red blood cells	<0.002	<*0.005	<0.19	<0.18	<3.78	<*11.88
Fat	0.058	0.008	<0.17	<*0.59	<*11.70	<*10.77
Brain	<*0.006	<0.002	<0.17	<0.19	<*12.00	<*11.68
Muscle	<0.002	<*0.006	<0.19	<0.18	<3.86	<*11.37
Lung	<0.002	<0.004	<0.18	<0.18	<3.98	<*11.40
Heart	<0.002	<0.002	<0.19	<0.18	<*11.46	<3.79
Spleen	<*0.006	<*0.006	<0.17	0.79	<3.80	<*11.17
Kidney	<*0.006	<*0.005	<0.17	<*0.59	<3.89	<*11.11
Liver	0.009	<*0.005	<0.18	<*0.51	<*11.48	<*11.11
Gonads, female		<*0.006		<*0.55		<4.65
Testes	<0.002		<0.19		14.92	

All data were calculated according to the following rules:

When two numbers are less than the limit of detection signified by a (<) symbol, these two numbers are averaged.

When two numbers are less than the limit of quantitation (0.006, 0.55 and 11.00 ppm in low, mid and high dose respectively) signified by a (<*) symbol, these two numbers are averaged.

When one number is less than the limit of detection (<) and one number is less than the limit of quantitation (<*), only the <* number is listed.

When one number is less than the limit of quantitation (<*) or detection (<) and one number is real, only the real number is listed.

The total recoveries of the seven day balance study were 100.7%, 107.2%, and 100.1% in rats administered a low, medium, or high dose of TAA.

Conclusion:

During the conditions used in this study, the absorption and elimination of orally administered TAA was rapid and occurred primarily via the urine. The tissue levels of radioactivity seven days post-administration were low.

Comments:

According to OECD TG 417, the correct way to analyse distribution pattern is to use either whole-body autoradiographic techniques or to measure tissue concentrations at different timepoints after exposure. Tissue distribution was only measured seven days after dosage therefore it is unknown if radioactivity initially showed a high distribution to specific tissues.

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Reference:	K. Lai, B. Simoneaux, L. 1986e. The metabolism of ¹⁴C-triazole acetic acid in the rat.
Guideline:	Checked for compliance to OECD TG 417 (4th April 1984) .
GLP:	No
Acceptability:	Yes
Test substance / purity:	[3, 5- ¹⁴ C]1, 2, 4-Triazole Acetic Acid (CL-V-92) / Purity > 99% (TLC)
Species / Strain:	Rat/Albino Sprague-Dawley
Doses / No. of animals:	Low: 0.58 mg/kg bw (22.6 uCi/mg) Mid: 58.63 mg/kg bw (0.227 uCi/mg) High: 1034.7 mg/kg bw (0.027 uCi/mg) Stock solutions of TAA (HCl salt) were prepared for each dose level. 2 rats/sex/dose
Administration:	Orally, by gavage Vehicle: water
Exposure time / Duration:	Single dose, 168 h observation period post dose (daily sampling of urine and faeces).

Materials and methods:

In the previous balance study (K. Lai, B. Simoneaux, L. 1986d), the primary route of excretion of triazole acetic acid (TAA) was demonstrated to be via urine. The major objective of this study was to characterize the metabolic profile of the excreted TAA in urine. The 0-24 hour urine containing the greatest percentage of recovered radioactivity (regardless of sex) at each dose level was analysed using TLC with five different solvent mixture systems. In order to achieve a more sensitive analysis of the metabolites, the 0-24 hour urine from one of the mid-dose female rats (see comments at end) was purified and analysed using gas chromatography and mass spectrometry. The urine sample was applied to an anion exchange resin (AG-1X8), washed with water and eluted with 1M Formic acid. The fraction was radioassayed and concentrated to dryness. After reconstitution in methanol and derivatisation by ethereal diazomethane, concentrated esterified samples of standard and urine metabolites were analysed by GC/MS. Radioassays were conducted by external standardization using a BacoTracer scintillation counter. The TLC separations were visualised by either development radioautograms or with a spark chamber.

Results:

Only one zone was detected in the autoradiography of TLC plates (data not shown).

The urine moiety consistently had a lower migration rate on TLC than the authentic standard used for dosing (Table 6.8.1.4-3). This effect could possibly be explained by differences in salt forms between dose compound and excreted compound.

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Table 6.8.1.4-3: Migration rates of authentic standard versus urine moiety in several TLC systems.

Compound	Solvent System				
	1	2	3	4	5
TAA	0.77	0.78	0.56	0.83	0.67
Urine Moiety	0.63	0.67	0.25	0.70	0.63

The spectra obtained in the GC/MS analysis of the esterified urine moiety and the esterified authentic standard were almost similar but the spectra of the esterified urine sample showed an additional major ion at m/z 128. The identity of this additional molecule present in urine was not presented in the study report.

Conclusion:

Triazole acetic acid was excreted mainly in unaltered form.

Comments:

In the study report, it is stated that the urine sample used for GC/MS was derived from a male mid-dose rat. However, according to the animal number reported, this is a female rat. The additional peak (m/z=128) observed in the GC/MS of the esterified urine sample was attributed to a co-eluting background peak. However, this was not verified by a GC/MS spectra of a urine sample from an untreated rat.

B.6.8.1.4.2 Acute Oral Toxicity

Reference:	P. Thevenaz (1984). CGA 142856 – Acute oral LD50 in the rat.
Guideline:	Performed in accordance with OECD TG 401 , checked for compliance with OECD TG 401 (24th February 1987) .
Deviations from TG 401:	There was no information regarding the randomisation and acclimatisation procedures included in the study report. Three rats of each sex were used instead of five.
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	CGA 142856 (batch RV-1508/3, 4-7) / Purity: >99%.
Species / Strain:	Rat/Tif:RAIf (SPF)
Doses / No. of animals:	5000 mg/kg bw / 3/sex/dose
Administration:	Orally by gavage Vehicle: distilled water containing 0.5% carboxymethylcellulose and 0.1% polysorbate 80
Exposure time / Duration:	Single dose / Observation period of 14 days post dose.
Expression of results:	Median lethal dose (LD ₅₀)

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Materials and methods:

Triazole acetic acid was orally administered to males and female rats of 7-8 weeks age. The test animals were monitored daily for mortality and clinical signs (twice on weekdays and once on weekends). Body weights were recorded at day 1, 7, 14, and at termination of study. Necropsy was performed on all animals at the end of the study.

Results:

All animals survived treatment. Clinical symptoms such as dyspnoea, exophthalmus, ruffled fur and curved body position were observed during 6-10 days. All symptoms were graded as slight except for dyspnoea and ruffled fur which were graded as moderate during the first five hours of treatment and were thereafter graded as slight. No other unusual symptoms considered to be related to treatment were observed. Body weight gain was reported to be normal (no reference data presented). There were no gross pathological findings.

Conclusion:

During the conditions of the study, the oral LD₅₀ of the test compound in rats was determined to be >5000 mg/kg.

Comments:

In OECD guideline 423, annex 3 (17th December 2001) testing of 5000 mg /kg bw in three animals is acceptable hence the lower number of animals used in this study is considered to be acceptable.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

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B.6.8.1.4.3 Subacute Oral Toxicity

14-day study in rat

Reference:	P. Thevenaz, 1986. CGA 142856 Technical: 14-Day subacute toxicity study in rats (dietary administration).
Guideline:	Performed in accordance with OECD 407 (12th May 1981) , checked for compliance with OECD 407 (27st July 1995) .
Deviations from TG 407:	Performed as a 14 day study. The thymus, spleen and heart were not weighed. Lymph nodes (mesenteric and axillary), sternum with bone marrow, trachea, prostate and uterus were preserved but not evaluated histopathologically. The blood clotting time, sodium, potassium and creatinine levels were not analysed. Reactivity to stimuli and assessment of grip strength and motor activity in the fourth exposure week was not conducted.
GLP:	Yes
Acceptability:	Yes (see comments) This study is not considered to be relevant to the assessment considering the representative uses supported by the dossier.
Test substance / purity:	CGA 142856 (batch RV 1508/8) / Purity: 99 %.
Species / Strain:	Rat/Tif:RAIf (SPF) rats
Doses / No. of animals:	0, 100, 1000 and 8000 ppm (equivalent to 10.6, 102.8, 788.3 mg/kg bw (males) and 10.1, 97.2, 703.5 mg/kg bw (females) 5/sex/dose level.
Administration:	Orally, in diet.
Exposure time / Duration:	Daily dose / Observation period of 14 days post dose.

Materials and methods:

Triazole acetic acid (TAA) was administrated daily via the diet to rats of approximately 4-5 weeks of age during two weeks.

The animals were acclimatised eight days prior to study and during this period they were weighed and subjected to the first eye and hearing examinations (day -6).

During treatment, the animals were observed daily for mortality and clinical signs of treatment. Body weights and food/water consumption were recorded weekly. The second eye and hearing examination was performed on day 12. At the termination of the study, an analysis of haematology, blood chemistry and organ weights was performed on all rats whereas only untreated rats and rats administered a high dose of TAA were subjected to a histopathological examination. A uni-variate statistical analysis was performed for each time point and parameter.

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Results:

An analysis of the homogenised and pelleted food showed that all samples were in agreement with the nominal concentrations. All animals survived treatment and there were no clinical symptoms or signs indicative of systemic toxicity observed during the study. The ophthalmic inspections and hearing examinations performed before and towards the end of the treatment revealed no evidence of a reaction to the treatment. The mean food consumption and the mean body weight values were similar between treated and untreated rats. There were no changes in hematology or clinical chemistry parameters observed that were attributable to the treatment. A significantly reduced level of reticulocytes was observed in females administered the high dose but this was not considered to be an adverse effect since the reduction was only observed in females at this dose level and no other blood parameters were affected. Analyses of organ weights and organ weight ratios revealed a significant negative trend from control to the highest dosage group in males regarding the liver to body ratio in males. This decrease was $\leq 10\%$ and since it was not accompanied by any histopathological findings and only present in males, it was not considered to be an adverse effect. There were no gross pathological findings or microscopic lesions observed that were considered to be related treatment.

Conclusion:

The NOAEL of CGA 142856 is >8000 ppm (788 and 704 mg/kg/bw/day for males and females respectively) based on the absence of any adverse findings at this dose level. The NOEL is 1000 ppm (103 and 97 mg/kg/bw/day for males and females respectively) based on the reduced liver/body weight ratio observed at 8000 ppm.

Comments:

The study was performed before the current TG 407 was adopted hence many parameters are missing and a full toxicological profile was not achieved. However, triazole acetic acid is a plant residue of difenoconazole and the exposure to this substance is presumed to be far below toxic doses during the conditions of the representative use considered in this DAR. Further studies are not required.

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B.6.8.1.4.4 Genotoxicity

Bacterial Reverse Mutation Test

Reference:	E. Deparade (1984). <i>Salmonella/mammalian-microsome mutagenicity test</i>.
Guideline:	Checked for compliance with OECD Guideline 471 (21st July 1997) .
Deviations:	There was no information regarding strain characteristics or the bacterial titer included in the study report neither was the presence of a background lawn confirmed. A strain able to detect cross-linking mutagens (<i>E. coli</i> WP2 <u>uvrA</u> , <i>E. coli</i> WP2 <u>uvrA(pKM101)</u> or <i>S. typhimurium</i> TA 102) was not included in the test. Positive controls in assays performed with metabolic activation was only included in the test of strain TA 98.
GLP:	Yes
Acceptability:	Yes (see comments)
Test substance / purity:	CGA 142856 (batch RV-1508/3, 4-7) / purity > 99%. Vehicle: Sodium phosphate buffer
Bacterial strains:	<i>Salmonella typhimurium</i> / TA98, TA100, TA1535, and TA1537.
Doses:	Mutagenicity test (original and confirmatory): 20, 80, 320, 1280, and 5120 ug/plate. +/- microsomal activation.
Exposure time:	48 hours at 37 °C

Materials and methods:

The mutagenic potential of triazole acetic acid (TAA) was investigated in the histidine-auxotrophic strains of *Salmonella typhimurium*: TA98, TA100, TA1535, and TA1537 in the presence and absence of a metabolic activation system (S9 fraction). The highest concentration applied was determined in a preliminary toxicity test (data not shown). Triplicate plates of all concentrations were tested both in the original and in the confirmatory test. The ability and adequacy of the test system to detect mutagenic activity was confirmed by positive control chemicals (see comments at end) and sodium phosphate buffer served as negative control.

Results:

The number of revertant colonies was comparable between the treated and untreated plates whereas an increase of the number of revertant colonies was observed in plates containing the positive control substances.

Conclusions:

CGA 142856 was not mutagenic during the conditions used in this assay.

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Comments:

Despite the lack of information regarding the titer used, the RMS considers that the titer was sufficient since the positive controls used showed results as expected.

***In vitro* Mammalian Cell Gene Mutation Test**

Reference:	Clare, G. (2002): Triazolyl Acetic Acid: Mammalian Cell Mutation Assay
Guideline:	Performed to comply with OECD 476 (21st July 1997) , US EPA (1998, EPA 712-C-98-221) and Commission Directive 2000/32/EC Annex 4E-B17 . Checked for compliance with OECD 476 (21st July 1997) .
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	1H-1, 2, 4 triazol-1-yl acetic acid (batch: FCT/T/197-01 (ex 20689/17) Purity 96.95%.
Cell line:	L5178Y mouse lymphoma cells (3.7.2c)
Doses:	Cytotoxicity test (+/- microsomal activation): 0.078, 0.156, 0.313, 0.63, 1.25, 2.5, 5, 10 mg/ml. Mutagenicity tests I and II (+/- microsomal activation): 0.63, 1.25, 2.5, 5, 10 mg/ml. Dissolved in water
Exposure time:	3 hours with and 24 hours without microsomal activation at 37 °C. Phenotypic expression time: 48 hours Incubation for cloning efficiency: 7 days Incubation for mutant frequency: 10-14 days

Materials and methods:

Two sets of independent mutagenicity studies were performed (I and II) in L5178Y mouse lymphoma cells to elucidate any mutagenic potential of Triazole acetic acid (TAA). The highest concentration applied was determined in a preliminary cytotoxicity test. Duplicate samples of cell suspensions were exposed to different concentrations of TAA, in presence or in absence of metabolic activation, during three or 24 hours. After exposure, the cells were washed once, resuspended and the cell density was measured. Fractions of the cell suspensions were plated to assess Day₀ relative survival and the remaining cell suspensions were diluted and further incubated for phenotypic expression and an assessment of the cloning efficiency (Day₂). The cultures were sampled after 24 and 48 hours to assess growth in suspension. The mutation frequency was calculated from the ratio of colonies formed in media with/without addition of the thymidine analogue trifluorothymidine (TFT). MMS and MC were included as positive controls in the absence and presence of metabolic activation respectively while water served as a negative control.

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Results:

Both tests showed that the treatment with TAA did not cause an increased mutation frequency compared to the negative control either in presence or absence of metabolic activation. The positive controls caused increased mutation frequencies that were statistically significant. The mean relative cell survival was high in both main tests performed.

Conclusion:

Triazole acetic acid did not induce gene mutations in L5178Y mouse lymphoma cells.

Comments:

The positive and negative controls included as historical control data are not identified. Since the report has been checked by a quality assurance unit, the RMS assumes that the historical data is relevant for the TAA study.

In Vitro Mammalian Chromosome Aberration Test in Human Lymphocytes

Reference:	L. Pritchard (2002): Triazole Acetic Acid: In Vitro Mammalian Chromosome Aberration Test
Guideline:	Performed to comply with OECD 473 (21st July 1997) , US EPA (1998, EPA 712-C-98-223) , Commission Directive 2000/32/EC Annex 4A-B10 , ICH (1995) Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests and ICH (1995) Genotoxicity: A standard Battery of Genotoxicity Testing of Pharmaceuticals. Checked for compliance with OECD 473 (21st July 1997).
Deviations from TG 473:	The stability of the test substance and of the test substance in solution was not performed nor was an analysis of the concentration achieved performed.
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	1H-1, 2, 4 triazol-1-yl acetic acid (batch: FCF/T/197-01 (ex 20689/17)) Purity 96.95%.
Cell line:	Human lymphocytes
Doses:	First and second test (+/- microsomal activation): Mitotic index: 0, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 mM Metaphase analysis: 2.5, 5 and 10 mM Dissolved in water
Exposure time:	First test (+/- microsomal activation): 3 hours treatment and 17 hours recovery Second test (- activation): 20 hours continuous treatment Second test (+ activation): 3 hours treatment and 17 hours recovery

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Materials and Methods:

The ability of Triazolyl Acetic Acid (TAA) to induce chromosomal aberrations was tested in human lymphocytes cultured *in vitro*. Pooled blood taken from healthy male non-smoking donors was incubated in medium supplemented with PHA to stimulate cell divisions. After 48 hours of growth, different concentrations of the test substance or positive/negative controls were added to cultures. Duplicate cultures were used at each concentration and in controls. Cells were exposed to the test substance during three hours and then incubated in fresh medium for 17 hours. Two hours before harvest, the mitotic inhibitor Colcemid[®] was added to arrest the cells at metaphase. The cell pellets were treated with a hypotonic solution, fixed and stained with Giemsa. In the second experiment, the cells were exposed continuously (20 hours) until harvest in the assays performed without activation. The proportion of mitotic cells per 1000 cells in each culture was recorded and 100 metaphase cells per culture were examined (50 in the positive controls). Chromosome aberrations were scored according to the classification of the ISCN (1985) and the incidence of polyploidy metaphase cells per 500 metaphase cells was determined for negative control cultures and the cultures treated with the highest dose. The number of aberrant metaphase cells was compared with the solvent control using Fischer's test.

Results:

The cytotoxicity of TAA was low according to the mitotic indices obtained. In the absence of metabolic activation, the mitotic indices in the treated cultures was reduced to 82 and 71% in the first and the second experiment respectively. In the metaphase analysis, the mean number of cells with aberrations (including gaps) was slightly elevated in cultures treated with the highest dose (3.5% compared to 1.5% in control). However, this effect was not statistically significant and according to the historical data included, the incidence (3.5%) was identical to the mean value of the negative control.

In assays performed without activation, chromosome breaks (csb) were observed in cells treated with TAA both in the first and in the second experiment. These effects were not statistically significant and they were only seen in one of the duplicate cultures analysed; 2 csb/100 cells in one culture exposed to 10 mM TAA (first experiment) and 1 csb/100 cells in one culture exposed to 5 mM and in one exposed to 10 mM TAA (second experiment).

Conclusion:

TAA did not show evidence of clastogenic activity during the experimental conditions used in this assay.

Comments:

It is unclear if fresh preparations of the test substance was used in the experiments.

It is further unclear if the test conditions and the controls used in the historical data were identical to those used in the TAA experiments. Since the report has been checked by a quality assurance unit, the RMS assumes that the historical data is relevant for the TAA study.

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B.6.8.1.5 Toxicology studies of CGA 205374**B.6.8.1.5.1 Acute Oral Toxicity**

Reference:	Ohba, K (1991a). Acute oral toxicity study of CGA 205374 in mice.
Guideline:	Performed in accordance with MAFF, Japan (59 NohSan No 4200) , checked for compliance with OECD TG 401 (24th February 1987) .
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	CGA 205374 tech. (batch 9106054) / purity: 99%. Vehicle: 0.5% CMC-Na
Species / Strain:	Mouse / Slc:ICR (~6 weeks old)
Doses / No. of animals:	5000 mg/kg bw / 5/dose and control group/sex
Administration:	Orally by gavage
Exposure time / Duration:	Single dose / Observation period of 14 days post dose.

Materials and Methods:

Vehicle or CGA 205374 tech. was administered to groups of male and female mice as a single dose. The dose level used was selected from a dose-finding study (data not shown). The animals were checked for mortality and clinical symptoms at 10 min, 20 min, 30 min, at 1, 2, 3, 4, 5 and 6 hours after treatment and thereafter daily during 14 days. Body weights were recorded immediately before treatment and on test days 3, 7, 10 and 14. Differences in mean values between treated and control groups were analysed statistically by Student's t-test. Necropsy was performed on all animals.

Results:

All animals survived treatment with CGA 205374 tech. and no clinical signs were observed. The treatment had no effect on body weights and there were no deviations from normal morphology found at necroscopy.

Conclusion:

The acute oral LD50 of CGA 205374 was > 5000 mg/kg in male and female mice.

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B.6.8.1.5.2 Genotoxicity

Bacterial Reverse Mutation Test

Reference:	Nakajima, M (1991b). Reverse mutation assay of CGA-205374.
Guideline:	Performed in accordance with MAFF, Japan (59 NohSan No 4200) , checked for compliance with OECD TG 471 (21st July 1997) .
Deviations from TG 471:	In this test, duplicate platings were used instead of triplicates. This is considered to be of low concern since the variation between the duplicates was low. The exclusion of a confirmatory experiment was not justified.
GLP:	Yes
Acceptability:	Yes (see comments)
Test substance / purity:	CGA 205374 tech. (batch 9106054) / purity 99.3%. Vehicle: Dimethylsulfoxide (DMSO).
Bacterial strains:	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 and <i>E. coli</i> WP2uvrA
Doses:	Toxicity test: 51.2-5000 µg /plate +/- microsomal activation. Mutagenicity test : 156, 313, 625, 1250 and 2500µg/plate +/- microsomal activation.
Exposure time:	48 hours at 37 °C

Materials and methods:

The mutagenicity of CGA 205374 tech. was investigated in four histidine-auxotrophic strains of *Salmonella typhimurium* and the tryptophan-auxotrophic strain *E. coli* WP2uvrA in absence and in presence of metabolic activation (S9 mix). The highest concentration applied was determined in a preliminary toxicity test. Duplicate plates were prepared according to the pre-incubation method and incubated 48 hours at 37°C. Thereafter they were evaluated by colony counting and the presence of a background lawn was confirmed. Positive controls were included in the test in order to demonstrate the sensitivity of the test system and solvent was used as a negative control.

Results :

Cell death was not observed in the preliminary toxicity test. However, the mixture became cloudy after adding the test substance solution at concentrations ≥ 128 µg/plate and a precipitate of the test substance was observed on the agar plates at ≥ 320 µg/plate without activation and ≥ 800 µg/plate with activation. Due to the precipitation, the background lawn was difficult to observe at 2000 µg/plate without activation and at 5000 µg/plate with activation. Despite this, 5000 µg/plate was selected as highest concentration for the main study.

In the main reversion test, cell death was only observed in strain TA 1537 at the highest dose level tested and there was no increased incidence of revertant colonies observed in any of the strains tested, with or without metabolic activation. However, cloudiness was observed at preincubation already in the lowest dose without

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activation and at 313 µg/plate with activation. The precipitate of the test substance was evident on the agar plates at 313 µg/plate and above with or without activation.

Conclusion:

In this study, CGA 205374 did not increase the reversion frequency in the *S. typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 or in the *E. coli* strain WP2 uvrA.

Comments:

Due to the insolubility and precipitation of test substance on agar plates it is unclear if bacteria have been exposed to the concentration intended or not. However, CGA 205374 is a postulated metabolite formed in the mammalian metabolism of difenoconazole hence a potential mutagenicity would most likely have been discovered in the mutagenicity tests of difenoconazole.

The positive controls used in assays without metabolic activation are not the strain-specific examples given in the guideline. In this study, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide was used in assays of *S. typhimurium* TA 98,100 and *E. coli* WP2 uvrA whereas 1-ethyl-3-nitro-1-nitrosoguanidine was used for TA 1535. Although no historical data was included, the controls are considered acceptable since they induced a high amount of revertant colonies. 2-Aminoanthracene was used as the sole indicator of the efficiency of the S9 mix.

B.6.8.1.6 Toxicology studies of CGA 205375

B.6.8.1.6.1 Acute Oral Toxicity-mouse

Reference:	Ohba, K (1991b). Acute oral toxicity study of CGA 205375 in mice.
Guideline:	Performed in accordance with MAFF, Japan (59 NohSan No 4200) , checked for compliance with OECD TG 401 (24th February 1987) .
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	CGA 205375 (batch 9106055) / purity: 99%. Vehicle: 0.5% CMC-Na
Species / Strain:	Mouse / Slc:ICR (~ 6 weeks old)
Doses / No. of animals:	0, 1000, 1300, 1600, 2000, 2500 mg/kg bw / 5 males/dose
Administration:	Orally by gavage
Exposure time / Duration:	Single dose / Observation period of 14 days post dose.
Expression of results:	Median lethal dose (LD ₅₀)

Materials and Methods:

A single dose of CGA 205375 or vehicle was administered to male mice. The dose levels used were selected from a dose-finding study (data not shown) in which both mice administered a dose of 2500 mg/kg died but none of the animals administered a dose of 1000 mg/kg did. Mortality and clinical symptoms were recorded at 10 min,

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20 min, 30 min and 1, 2, 3, 4, 5 and 6 hours after treatment and thereafter at daily intervals for a total of 14 days. The body weights of the animals were recorded immediately before treatment and on test days 3, 7, 10 and 14 and when animals were found dead. Differences in mean values between surviving animals of treated and control groups were analysed statistically by Student's t-test. Necropsy was performed on all animals when found dead or at the end of study. The LD₅₀ value was calculated from the cumulative number of deaths at the end of the observation period by the Probit method.

Results:

All animals in the control-group and in the 1000, 1300 or 1600 mg/kg groups survived treatment whereas two and three animals died in the 2000 and 2500 mg/kg groups respectively (Table 6.8.1.6.1-1). The clinical signs noted on the day of treatment included reduced locomotor activity in all treated animals, prone position in most animals administered 1300, 1600, 2000 and 2500 mg/kg, crawling in one or two animals administered 1300, 1600 or 2000 mg/kg animals and lateral position in one to three of the animals administered 2000 or 2500 mg/kg. Decreased movement and prone and/or lateral position were still seen in a few animals on the first or second day after treatment but all surviving animals appeared normal by the third day. The mean body weights were reduced in animals administered 2000 and 2500 mg/kg on the third day of study but returned to normal by the tenth day. The necropsy examinations revealed haemorrhage of the glandular stomach in all animals that died and a thickening of the anterior stomach was observed in nearly all of the treated animals that survived.

Table 6.8.1.6.1-1: Mortality in male mice after single oral administration of CGA 204375

Dose (mg/kg)	Males	
	dead/treated	time to death
0	0/5	-
1000	0/5	-
1300	0/5	-
1600	0/5	-
2000	2/5	day 2 and day 3 after dosing
2500	3/5	day of dosing (1), day 1 after dosing (2)

Conclusion:

The acute oral LD₅₀ of CGA 205375 was 2309 mg/kg (C.I : 1911-4118) in male mice. Classification is not required according to the Council Directive 67/548/EEC.

WARNING: This document forms part of an EC evaluation data package and should not be used in isolation. Registration must not be granted on the basis of this document.

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B.6.8.1.6.2 Genotoxicity

Bacterial Reverse Mutation Test

Reference:	Nakajima, M (1991c). Reverse mutation assay of CGA-205375.
Guideline:	Performed in accordance with MAFF, Japan (59 NohSan No 4200) , checked for compliance with OECD TG 471 (21st July 1997) .
Deviations from TG 471:	There is no information regarding the bacterial titre but this is considered to be of low concern since the presence of a background lawn was confirmed. Duplicate platings were used instead of triplicates but as the degree of variation between the duplicates was low, this is considered to be of low concern. In this study, 2-Aminoanthracene was used as the sole indicator of the efficiency of the S9 mix. The exclusion of a confirmatory experiment was not justified.
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	CGA 205375 (batch 9106055) / purity 99.8%. Vehicle: Dimethylsulfoxide (DMSO).
Bacteria / Strain:	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 and <i>E. coli</i> WP2uvrA
Doses:	Toxicity test: 51.2-5000 µg /plate +/- microsomal activation. Mutagenicity test : Without microsomal activation: 2.5-80 µg /plate. With microsomal activation : TA 98: 2.5-80 µg /plate TA 100, TA 1535: 5-160 µg /plate TA 1537, <i>E. coli</i> : 10-320 µg /plate
Exposure time:	48 hours at 37 °C

Materials and methods:

CGA 205375 was tested for mutagenicity in four histidine-auxotrophic strains of *S. typhimurium* and the tryptophan-auxotrophic strain *E. coli* WP2uvrA. All assays were performed with and without metabolic activation (S9 mix). The highest concentration applied was determined in a preliminary toxicity test. After preparation using the pre-incubation method, the plates were incubated during 48 hours at 37°C. The plates were evaluated by colony counting and the presence of a background lawn was confirmed. Positive controls were included in the test in order to demonstrate the sensitivity of the test system.

Results :

In the preliminary toxicity test performed with all strains, cell death was observed at all concentrations, with or without activation. The mixture became cloudy upon addition of the test substance solution at a concentration of

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5000 µg/plate and a precipitate of the test substance was observed at doses ≥ 2000 µg/plate and ≥ 800 µg/plate with or without activation respectively.

In the main reversion test, cell death was observed at 80 µg/plate in assays of all strains performed without activation (in TA 100 also at 40 µg/plate). In assays performed with activation, cell death was observed at the two highest doses tested in strains TA 100, TA 98 and TA 1537 and in the highest dose tested in TA 1535 and *E. coli* WP2uvrA. The results showed no increased incidence of back mutations indicative of a mutagenic response in any of the strains tested, with or without metabolic activation.

In this study, CGA 205375 did not increase the reversion frequency in the *S. typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 or *E. coli* strain WP2 uvrA.

Comments:

The cytotoxicity of CGA 205375 only allowed low concentrations of the substance to be tested. However, CGA 205375 is a metabolite formed in the mammalian metabolism of difenoconazole hence its potential mutagenicity would most likely have been discovered in mutagenicity tests of difenoconazole.

B.6.8.2 Supplementary studies on the active substance

B.6.8.2.1 Exploratory short-term study in male mice

Reference:	Thomas, H. (1992). The effect of CGA 169374 tech. on selected biochemical and morphological liver parameters following subchronic administration to male mice.
Guideline:	No specific guideline
GLP:	No
Acceptability:	As supportive information
Test substance / purity:	Difenoconazole (CGA 169374) technical (batch P. 807002) / purity 91.8%
	Vehicle:
Species / Strain:	Mouse/ Tif:MAGf (SPF)
Doses/ No of animals:	0, 1, 10, 100 and 400 mg/kg bw / 9 /dose level (6 or 3 in controls).
Administration:	Orally (gavage): difenoconazole treatment and reference compound nafenopin Intraperitoneally: reference compounds phenobarbitone (PB) and 3-methylcholanthrene (3-MC)
Exposure time:	14 days
	Reversibility was studied in animals administered 400 mg/kg bw during 14 days followed by a four-week recovery period.

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Materials and methods:

Groups of male mice were administered either the test substance or the reference compounds phenobarbitone (PB), 3-methylcholanthrene (3-MC) or nafenopin (NAF) during two weeks. Animals were checked for clinical signs or reactions to treatment prior to administration, between two and four hours post-administration and twice a week during the recovery period. The body weights were recorded daily during the treatment period and twice per week during the recovery period.

Table B.6.8.2-1

Group	Test material	Vehicle	Route	Dose (mg/kg)	n for biochemistry	n for electron microscopy	Treatment
A	none	CMC	gavage	0	6	3	14 d
B	none	CMC	gavage	0	6	3	14 d + 28 d recovery
C	difenoconazole	CMC	gavage	1	6	3	14 d
D	difenoconazole	CMC	gavage	10	6	3	14 d
E	difenoconazole	CMC	gavage	100	6	3	14 d
F	difenoconazole	CMC	gavage	400	6	3	14 d
G	difenoconazole	CMC	gavage	400	6	3	14 d + 28 d recovery
H	phenobarbitone	saline	i.p	40	6	0	4 d
I	3-methylcholanthrene	corn oil	i.p	80	6	0	2 d
J*	nafenopin	none	i.p	100	-	-	4 d
K	nafenopin	CMC	gavage	50	3	0	6d

*4/6 animals died on the third or fourth day; group discontinued and replaced by group K

After administration of the last dose, the animals were fasted during 16 hours (32 hours in animals administered 3-MC) and thereafter sacrificed. The livers from six animals/group were weighed and then frozen prior to biochemical analyses. Frozen samples were thawed and ultracentrifuged in order to obtain microsomal and cytosolic fractions and the following biochemical parameters were determined:

- protein content of supernatant, microsomal and cytosolic fractions
- microsomal cytochrome P-450 content
- immunoblot analyses using monoclonal antibodies directed toward the CYP-450 isoenzymes (CYP2B1, CYP2B2, CYP1A1, CYP1A2, CYP2C1, CYP4A1, CYP4A2, CYP4A3, CYP3A1, CYP3A2)
- microsomal 7-ethoxyresorufin O-de-ethylase (EROD) and 7-pentoxaresorufin O-depentylase (PROD) activities
- regio and stereoselective microsomal testosterone hydroxylation
- cyanide-insensitive peroxisomal β -oxidation
- microsomal hydroxylation of lauric acid
- microsomal epoxide hydrolase activity
- microsomal UDP-glucuronosyltransferase activity
- cytosolic glutathione S-transferase activity
- spectral interaction of difenoconazole with microsome CYP 450

Liversamples from the remaining 3 animals/group (difenoconazole) were fixed, sectioned and stained for electron microscopy. Differences between treated and untreated groups was assessed statistically using Dunnett's pairwise comparison.

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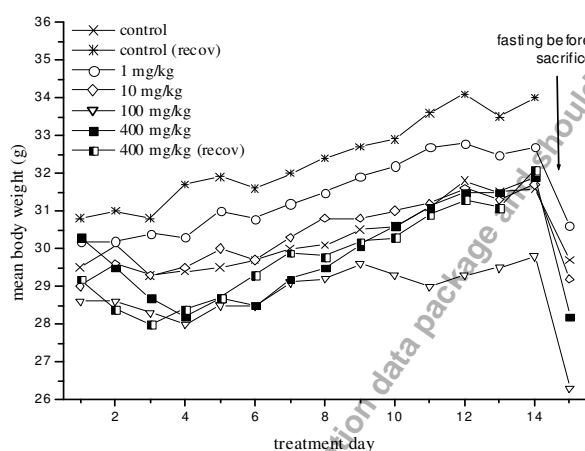
Results:

Analyses of the different dosing suspensions of difenoconazole showed concentrations close to the nominal values except for the 1 mg/kg suspensions which were up to 3-fold higher than intended. All suspensions were relatively stable during a period of four hours.

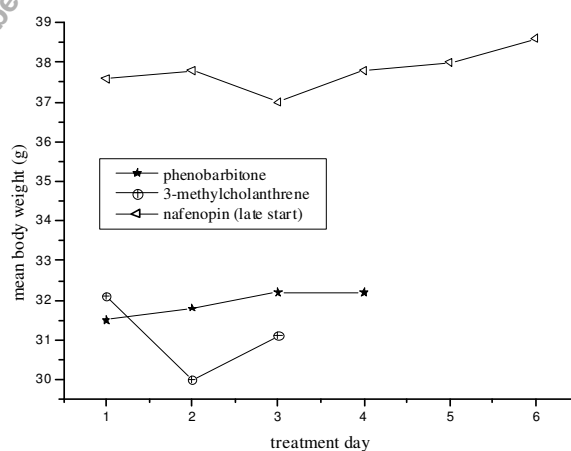
Five animals died during the study due to dosing accidents (one in the control group, two in the control-recovery group, one in the difenoconazole/400 mg/kg group and one in the difenoconazole-recovery/400 mg/kg group). These deaths occurred between the second and fourth day of the study and they were replaced with substitute animals that were treated for the rest of the experimental period. An additional control group animal died spontaneously on the day of sacrifice. Since four of the animals treated intraperitoneally with NAF (100 mg/kg bw) died during the third or fourth day of the study, this group was discontinued and a group of three animals was instead treated orally with a lower dose (50 mg/kg bw) of NAF.

There were no remarkable clinical signs observed and the body weight gains in the difenoconazole-treated mice were not markedly different from the control-group mice (Figure B.6.8.2-1). The body weights of mice administered 100 mg/kg/day were slightly lower compared to the control animals but in the absence of a dose-response, this decrease is not considered to be related to treatment.

Figure B.6.8.2-1: Body weights, exploratory short-term study in male mice
difenoconazole



reference substances



The liver weight analysis revealed a marked (+79%) increase in animals treated with 400 mg/kg bw of difenoconazole compared to the control animals (Table B.6.8.2-1). However, after the 28-day recovery period, the liver weights of control and 400 mg/kg/day mice were equivalent. The short treatment with the reference compounds 3-MC and NAF also resulted in significantly higher liver weights.

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Table B.6.8.2-1: Liver weights in male mice treated with difenoconazole, PB, 3-MC or NAF

test substance	difenoconazole					difenoconazole		PB	3-MC	NAF
sacrifice day	day 15					day 43		day 5	day 4	day 7
dose level (mg/kg/day)	control	1	10	100	400	control	400	40	80	50
body (carcass)	29.7	30.6	29.2	26.3*	28.2	34.8	34.5	29.5	28.3	35.0
liver	1.17	1.25	1.34	1.32	2.09***	1.41	1.40	1.33	1.50***	2.20***

All weights in grams

* = $p \leq 0.05$, *** = $p \leq 0.001$, two-sided Dunnett test

The protein content of the liver supernatant fractions were not affected by treatment with difenoconazole or the reference substances (Table B.6.8.2-2). There was a dose-dependent increase in the protein content of the microsomal liver fractions in mice treated with difenoconazole at 100 mg/kg/day and 400 mg/kg/day but this increase was no longer apparent in animals experienced a 28-day recovery period. An increased microsomal protein content was also observed in animals treated with the reference substances.

Table B.6.8.2-2: Protein parameters, exploratory short-term study in male mice with difenoconazole

test substance	difenoconazole					difenoconazole		PB	3-MC	NAF
sacrifice day	day 15					day 43		day 5	day 4	day 7
dose level (mg/kg/day)	control	1	10	100	400	control	400	40	80	50
supernatant protein (mg/g liver)	192	189	199	188	171	193	195	196	188	205
microsomal protein (mg/g liver)	27.2	28.5	28.1	33.9**	39.6***	33.3	28.9	34.1**	33.8**	41.3***
cytosolic protein (mg/g liver)	100.2	98.6	97.3	92.3	91.7	106.6	101.4	99.3	90.6	98.2

** = $p \leq 0.01$, *** = $p \leq 0.001$, two-sided Dunnett test

The microsomal cytochrome P-450 content was significantly elevated in mice treated with 100 mg/kg/day and 400 mg/kg/day difenoconazole as well as in mice treated with the PB and 3-MC. The levels of cytochrome P-450 returned to the control level after the 28-day recovery period (Table B.6.8.2-3).

Table B.6.8.2-3: Microsomal cytochrome P-450 content and gene families, exploratory short-term study in male mice with difenoconazole

test substance		difenoconazole					difenoconazole		PB	3-MC	NAF
sacrifice day		day 15					day 43		day 5	day 4	day 7
dose level (mg/kg/day)		control	1	10	100	400	control	400	40	80	50
cytochrome P-450 (nMol/g)		18.5	17.5	16.9	48.2***	78.3***	15.1	15.3	33.3***	45.8***	26.0
antibody	P-450 gene family										
MAB d15	CYP1A	100 ^a	95	109	123	135	100	118	143	4532	61
MAB be4	CYP2B	100	88	62	51	57	100	97	95	66	72
MAB p6	CYP3A	100	80	63	215	416	100	91	387	67	327
MAB clo4	CYP4A	100	63	49	47	83	100	78	67	50	910

^a units are Relative Area Units, from densitometric scans of single Western blots (samples pooled)

*** = $p \leq 0.001$, two-sided Dunnett test

The western blot analysis showed enhanced levels of the CYP1A and CYP3A families in mice administered 100 or 400 mg/kg difenoconazole and a dose dependent decrease in the level of CYP2B isoenzymes (Table B.6.8.2-3). A similar profile was obtained with PB but not with 3-MC and NAF which induced the CYP1A and CYP4A families 45-fold and 9-fold respectively.

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The microsomal epoxide hydrolase activity was markedly elevated in mice administered 100 and 400 mg/kg/day difenoconazole compared to the control animals (Table B.6.8.2-4) but after the 28-day recovery period, these values had returned to normal. A similar profile was obtained with PB and NAF but not with 3-MC.

The EROD (7-ethoxyresorufin O-de-ethylase) and PROD (7-pentoxaresorufin O-depentylase) activities were increased approximately 4 and 33-fold in a dose-related manner in mice treated with 400-mg/kg difenoconazole in similar to treatment with PB. In contrast to mice treated with difenoconazole, much greater changes were noted in EROD than PROD in mice treated with 3-MC. All changes were reversible after the 28-day recovery period (Table B.6.8.2-4).

Table B.6.8.2-4: Enzyme activities, exploratory short-term study in male mice with difenoconazole

test substance	difenoconazole					difenoconazole	PB	3-MC	NAF	
sacrifice day	day 15					day 43	day 5	day 4	day 7	
dose level (mg/kg/day)	control	1	10	100	400	control	400	40	80	50
microsomal epoxide hydrolase	61.8	73.7	58.6	92.8	213.7***	68.3	60.0	132.9** *	71.5	205.9***
microsomal morphine UDP-glucuronosyl transferase	684	797	724	882	1090	669	715	1021	972	994
microsomal 1-naphthol UDP-glucuronosyl transferase	815	897	972	1013	983	914	911	1478***	1245***	1791***
microsomal 7-ethoxyresorufin O-de-ethylase (EROD)	2.27	2.98	3.56	8.76***	7.52***	4.17	2.59	7.67***	105***	3.55
microsomal 7-pentoxresorufin O-depentylase (PROD)	0.89	1.99**	2.14**	17.26***	29.78***	1.07	1.05	14.4***	2.34***	1.47
cytosolic glutathione S-transferase	129.4	137.8	117.2	133.5	182.6	153.6	167.6	179.5	98.0	164.0

units are nMol/min/g liver

** = $p \leq 0.01$, *** = $p \leq 0.001$, two-sided Dunnett test

The level of total testosterone hydroxylation, which is dependent on cytochrome P-450, was induced in a dose-related manner to about 6-fold the control level in mice treated with 400 mg/kg difenoconazole (Table B.6.8.2-5). Testosterone hydroxylation rates were also significantly increased with all three reference compounds. Except for 7 α -hydroxy-testosterone, all testosterone metabolites were increased between 3 and 120 fold in mice treated with 400mg/kg of difenoconazole compared to the control animals. The increases of 6 β - and 15 β -hydroxy-testosterone indicate either a strong barbiturate- or steroid-type induction. Since the profiles of the testosterone metabolites were different in magnitude and distribution in animals treated with 3-MC and NAF it is indicated that difenoconazole is not likely to be a 3-MC- or NAF-type inducer.

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Table B.6. 8.2-5 Testosterone hydroxylation

test substance	difenoconazole					difenoconazole		PB	3-MC	NAF
sacrifice day	day 15					day 43		day 5	day 4	day 7
dose level (mg/kg/day)	control	1	10	100	400	control	400	40	80	50
Total activity	82	114	117*	407***	544***	107	97	281***	124**	370***
2 β -hydroxy-testosterone	10	6.2	5.3*	20**	33***	9.5	4.7**	15	7.0	22
6 β -hydroxy-testosterone	23	43**	38*	194***	288***	38	33	150***	32	220***
15 β -hydroxy-testosterone	2.4	4.6**	4.1*	15***	23***	3.6	4.0	11***	4.0*	15***
16 β -hydroxy-testosterone	3.9	1.7	3.0	6.4	8.9*	3.3	1.7	4.0	3.9	3.9
2 α -hydroxy-testosterone	1.4	2.5	1.7	4.8	5.8**	3.3	1.8	2.0	1.0	1.7
6 α -hydroxy-testosterone	3.3	5.9***	7.6***	26***	29***	4.1	6.2*	16***	7.3***	6.8***
7 α -hydroxy-testosterone	9.6	10	11	17	15	9.6	8.8	17	9.1	20*
16 α -hydroxy-testosterone	6.3	7.7	7.7	16***	19***	6.4	6.7	13***	14**	24***
androstenedione	17	24*	32**	91***	105***	21	23	41***	37***	41***
unidentified test. metabolite	5.2	7.7	7.0	18***	19***	8.3	8.5	13***	9.0**	17***

units are nMol/g liver/min

* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, two-sided Dunnett test

Mice treated with 100 and 400 mg/kg/day of difenoconazole showed approximately a 2-fold increased lauric acid 11-hydroxylation whereas lauric acid 12-hydroxylation decreased at all dose levels (Table B.6.8.2-6). Mice treated with PB also showed increased lauric acid 11-hydroxylation whereas the 12-hydroxylation remained unchanged. The reference compound 3-MC did reduced the 12-hydroxylation, while NAF increased both 11- and 12-hydroxylation markedly.

Table B.6.8.2-6 Lauric acid hydroxylation, exploratory short-term study in male mice with difenoconazole

test substance	difenoconazole					difenoconazole		PB	3-MC	NAF
sacrifice day	day 15					day 43		day 5	day 4	day 7
dose level (mg/kg/day)	control	1	10	100	400	control	400	40	80	50
lauric acid 11-hydroxylase	24.1	25.9	26.2	43.0***	55.4***	20.6	18.4	48.5***	28.1	83.7***
lauric acid 12-hydroxylase	45.8	23.5**	14.9***	28.4	37.8	19.3	15.6	43.9	23.4**	391***

units are nMol/min/g liver

** = $p \leq 0.01$, *** = $p \leq 0.001$, two-sided Dunnett test

A slight and dose-dependent decrease of the Cyanide-insensitive peroxisomal fatty acid β -oxidation was observed in mice treated with difenoconazole. However, this change was not statistically significant and it was reversible after 28 days of recovery (Table B.6.8.2-7). PB and 3-MC did not affect the peroxisomal β -oxidation whereas the treatment with NAF resulted in a more than 4-fold increase in this process.

Table B.6. 8.2-7: Peroxisomal fatty acid β -oxidation, exploratory short-term study in male mice with difenoconazole

test substance	difenoconazole					difenoconazole		PB	3-MC	NAF
sacrifice day	day 15					day 43		day 5	day 4	day 7
dose level (mg/kg/day)	control	1	10	100	400	control	400	40	80	50
peroxisomal fatty acid β -oxidation	1298	1247	1083	1055	892	1150	1167	1233	932	5657***

units are nMol/min/g liver

*** = $p \leq 0.001$, two-sided Dunnett test

Filtration of liver microsomal suspensions from mice treated with difenoconazole, PB, 3-MC or NAF revealed a type II binding spectra which is indicative for an inhibitory action of the test article on microsomal cytochrome

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P-450 (Table B.6.8.2-8). All spectra indicated two microsomal binding sites for difenoconazole, which was interpreted as two different isoenzymes. The dissociation constants (K_{S1} and K_{S2}) obtained were of comparable magnitude between all compounds (Table B.6.8.2-8). A unique binding site for difenoconazole ($K_{S1}=1.99 \mu\text{M}$) was identified in mice administered 400 mg/kg.

Table B.6.8.2-8: Spectral titration of liver cytochrome P-450

test substance	difenoconazole					difenoconazole		PB	3-MC	NAF
sacrifice day	day 15					day 43		day 5	day 4	day 7
dose level (mg/kg/day)	control	1	10	100	400	control	400	40	80	50
amplitude ($A_{427\text{nm}}-A_{394\text{nm}}$) of absorbance at 100 μM CGA 169374 (RAU)	19.6	20.6	20.8	29.2	29.6	19.2	21.8	36.8	35.0	19.2
high affinity dissociation constant K_{S1} (μM)	0.23	-	-	-	1.99	-	-	0.43	0.43	0.22
low affinity dissociation constant K_{S2} (μM)	3.96	-	-	-	5.27	-	-	3.00	3.63	1.73

RAU = relative absorbance units per mg microsomal protein

*** = $p \leq 0.001$, two-sided Dunnett test

Conclusion:

Difenoconazole is considered to be a reversible barbiturate-type inducer of metabolising enzymes in the mouse liver. The lowest level without an inductive effect on metabolising enzymes and other parameters in the mouse liver is 10 mg/kg bw.

Comments:

The results of the electron microscopy slides cannot be adequately evaluated due to the poor quality of the copies. According to the study author, the ultramorphological analysis of liver sections from the animals treated with 400 mg/kg difenoconazole revealed a distinct proliferation of smooth and rough endoplasmic reticulum membranes and a disorganisation of rough endoplasmic reticulum membranes, leading to a mixture of smooth and rough endoplasmic reticulum elements, mostly in vesicular form. Following the 28-day recovery period, hepatocytes from control and treated mice were essentially the same.

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B.6.8.2.2 Cataract study in chickens

Reference:	Schoch, M. and Schneider, M. (1989). 56 day feeding cataractogenicity in young chicken.
Guideline:	No specific guideline
GLP:	No
Deviations:	The study was prolonged from 28 days (as was initially proposed in the protocol) to 56 days since only marginal lens alterations were observed in the treated animals after 28 days.
Acceptability:	As supportive information
Test substance / purity:	Difenoconazole (CGA 169374) technical (batch: RL 1389) / Purity $\geq 95\%$. Dissolved in Ol. Arachidis Ph. H. VI (10%).
Species / Strain:	Chicken / Hisex
Doses/ No of animals:	5000 ppm /5 /sex (3/ sex in positive controls).
Administration:	Orally via the diet
Exposure time:	56 days

Materials and methods:

Young chickens of each sex were administered Difenoconazole in the diet during 8 weeks. Additional chickens received either the basal diet as a negative control or a diet admixed with 2,4-dinitrophenol at 2500 ppm as a positive control.

The animals were checked once or twice daily for clinical signs and mortality. Eye examinations were conducted on all animals twice weekly, body weights were recorded weekly and cage-wise food consumption was recorded daily. All surviving animals were sacrificed on day 57 of the study. Eyes were removed and fixed in buffered 10% neutral formalin and subsequently processed for histopathological examination using a light microscope. The bodyweights were compared for both sexes separately and the mean weekly food consumption was compared by a two sample t-test.

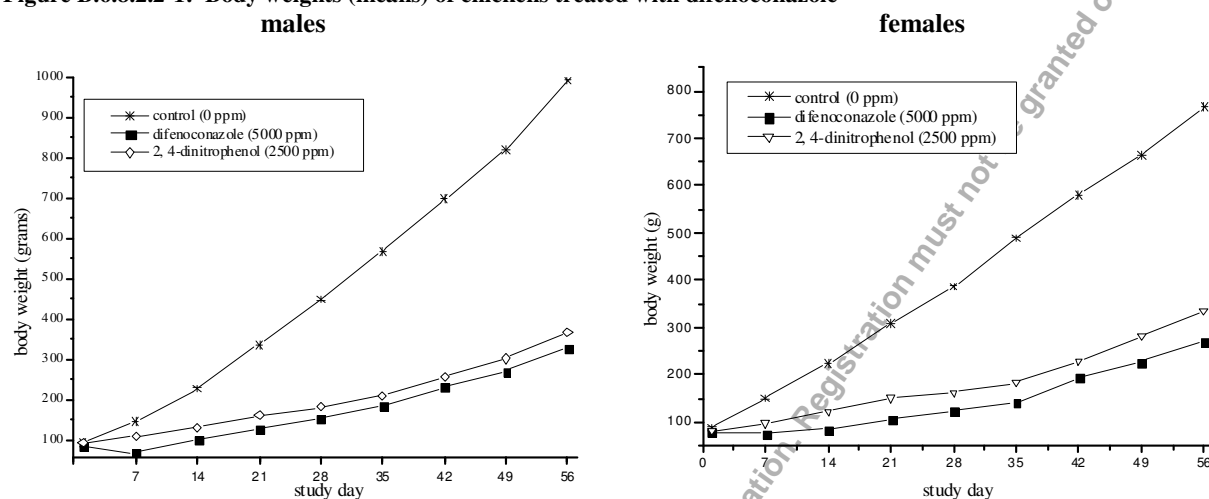
Results:

All animals in the negative or positive control groups survived treatment but one of the female test animals died on day 36 of the study. This animals showed a reduced locomotor activity and ventral recumbency immediately before death. There were no clinical signs observed among the untreated animals whereas ruffled feathers was seen in all animals of the test group and the positive control group from day 7 until termination. Additionally, a reduced locomotor activity was observed on day 9 and 10 and between day 13 and 23 in chickens treated with difenoconazole. The body weights of both male and females treated with difenoconazole were significantly ($p < 0.05$) lower than in the untreated animals (Figure B.6.8.2.2-1). Mean body

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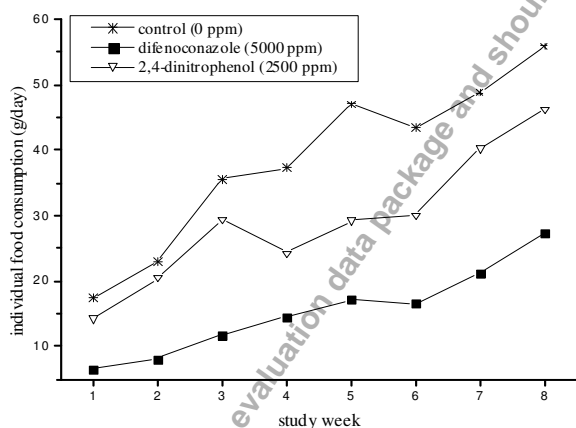
weights of the positive control group males and females were also consistently lower than the weights of the control group animals but slightly higher than those of the test group animals (Figure B.6.8.2.2-1).

Figure B.6.8.2.2-1: Body weights (means) of chickens treated with difenoconazole



The food consumption was measured separately for females and males within each group. The difenoconazole treated animals consumed markedly less than the control animals throughout the study (Figure B.6.8.2.2-2).

Figure B.6.8.2.2-2: The mean food consumption in chickens with difenoconazole



During the study, lens alterations were observed in all difenoconazole-treated males, in 2/5 of the treated females, in all positive control animals and in one untreated male (Table B.6.8.2.2-1). The animals treated with 2,4-dinitrophenol showed marked lens opacities on day 3, which diminished in severity on day 7 and persisted as slight alterations through study termination (except for one female which had no findings after day 38). In contrast, most animals treated with difenoconazole showed slight-to-moderate lens alterations after day 45 that were irreversible (Table B.6.8.2.2-1).

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Table B.6.8.2.2-1: In-life ophthalmoscopic findings in chickens treated with Difenoconazole

			study day															
grade	sex	animal	3	7	10	14	17	21	24	28	31	35	38	42	45	49	52	56
negative control (basal diet only)																		
1	male	a				+		•	•	+								
test group (difenoconazole 5000 ppm)																		
1	male	d e f g h							+	+	+				•	•	•	•
	female	o q										+			•	•	•	+
2	male	f g h			+	+					+				+	•	•	•
	female	o															+	+
3	male	g													+			
positive control (2,4-dinitrophenol 2500 ppm)																		
1	male	i j k		•			+	•	•	•	•	•	•	•	•	•	•	•
	female	t u v		•	•	•	•	•	•	+	•	•	•	•	•	•	•	•
2	male	i j k	•		•	•					•	•						•
	female	t u v	•				•	•	•		•	•						
3	male	i j k	•															
	female	t u v	•															

grade 1 = slightly diffuse alteration within a very small area of the lens

grade 2 = more prominent and/or darker lens alteration

grade 3 = marked alteration \geq 1/3 of lens area

• = one eye; + = both eyes

^aonly animals/eyes with positive findings

The histopathological examination revealed initial changes in the lens, indicative of cataract, in 3/5 males and 1/5 females treated with difenoconazole (Table B.6.8.2.2-2). The lesions comprised slight swelling of the epithelial cells either at the equator or anteriorly, and/or necrosis of the lens fibres posteriorly, under the capsule or in the outer cortex. No changes were seen in the lenses of the animals in the negative control group.

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Table B.6.8.2.2-2: Histopathological findings indicative of cataracts in the lenses of chickens treated with difenoconazole

group	sex	animal	left eye	right eye
negative control	m	a	no changes	no changes
	m	b	no changes	no changes
	m	c	no changes	no changes
	f	l	no changes	no changes
	f	m	no changes	no changes
	f	n	no changes	no changes
difenoconazole	m	d	no changes	no changes
	m	e	no changes	no changes
	m	f	slight changes	no changes
	m	g	moderate changes	slight changes
	m	h	slight changes	no changes
	f	o	slight changes	no changes
	f	p	no changes	no changes
	f	q	no changes	no changes
	f	r	no changes	no changes
	f	s	no changes	no changes
positive control	m	i	no changes	no changes
	m	j	slight changes	no changes
	m	k	slight changes	no changes
	f	t	no changes	slight changes
	f	u	no changes	no changes
	f	v	no changes	no changes

Conclusion:

Dietary treatment of chickens with 5000 ppm difenoconazole during 8 weeks led to cataract development in some animals after one month of treatment.

Comments:

A dose level of 5000 ppm was chosen based on the dose level that caused cataracts in dogs (6000 ppm, O'Connor *et al*, 1987). The intake of test substance was assumed to be significantly higher compared to the dog study (due to a higher food consumption/kg bw). No fatalities were observed in the pre-test in which chickens were administered 5000 ppm in the diet.

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B.6.8.2.3 18-week cataractogenicity study in dogs

Reference:	Janiak T, Frei T, Luetkemeier H, Vogel O, Pappritz G, Mladenovic P. (1989). CGA 169374 technical: Oral toxicity (feeding) study in dogs; assessment of cataractogenic potential.
Guideline:	No specific guideline
GLP:	Yes
Acceptability:	As additional information
Test substance / purity:	Difenoconazole (CGA 169374) technical (batch: P 509003/4) / Purity 95%. Dissolved in acetone p.a.
Species/strain	Dog / beagle
Doses/ No of animals:	See table B.6.10-13/
Administration:	Orally via the diet
Exposure time:	Group 1: 127 days, group 2: 21 days followed by a 136 day recovery period

Materials and methods:

Two groups of purebred beagle dogs (4-6 months old) were daily administered difenoconazole in the diet according to table B.6.8.2.3-1. This rationale allows delayed adverse eye effects and reversibility of treatment-related changes to be discovered. The animals were checked twice daily for viability and at least once a day for clinical signs. The food consumption was recorded daily and bodyweights were recorded weekly.

Ophthalmoscopic examinations were performed every second week. Blood samples were collected from all animals during the pre-test period and during week 3, 13 and 19 of the study. To reduce the biological variation, blood samples were collected during fixed time intervals in the morning. All parameters listed in table B.6.8.2.3-2 were analysed. The stability, homogeneity and content of the test article in feed was determined prior to the start of the study and the concentration was thereafter analysed at monthly intervals.

Table B.6.8.2.3-1

Sex/number of animals	Group 1 (reference)		Group 2 (recovery)	
	Male/1	Female/1	Male/2	Female/2
week 1	6000 ppm	6000 ppm	6000 ppm	6000 ppm
weeks 2-3	3000 ppm	3000 ppm	3000 ppm	3000 ppm
weeks 4-9	3000 ppm	3000 ppm	0 ppm	0 ppm
weeks 10-18	4000 ppm	4000 ppm	0 ppm	0 ppm

The doses were selected to be consistent with the doses tested in previous dog studies (see O'Connor et al, 1987 and Rudzki et al, 1988).

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Table B.6.8.2.3-2

Haematology:

<i>Red blood cells</i>	<i>White blood cells</i>	<i>Clotting Potential</i>
erythrocyte count (RBC)	total leukocyte count	platelet count
haemoglobin (Hb)	absolute and relative differential:	thromboplastin time
haematocrit (Hct)	band neutrophils	
mean corp. volume (MCV)	segment neutrophils	
mean corp. haemoglobin (MCH)	eosinophils	
mean corp. Hb. conc. (MCHC)	basophils	
reticulocyte count	lymphocytes	
nucleated erythrocytes	monocytes	
red cell morphology	plasma cells	
	blast cells	

Clinical chemistry:

<i>Electrolytes</i>	<i>Metabolites, Protein, Hormones</i>	<i>Enzymes</i>
calcium	bilirubin (total)	alanine aminotransferase (ALT)
chloride	cholesterol (total)	aspartate aminotransferase (AST)
phosphorus (inorganic)	creatinine	alkaline phosphatase (AP)
potassium	glucose	creatine kinase (CK)
sodium	urea	gamma glutamyl-transferase (GGT)
	protein (total)	lactate dehydrogenase (LDH)
	aldosterone	ornithine carbamyl-transferase (OCT)
	calcitonin	
	cortisol	

All animals were sacrificed in week 19 and subjected to a gross pathological examination. Eyes and tissues with macroscopic findings were examined histopathologically and selected organs were weighed (Table B.6.8.2.3-3).

Table B.6.8.2.3-3

Pathology:

The following organs were collected (column C), weighed (W) and examined histopathologically (H)

C	W	H	C	W	H	C	W	H
✓	✓	adrenals	✓	✓	kidneys	✓		seminal vesicles
✓		aorta	✓	✓	liver	✓		skeletal muscle
✓		bone (femur, incl. artic. surface)	✓		lung	✓	✓	spleen
✓		bone marrow (sternum, femur)	✓		lymph node (mesenteric)	✓		stomach
✓	✓	brain	✓		lymph node (retropharyngeal)	✓	✓	testes
✓		caecum	✓		mammary gland area (females)	✓	✓	thymus
✓		colon	✓		oesophagus	✓	✓	thyroid w. parathyroid
✓		duodenum	✓		ovaries	✓		trachea
✓		epididymides	✓		pancreas	✓		urinary bladder
✓	✓	eyes incl. lens	✓		pituitary	✓		uterus
✓		gall bladder	✓	✓	prostate			others:
✓	✓	heart	✓		rectum			
✓		ileum	✓		salivary glands (mandibular)	✓	✓	gross lesions/masses
✓		jejunum	✓		sciatic nerve	✓		body (exsanguinated)

✓: all animals

Results:

The diet analyses showed that the distribution and concentration of test substance in the diet were within an acceptable variation throughout the study.

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All animals survived treatment. Vomiting was observed in the group 1 male and in one of the group 2 females during the first study week. Other clinical signs observed included faecal changes such as the presence of mucus and worms/red areas in the faeces of the group 1 female during weeks 6 and 9 respectively, the presence of mucus in the faeces of one of the group 2 males during week 10 and diarrhea in one of the group 2 females during week 14.

The food consumption was markedly depressed during the first week of treatment in all animals (Figure B.6.8.2.3-1) which was accompanied by body weight losses of 13-14% from pretest values in the males and 11-15% in the females (Figure B.6.8.2.3-2). The dietary concentration was then reduced to 3000 ppm whereupon the food consumption increased and all animals began to gain weight. By the third study week, all males and the group 2 females were consuming all of the diet offered. All males gained weight at approximately the same rate, but the group 1 female consistently consumed less than the other females and gained weight at a slower rate. When the dietary concentration was increased to 4000 ppm (at week 10), it lost weight again.

Figure B.6.8.2.3-1: Mean food consumption in dogs administered difenoconazole

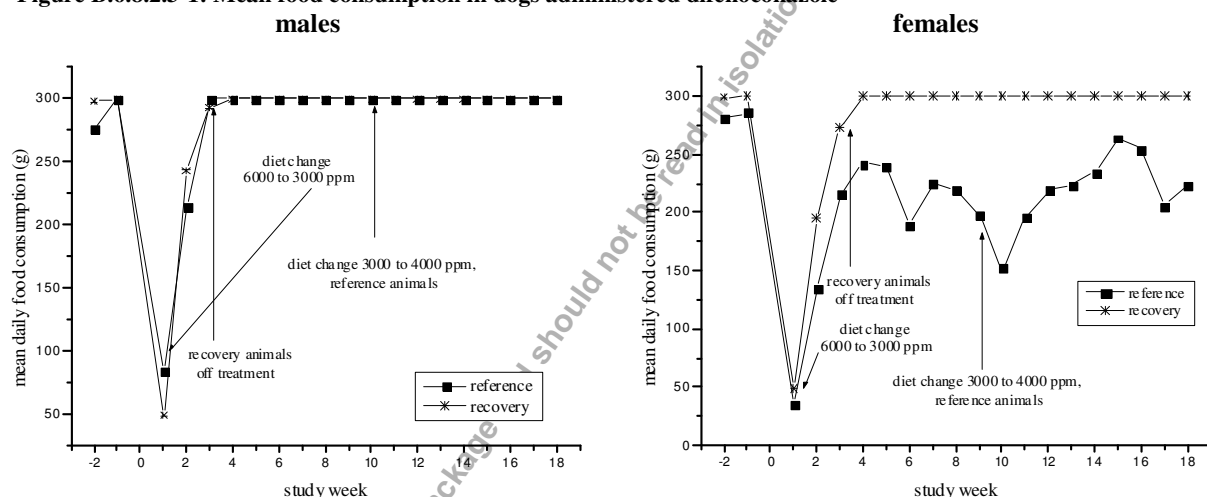
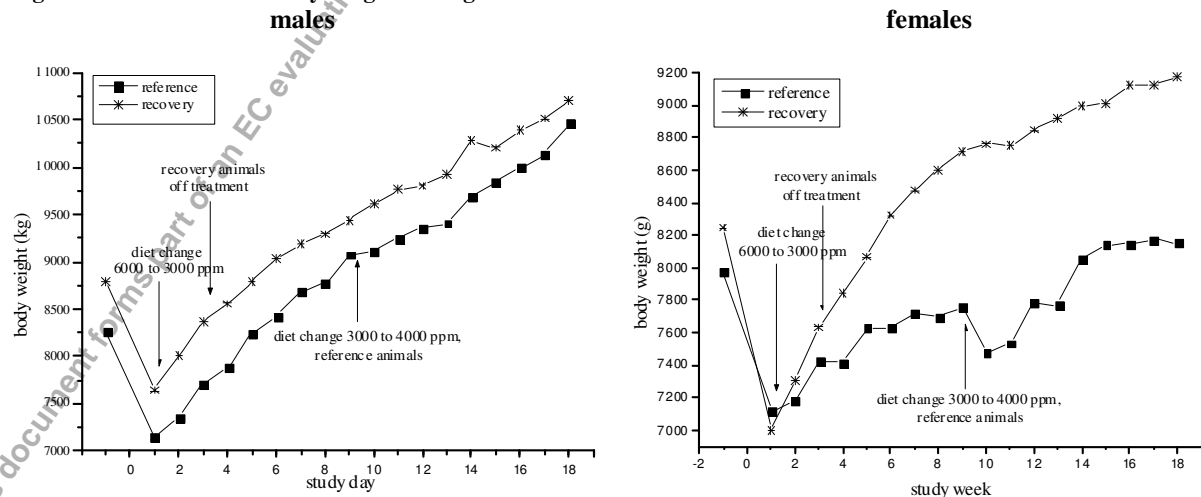


Figure B.6.8.2.3-2: Mean body weights of dogs administered difenoconazole



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According to the ophthalmoscopic examinations performed, treatment with difenoconazole did not cause any alterations in the lenses of any animals. Some differences in the haematology parameters were observed between group 1 and 2 animals but as almost all values were within the biological variation according to the historical data included in the report, these differences were not attributed to treatment. However, the leucocyte and the erythrocyte levels obtained in the group 1 male and the group 1 female, respectively, were slightly above the 95% upper limit of the historical control range.

Table B.6.8.2.3-4 Haematological parameters (means) in dogs treated with difenoconazole

	Males				Females			
	Reference (n=1)		Recovery (n=2)		Reference (n=1)		Recovery (n=2)	
	pretest	week 19	pretest	week 19	pretest	week 19	pretest	week 19
erythrocyte count (T/l)	6.2	6.7	6.3	7.0	7.9	8.5	6.6	7.1
haemoglobin (mmol/l)	8.5	9.4	9.7	10.5	10.6	11.0	9.7	10.7
haematocrit (%)	41	46	46	51	51	54	48	51
leukocyte count (g/l)	10.2	21.2	14.1	13.0	11.0	15.1	12.6	13.2
platelet count (g/l)	298	253	353	271	266	353	330	336
prothrombin time (sec)	6.6	6.6	6.4	6.4	6.8	7.0	7.4	7.5

The differences observed in clinical chemistry parameters between the group 1 and 2 animals were almost all within the biological variation according to the historical data included in the report (Table B.6.8.2.3-5). The alkaline phosphatase activity was increased above the 95% upper limit of the historical control range in the group 1 male, an effect that was also observed in the 6-month and 12-month dog feeding studies (O'Connor et al, 1987 and Rudzki et al, 1988).

Table B.6.8.2.3-5: Clinical chemistry parameters in dogs treated with difenoconazole (means)

	Males				Females			
	Reference (n=1)		Recovery (n=2)		Reference (n=1)		Recovery (n=2)	
	pretest	week 19	pretest	week 19	pretest	week 19	pretest	week 19
glucose (mmol/l)	5.54	7.06	5.37	7.23	5.13	6.12	6.27	7.41
BUN (mmol/l)	3.83	4.44	3.99	6.04	6.22	5.41	4.46	5.70
creatinine (μmol/l)	61	61	68	78	93	74	84	82
total bilirubin (μmol/l)	1.3	2.9	3.2	4.7	5.0	4.9	4.0	5.3
cholesterol (mmol/l)	3.69	3.99	3.39	4.0	3.03	2.87	3.03	3.81
ASAT (μkat/l)	0.58	0.37	0.53	0.41	0.74	0.47	0.76	0.48
ALAT (μkat/l)	0.57	0.72	0.55	0.45	0.70	0.62	0.56	0.48
LDH (μkat/l)	3.22	0.59	3.32	0.77	3.31	1.40	3.43	0.98
CK (μkat/l)	1.48	1.32	1.29	2.17	0.67	2.53	1.89	2.52
ALP (μkat/l)	13.31	19.73	11.81	6.84	3.39	6.56	5.04	2.61
GGT (nkat/l)	125.03	62.17	142.53	74.71	135.03	77.39	95.86	64.18
OCT (nkat/l)	42.18	53.01	43.85	51.18	59.01	49.51	46.35	39.26
calcium (mmol/l)	2.72	2.77	2.61	2.86	2.58	2.76	2.49	2.80
phosphorus (mmol/l)	2.98	1.97	2.35	1.14	2.01	1.35	2.10	1.04
sodium (mmol/l)	152.3	150.5	150.1	153.7	150.7	152.9	149.8	154.0
potassium (mmol/l)	4.48	3.94	4.48	3.57	4.09	3.77	4.38	3.74
chloride (mmol/l)	113.3	119.2	115.3	123.0	118.3	125.0	119.3	125.0
total protein (g/l)	51.9	59.4	52.8	63.9	56.2	61.1	54.9	64.6
calcitonin (pg/ml)	24	34	85	113	35	32	23	35
cortisol (μg/ml)	2.1	5.6	2.4	9.4	1.9	5.7	8.1	7.7
aldosterone (pg/ml)	187	112	208	57	140	121	728	203

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The liver weight of the group 1 male was increased compared to the group 2 animals (Table B.6.8.2.3-6). This was not observed in the female subjected to the same treatment.

Table B.6.8.2.3-6: Organ weights (means) of dogs treated with difenoconazole

	Males				Females			
	Reference (n=1)		Recovery (n=2)		Reference (n=1)		Recovery (n=2)	
	absolute	relative ^b	absolute	relative	absolute	relative	absolute	relative
carcass	9979	-	10192	-	7610	-	8725	-
brain	80.4	0.8	74.3	0.7	70.4	0.9	70.1	0.8
heart	80.5	0.8	83.0	0.8	68.3	0.9	75.5	0.9
liver	464.7	4.7	307.3	3.0	285.5	3.8	298.2	3.4
thyroids [(r+1)/2]	0.62	0.01	0.51	0.01	0.31	0.00	0.28	0.00
thymus	4.01	0.04	7.28	0.07	5.42	0.07	6.31	0.07
kidneys [(r+1)/2]	30.39	0.31	30.22	0.25	20.11	0.27	17.67	0.20
adrenals [(r+1)/2]	0.72	0.01	0.60	0.01	0.67	0.01	0.62	0.01
spleen	170.96	1.71	185.99	1.82	62.32	0.82	142.21	1.63
testes [(r+1)/2]	7.28	0.08	9.57	0.09	-	-	-	-
prostate	5.00	0.05	4.97	0.05	-	-	-	-

^aall weights in g

^bRelative weights = (organ/body)*100

The macroscopical findings noted (congestion in the spleen, worms in the small intestine, foci in the lungs, discoloration in the cervical lymph node, cysts in the ovaries and thickening in the vagina) and the histopathological findings (Table B.6.8.2.3-7) were observed in both groups. Since no historical control data was included in the study report, it is unclear whether these findings are common lesions in dogs of this strain and age or if they were related to treatment. There were no histological alterations of any sort observed in the eye.

Table B.6.8.2.3-7: Histopathology findings in dogs treated with difenoconazole

	Males			Females		
	Reference	Recovery		Reference	Recovery	
	Male-1	Male-2	Male-3	Female-1	Female-2	Female-3
lungs						
interstitial pneumonia	slight	slight	-	slight	-	slight
Bronchopneumonia	-	-	-	-	moderate	-
small intestine						
follicular hyperplasia	severe	severe	moderate	severe	severe	severe
large intestine						
follicular hyperplasia	moderate	severe	severe	moderate	severe	severe
Spleen						
Congestion	severe	severe	severe	severe	severe	severe
cervical lymph node						
Erythrophagocytosis	moderate	slight	-	-	-	-
sinus oedema	moderate	moderate	severe	-	-	moderate
lymphoid hyperplasia	-	-	slight	-	-	moderate
Ovaries						
cyst	-	-	-	present	present	-

Conclusion:

Treatment of single male and female dogs with difenoconazole at concentrations between 3000 and 6000 ppm for 18 weeks did not result in the formation of cataracts.

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Comments:

The low number of animals used in this study and the exclusion of untreated control dogs as reference animals weaken this study since the significance of the results obtained (organ weights, histopathological findings) cannot be adequately evaluated. A table demonstrating the test substance intake in mg/kg bw would have facilitated a comparison of the exposure to difenoconazole in this study and in the 6-month study by O'Connor *et al*, 1987.

B.6.9 Medical data and information (Annex IIA 5.9)**B.6.9.1 Medicinal surveillance on manufacturing plant personnel: Medical examination of factory workers****Methods:**

Manufacturing employees are medically examined by company physicians at the beginning of their employment and then routinely once a year. In Switzerland, routine medical examinations according to the criteria of the Swiss Accident Insurance Institution (SUVA) include:

Anamnesis,

Physical examination including blood pressure measurement

Blood analysis: haemoglobin, erythrocytes, leukocytes, thrombocytes, complete blood count, blood sedimentation rate, blood sugar, blood pressure, cholesterol, triglycerides, ALAT, ASAT, alkaline phosphatase, bilirubin, creatinine, uric acid

Urine analysis

Difenoconazole (CGA 169374) has been manufactured in production plants at Monthey in Switzerland and formulated at various sites around the world. Data was sought from all sites handling either the technical active ingredient or formulated product. A request for adverse health data covering the years 1992 to 2002, carried out in December 2002, provided the following information: Questionnaires have been sent to those responsible for the production sites and company physicians:

Results:

Monthey (CH; production); Reporting period 1992 until December 2002

The a.i. has been handled since 1992, in 3 buildings, with an estimated 5300 tonnes being produced over the decade. In 2002 about 2/3 of the product was handled and packed as powder. The remaining 1/3 was used in two premixes, which were either formulated in Monthey or sent in bulk to Aigues Vives, France. The exact number of people potentially in contact with the material is unknown but it is estimated that 'several hundred' temporary workers have been involved with difenaconazole over the ten-year period.

No adverse health effects have been recorded within the 3 buildings concerned or within the medical service based at Monthey.

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Roosendaal (NL; formulation); Reporting period 1992 until December 2002

The a.i. has been handled at this site since 1997, with 60-70 tonnes being handled annually. Up to 16 people in QC roles and 34 people in the formulation plant and warehouse have been involved in handling difenaconazole. No adverse health effects have been reported.

Aigues Vives (F; formulation); Reporting period early 1990s until December 2002

The active ingredient has been handled since the early 1990s, with 132 tonnes being handled in the years 1998-2003. Up to 19 people have been involved with the formulation and packing activities. One case of allergic reaction to a formulated product, 'Score', was reported in 1995.

Iksan (Korea; formulation); Reporting period early 1990s until December 2002

The technical material and formulated products ('Bogard' WG – approximately 30 tonnes produced annually in 2001 and 2002) have been handled at the Iksan site without any reports of adverse health effects.

Pendle Hill (Australia; formulation); Reporting period 1995 until December 2002

First handled difenaconazole in 1995. During 8 years experience of formulating difenaconazole, no adverse health effects have been reported.

Gunung Putri (Indonesia; formulation); Reporting period 2002

The facility in Indonesia began to handle difenaconazole in 2002 for the production of the 'Score' formulation. No reported adverse health effects have been reported to date.

Bien Hoa (Vietnam; repacking); Reporting period not given

Repacking activities only, no adverse health effects have been reported.

Update April 2004

Since December 2002, all the above sites have been required to report any adverse reactions or occupational illnesses related to chemical exposure. A review of the database reveals no such adverse reactions or occupational illnesses have been reported.

Other sites (repacking); Reporting period not given

Two third-party sites in Malaysia and Taiwan also repack difenaconazole and have reported no adverse reaction during the handling of difenaconazole-containing products.

Conclusion:

Large quantities of difenaconazole, both as technical material and formulated products, have been handled within Syngenta (and its legacy companies) for over 10 years, without any associated adverse health effects of the workforce being noted. The average production volume per year is in the range of 100 to 1000 tons. The

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production is performed in 1 campaign per year with a duration of 8 months. Twenty-seven people work on each campaign.

B.6.9.2 Direct observations, e.g. clinical cases and poisoning incidents

No cases of poisoning have been reported to the company and no cases of sensitisation have been observed. A literature search on human data / human exposure for difenoconazole has been performed. The search was done according to the following criteria:

Period of publication: 1967 – 2001

Key words: {human, man, woman, boy, girl, child, patient} + {health effect, incident, accident, poisoning, intoxication, allergy, sensitisation, skin reaction, eye} + {95266-40-3, SCORE, TASP, SPYRALE, DIVIDEND, CGA 169374, difenoconazole}.

Data bases: AGRICOLA, ANABSTR, BEILSTEIN, BIOBUSINESS, BIOSIS, CA, CABA, CANCERLIT, CAPLUS, CASREACT, CBNB, CHEMCATS, CHEMLIST, CIN, CROPU, CSCHEM, DDFU, DRUGU, EMBASE, HSDB, IFICDB, IFIPAT, IFIUDB, LIFESCI, MEDLINE, MRCK, NIOSHTIC, PIRA, PROMT, RTECS, SCISEARCH, TOXLINE, TOXLIT, ULIDAT, USPATFULL

The search for publications resulted in 0 hits. There are no published cases of human poisoning with difenoconazole or its formulations.

B.6.9.3 Observations on exposure of the general population and epidemiological studies

No epidemiological study has been performed by the company. No reports from the open medical literature are on record.

B.6.9.4 Clinical signs and symptoms of poisoning and details of clinical tests

Difenoconazole is of low acute toxicity. Intoxications are only likely, if large quantities are ingested. In animal studies, symptoms of acute intoxication were non-specific and transient. The same may be expected for humans. There are no specific signs of poisoning with difenoconazole.

Clinical tests

No specific monitoring programs in humans have been performed.

Determination of active substance, metabolites

Due to the fact that difenoconazole has an acute oral toxicity LD50 (rat) of 1453 mg/kg bw, it can be classified as slightly hazardous (WHO class III) and as Harmful (R22) according to Commission Directive 2001/59/EC. No analytical method for the determination of residues in body fluids and tissue is required (Guideline 8064/VI/97-rev4); since difenoconazole is not classified as toxic or very toxic.

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B.6.9.5 First aid measures

General: Terminate exposure; remove person from scene of spillage or other contamination.

In case of skin contact:	Remove contaminated clothing and thoroughly wash the affected parts of the body with soap and water.
In case of eye contact:	Rinse eyes with clean water for several minutes. Obtain medical advice.
In case of ingestion:	If the patient is unconscious, do not give anything by mouth. Do not induce vomiting. Obtain medical advice.

The therapy for difenoconazole poisoning is symptomatic and supportive.

Therapeutic regimes

Antidote	No antidote is known, apply symptomatic and supportive treatment
In case of skin/eye contact:	Decontamination
In case of ingestion:	If the amount ingested is judged less than a potentially toxic dose, employ general supportive measures only. Use activated charcoal for gastrointestinal decontamination. If gastric lavage is considered necessary, prevent aspiration.

B.6.9.6 Expected effects and duration of poisoning

No specific effects can be deduced from animal data. No human data are available.

B.6.10 Summary of mammalian toxicology and proposed ADI, AOEL, ARfD and drinking water limit (AnnexIIA 5.10)

B.6.10.1 Absorption, distribution, excretion and metabolism (toxicokinetics)

The **oral absorption** of difenoconazole is **80 - 90%** at lower (0.5 mg/kg bw) dose levels and **40-60%** at higher (300 mg/kg bw) dose levels. Therefore an oral absorption of 100 % is used in further calculations.

A single oral dose of **0.5 mg** [¹⁴C-phenyl]-difenoconazole/kg was **almost completely absorbed** by male and female rats and eliminated predominantly *via bile* (accounting for **73 and 76%** of a 0.5 mg/kg dose in males and females, respectively). **Urinary** excretion in bile duct cannulated rats accounted for **14 and 9%** in males and females, respectively and **less than 4% faecal** excretion which confirm the high absorption. Corresponding

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results in non-cannulated rats were 13 - 22% in urine and 81 - 87% in faeces. An **entero-hepatic recirculation** was demonstrated experimentally. Biliary metabolites were largely excreted in faeces. There was **no difference in excretion profiles** between the sexes or between two radiolabelled forms (^{14}C -phenyl- or ^{14}C -triazole-difenoconazole). Furthermore, there was neither a sex difference nor differences in excretion profiles in rats given the same radiolabelled dose (0.5 mg/kg) following pre-treatment with multiple oral doses of radiolabelled difenoconazole (0.5 mg/kg), from the rats with no pre-treatment. At the 0.5 mg/kg bw- dose level, the **half life** of excretion was approximately **20 hours**.

At 2 and 24 hours, most of the radioactivity was present in the **gastrointestinal tract contents** and in **bile** (as shown by whole body autoradiography sections) and only **liver and kidney** had higher tissue concentrations than plasma, after a dose of 0.5 mg [^{14}C -phenyl]-difenoconazole/kg bw. After 7 days, only **fat** had comparable concentrations with those present in plasma. Residues in female tissues tended to be lower than in males. Pre-treatment with unlabelled test substance had no effect on tissue distribution.

A single oral dose of **300 mg** [^{14}C -phenyl]-difenoconazole/kg was **less extensively absorbed**, however still eliminated predominantly *via bile* (accounting for **56 and 39%** of the dose in males and females, respectively). **Urinary** excretion in bile duct cannulated rats accounted for **1%** (both sexes) and **faecal** excretion for **17 and 22%** in males and females, respectively which confirm the **lower absorption**. Corresponding results in non-cannulated rats were 8 - 15% in urine and 85 - 95% in faeces. As seen at the low dose level, there was **no difference in excretion profiles** between the sexes or between two radiolabelled forms. Non-cannulated rats excreted more of the dose in urine than bile duct cannulated rats, apparently following reabsorption and further metabolism of some biliary metabolites. However, the predominant route of excretion of biliary radioactivity was in faeces, as observed at the low dose level. At the 300 mg/kg bw- dose level, the **half life** of excretion was **33 - 48 hours**.

At 4 hours, the highest tissue concentrations were present in **fat** (both sexes) with progressively lower levels in **liver, Harderian glands, adrenal glands, kidney and pancreas**, after a dose of 300 mg [^{14}C -phenyl]-difenoconazole/kg bw. After 7 days, only **fat** had higher concentrations with those present in plasma. Residues in female tissues tended to be lower than in males.

Tissue depletion results showed that at 4 hours after a 300 mg/kg [^{14}C -phenyl]-dose, most tissue concentrations were similar to or higher than in plasma in both sexes. All other tissues that initially showed concentrations higher than in plasma declined rapidly by 48 hours after dosing and by 168 hours all [^{14}C -phenyl] tissue levels had declined markedly with only fat showing residues higher than in plasma. [^{14}C -triazole]-tissue residues were significantly lower than [^{14}C -phenyl]-residues and by 168 hours were measurable only in the liver. Measurements in the gastrointestinal tract contents were consistent with the observed absorption and elimination profiles.

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Metabolites were isolated from urine and faeces of male and female rats administered a single oral dose of 0.5 or 300 mg [^{14}C -phenyl] and [^{14}C -triazole]-difenoconazole/kg, or a 0.5 mg/kg dose after 14 daily oral doses of 0.5 mg unlabelled difenoconazole/kg. **Three main metabolites, A, B and C** were isolated from faeces and together **accounted for** an average of **68% of the dose**. The urinary metabolite profile was more complex and showed more variability between the two radiolabelled forms (^{14}C -phenyl and ^{14}C -triazole). Other urinary metabolites included metabolite C. One metabolite - CGA 189138 (chlorophenoxy-chlorobenzoic acid) - was also isolated from liver.

Hence, at both dose levels, elimination kinetics were independent of sex and radiolabel position. The difenoconazole molecule was **extensively metabolised**, although with limited cleavage of the triazole and dioxolane rings. The extensive **biliary elimination** was **consistent with** the relatively **high molecular weights** of the major **metabolites**.

B.6.10.2 Acute studies

A classification as "Harmful if swallowed" (R22) is considered warranted according to Commission Directive 2001/59/EC based on the acute oral LD₅₀ of 1453 mg/kg. No signs of dermal irritation were noted. Signs of ocular irritation were observed in the rabbit. However, as the mean values of the readings were below the thresholds defined in Directive 2001/59/EC, no classification is required. In a modified Buehler test no sensitisation effects were detected and therefore no classification is considered necessary.

Study author	parameter	species	Dose levels	Results (incl ev classification)
Argus et al., 1987	Acute oral LD ₅₀	rat	1000, 2000 and 3000 mg/kg	male and female 1453 mg/kg Xn, R22
Hartmann, 1990	Acute oral LD ₅₀	mouse	1000 and 2000 mg/kg	>2000 mg/kg
Mastrocco et al., 1987	Acute dermal LD ₅₀	rabbit	Limit test, 2010 mg/kg	>2010 mg/kg bw
Hartmann H.R., 1991	Acute inhalation LC ₅₀	rat	Limit test, 3458 mg/m ³	>3300 mg/m ³
Glaza, S.M., 1991	Skin irritation	rabbit	0.5g	non-irritating
Glaza, S.M., 1991	Eye irritation	rabbit	0.05g	non-irritating
Mastrocco et al., 1987	Skin sensitisation (modified Buehler)	female Guinea pigs	0.5g	non-sensitising

B.6.10.3 Short-term toxicity

The short term oral toxicity was investigated in rats, dogs and mice. One 28 day study and two 90 day studies were performed in rats. Two studies were performed in dogs, one 6 month study and one 1 year study. A 90 day study was performed in mice. A short term dermal toxicity study was also performed in rats, but a 90-day dermal study was not performed because of the limited effects seen in the 28-day study. All of these studies were considered acceptable. All of the studies were performed according to GLP principles with the exception of the

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28 day study in rats. A 90 day dog study was not performed since studies of 6 and 12 months duration, which look at effects at 3 months either side of this timepoint, were conducted. Furthermore, there were no indications that dogs were more sensitive to Difenoconazole than rats. No short-term inhalation toxicity studies were performed because difenoconazole is not volatile (vapour pressure 0.0000332 mPa [25 °C]) and is not used as a fumigant or an aerosol.

Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
ORAL:					
28-day in rat					
Suter, P., 1986a	27/27, 156/166 and 914/841 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 156/166 mg kg⁻¹ day⁻¹ 1500 ppm	LOAEL: M/F: 914/841 mg kg⁻¹ day⁻¹ 10 000 ppm	↓ Body weight ↓ Carcass weight ↓ Organ weight
	0, 250, 1 500, 10 000 ppm		NOEL: M/F: <27/27 mg kg ⁻¹ day ⁻¹ <250 ppm	LOEL: M/F: 27/27 mg kg ⁻¹ day ⁻¹ 250 ppm	10 000 ppm: Altered clinical chemical parameters Altered blood parameters ↓ PT time Dysproteinemia
90-day in rat					
Suter, P., 1986b (Wistar rats)	0, 3.3/3.5, 19.9/21.4 and 120.9/ 128.5 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 20/21 mg kg⁻¹ day⁻¹ 250 ppm	LOAEL: M/F: 120.9/ 128.5 mg kg⁻¹ day⁻¹ 1500 ppm	↓ Body weight ↓ Carcass weight ↓ Heart weight (11%) ↓ Food consumption
	0, 40, 250 and 1500 ppm		NOEL: M/F: 3.3/3.5 mg kg ⁻¹ day ⁻¹ 40 ppm	LOEL: M/F: 20/21 mg kg ⁻¹ day ⁻¹ 250 ppm	1500 ppm: Altered blood parameters Altered clinical chemistry parameters Dysproteinemia ↑ Liver weight ↑ Serum albumin
Cox, R.H., 1987a (Sprague Dawley rats)	0, 1.3/1.7, 13/17, 51/66, 105/131 and 214/275 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 51/66 mg kg⁻¹ day⁻¹ 750 ppm NOEL: M/F: 1.3/1.7 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 105/131 mg kg⁻¹ day⁻¹ 1500 ppm LOEL: M/F: 13/17 mg kg ⁻¹ day ⁻¹	↓ Body weight ↓ Body weight gain 1500 ppm: ↓ Carcass weight Altered clinical chemistry parameters Hepatocellular enlargement 750 ppm: ↓ RBC parameters dysproteinemia ↑ Liver weight
	0, 20, 200, 750, 1500 and 3000 ppm		20 ppm	200 ppm	↓ Body weight gain (F)
90-day in mouse					
Cox, R.H., 1987b	0, 3.3/4.6, 34.2/45.2 and 440/639	Orally via the diet	NOAEL: M/F: 34.2/45.2 mg kg⁻¹ day⁻¹	LOAEL: M/F: 440/639 mg kg⁻¹ day⁻¹	↓ Ovary weight ↓ Body weight gain

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
	mg kg ⁻¹ day ⁻¹ (M/F)		200 ppm	2 500 ppm	
	0, 20, 200, 2500, (7500 and 15000) ppm		NOEL: M/F: 3.3/4.6 mg kg ⁻¹ day ⁻¹	LOEL: M/F: 34.2/45.2 mg kg ⁻¹ day ⁻¹	2500 ppm: ↑ Liver weight Macroscopic liver enlargement Hepatocellular vacuolization Hepatocellular coagulative necrosis
			20 ppm	200 ppm	Hepatocellular enlargement
6 months in dog					
O'Connor et al., 1987	0, 3.6/3.4, 31.3/34.8, 96.6/110.6 and 157.8/203.7 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 31.3/ 34.8 mg kg⁻¹ day⁻¹	LOAEL: M/F: 96.6/110.6 mg kg⁻¹ day⁻¹	6000 ppm: ↓ Body weight ↓ Food consumption ↓ Carcass weight ↓ Prostate weight
	0, 100, 1000, 3000 and 6000 ppm		1000 ppm	3000 ppm	Cataract
			NOEL: M/F: 3.6/ 3.4mg kg ⁻¹ day ⁻¹	LOEL: M/F: 96.6/110.6 mg kg ⁻¹ day ⁻¹	6000 ppm: ↓ Ovary weight (n.s.) ↓ Uterus weight (n.s.) ↑ Platelet count ↓ Calcium Dysproteinemia
					3000 ppm: ↑ Liver weight (F) ↑ ALP (F)
			100 ppm	1000 ppm	↓ Food consumption (M)
1-year in dog					
Rudzki et al., 1988	0, 0.71/0.63, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: ≥ 51.2/44.3 mg kg⁻¹ day⁻¹	LOAEL: Could not be established	
	0, 20, 100, 500 and 1500 ppm		NOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	LOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	1500 ppm: ↓ Food consumption ↓ Body weight gain
			100 ppm	500 ppm	↑ ALP (M)
DERMAL: 28-day in rat					
Gerspach R., 2000	0, 10, 100 or 1000 kg ⁻¹ bw day ⁻¹	Dermal	NOAEL 1 000 kg⁻¹ bw day⁻¹	LOAEL >1 000 kg⁻¹ bw day⁻¹	

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
			NOEL: 100 kg ⁻¹ bw day ⁻¹		1000 ppm: ↑ Liver weight ↓ Bilirubin levels Hepatocellular hypertrophy ↓ Food consumption Hypertrophy of thyroid gland Hyperkeratosis of application site
<i>M = male; F = female</i>					

The body weight was the most sensitive parameter in the oral short term toxicity studies in rats with a NOAEL for body weight reduction at 20 and 21 mg/kg bw for males and females respectively, derived from the 90 day study (Suter 1986b). The liver was identified as target organ in the rat studies and the effects were mostly expressed as increases in relative and absolute liver weights, but these changes were not associated with histological changes except in the second 90 day study where an increased incidence and severity of hepatocellular enlargement were noted at $\geq 100/130$ mg/kg bw for males and females respectively. The liver findings were not considered as adverse in the first 90 days study (Suter 1986b) and were reversible.

The dogs in the 28 day study also responded to Difenoconazole treatment by body weight loss (NOAEL 97/111 mg/kg bw males and females respectively). In the 6 month study the target organ was the liver with increased liver weights seen in the 6000 and 3000ppm animals. There was no associated pathological change. The NOAEL was set at 1000 ppm (31/35 mg/kg bw for males and females respectively) based on the findings of cataracts in animals at 3000 ppm. The increase in absolute liver weight correlated with elevated ALP levels in 3000 ppm females which also supports adverse effects at this level although no histological liver changes were found. In the 1 year dog study the only treatment related effects were increased alkaline phosphatase activity in animals given 1500 and 500 ppm in the diet and reduced food consumption at 1500 ppm. There were no effects on liver weight or histopathology, nor any evidence of cataracts.

Dermal application of difenoconazole resulted in a NOAEL of ≥ 1000 mg/kg/day – the highest dose tested. The target organ was again the liver, with increased liver weights and decreased bilirubin levels in animals treated with 1000 mg/kg/day showing associated pathological change manifested as centrilobular hepatocellular hypertrophy. Histopathology revealed hyperkeratosis at the skin application site in the 1000 mg/kg animals, and follicular cell hypertrophy of the thyroid gland was diagnosed in high-dose males and females. None of these changes were considered adverse but to represent reactions of adaptation.

B.6.10.4 Genotoxicity

In vitro, difenoconazole was negative in both bacterial and mammalian cell assays for gene mutation, negative for chromosomal damage in cytogenetic assays using isolated human lymphocytes and negative for DNA

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damage/repair in the unscheduled DNA synthesis assay. *In vivo*, difenoconazole was negative for chromosomal damage in the mouse bone marrow micronucleus assay. Increases in chromosomal aberrations were reported in CHO cells treated *in vitro* with difenoconazole, but only at high concentrations inducing cytotoxicity and they were not clearly reproducible either between repeat examinations of the same slides, between experiments or across studies. These observations are not considered of significance in light of the negative results in the other genotoxicity assays, including other *in vitro* and *in vivo* cytogenetic assays.

Study	Concentrations/ Dose levels	Results	Reference
Salmonella/E. coli <i>in vitro</i>	0 to 5447 µg/plate, +/-activation	negative	Ogorek, 1990
Gene mutation in mouse lymphoma L5178Y/TK ⁺ cells <i>in vitro</i>	0 to 150 µg/ml, - activation 0 to 50 µg/ml, + activation	negative	Dollenmeier, 1986a
Cytogenetic test on Chinese hamster cells <i>in vitro</i>	0 to 105 µg/ml, - activation 0 to 105 µg/ml, + activation	equivocal and non-reproducible positive response without and with metabolic activation at cytotoxic concentrations	Lloyd, 2001
Cytogenetic test on Chinese hamster cells <i>in vitro</i>	0 to 200 µg/ml, - activation 0 to 200 µg/ml, + activation	non-reproducible positive response with metabolic activation at one concentration	Ogorek, 2001
Cytogenetic test in human lymphocytes <i>in vitro</i>	0 to 40 µg/ml, - activation 0 to 40 µg/ml, + activation	negative	Strasser, 1985
Cytogenetic test in human lymphocytes <i>in vitro</i>	0 to 75 µg/ml, - activation 0 to 75 µg/ml, + activation	negative	Fox, 2001
DNA repair on rat hepatocytes <i>in vitro</i>	0 to 50 µg /ml	negative	Hertner, 1992
Micronucleus test mouse bone marrow <i>in vivo</i>	0, 400, 800, 1600 mg/kg bw	negative	Ogorek, 1991

From the results obtained in these tests *in vitro* and *in vivo* it is concluded that difenoconazole is not genotoxic.

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B.6.10.5 Long-term toxicity and carcinogenicity

A combined chronic toxicity and carcinogenicity study was performed in rats and an oncogenicity study was performed in mice. A one year study in dogs was also performed and is presented under B.6.3.1.6.

Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
ORAL:					
2-year combined chronic toxicity/oncogenicity in rat					
Cox, 1989a	0, 0.5/0.6, 1.0/1.3, 24.1/32.8 and 124/170 mg kg ⁻¹ day ⁻¹ (M/F)	Orally, via the diet	NOAEL: M/F: 1.0/1.3 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 24.1/32.8 mg kg ⁻¹ day ⁻¹	2500 ppm: ↓ Body weight ↓ Body weight gain ↓ Food consumption (F) ↓ Carcass weight
Report Supplement: Saunders, 1992	0, 10, 20, 500, 2500 ppm		500 ppm	2500 ppm	
			NOEL: M/F: 1.0/1.3 mg kg ⁻¹ day ⁻¹	LOEL: M/F: 24.1/32.8 mg kg ⁻¹ day ⁻¹	500 ppm: ↓ Body weight gain 2500 ppm: ↓ RBC parameters, ↓ WBC parameters Dysproteinemia Altered clinical chemistry parameters 2500 ppm
			20 ppm	500 ppm	500 ppm: ↓ Body weight (F) ↓ Hb (F) ↓ Platelet count (M) ↑ ALAT (M) Hepatocellular hypertrophy
18 months oncogenicity study in mice					
Cox, 1989b	0, 1.5/1.9, 4.7/5.6, 46.3/57.8 and 423/513 mg kg ⁻¹ day ⁻¹ (M/F)	Orally, via the diet	NOAEL: M/F: <46.3 /57.8 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 46.3 /57.8 mg kg ⁻¹ day ⁻¹	4500 ppm: ↑ ALP (M)
	and 819 mg kg ⁻¹ day ⁻¹ for males at 4500 ppm		30 ppm	300 ppm	2500 ppm: ↑ ALAT ↑ Liver weight Hepatocellular carcinoma
	0, 10, 30, 300, 2500 (3000 1 st two weeks)				300 ppm: ↑ Liver weight (F) ↑ Sorbitaldehydrogenas (SDH) (M) Hepatocellular necrosis (M, F)

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
			NOEL: M/F: 4.7/5.6 mg kg ⁻¹ day ⁻¹ 30 ppm	LOEL: M/F: 46.3 /57.8 mg kg ⁻¹ day ⁻¹ 300 ppm	4500 ppm: ↓ Brain weight (6%) ↓ Testis weight (no dose- response) 2500 ppm: ↑ Carcass weight (not at term.) Altered WBC parameters Macroscopic hepatocellular enlargement Macroscopic hepatocellular masses Bile stasis Hepatocellular fatty change ↓ Body weight ↓ Body weight gain ↑ Liver weight (only females at interim) Hepatocellular hypertrophy Hepatocellular adenoma
<i>1-year in dogs</i>					
Rudzki et al., 1988	0, 0.71/0.63, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg kg ⁻¹ day ⁻¹ (M/F) 0, 20, 100, 500 and 1500 ppm	Orally via the diet	NOAEL: M/F: ≥ 51.2/44.3 mg kg⁻¹ day⁻¹ ≥ 1500 ppm NOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹ 100 ppm	LOAEL: Could not be established LOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹ 500 ppm	1500 ppm: ↓ Food consumption ↓ Body weight gain ↑ ALP (M)
M = male; F = female					

Dietary administration of difenoconazole technical to rats resulted in a decrease (8-23%) in absolute body weight values in the 2 500-ppm dose group and a dose-related decrease (6-40%) in body weight gain in the 500 and 2 500-ppm dose groups. Mean food consumption was consistently lower (1-14%) for the 2 500-ppm animals as compared with controls. There was a negative effect on the red cell mass in the 2 500-ppm females however the effect is **not regarded to be adverse**. Relative liver weight was increased (14-48%) in the 2 500-ppm animals at weeks 53 and 105, but in the 2 500-ppm recovery animals it was similar to the control animals, indicating that **the liver enlargement is adaptive** during exposure and that the effect is reversible after exposure cessation. There were **no treatment-related macroscopic findings**; increased incidence and severity of hepatocellular hypertrophy were noted in 500 and 2 500-ppm males and females. **No treatment-related increases in neoplastic findings occurred during the study**. The dietary concentration of 20 ppm was considered to be the NOEL/NOAEL.

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Dietary administration of difenoconazole technical to mice for up to 18 months at dose levels of 0, 10, 30, 300, 2500 and 4500 ppm resulted in **100% mortality/morbidity** among the 4500 ppm females and **high mortality** among the 4500 ppm males during the first study weeks: survival to termination was therefore decreased for the 4500 ppm males. **Body weight losses** were noted for the high-dose animals during the first study weeks; body weight gains thereafter approached control values, but terminal body weights were reduced. **Liver enzyme levels** were elevated in the 4 500-ppm males and in the 2 500-ppm animals. **Liver weights** were increased in the 4 500-ppm males, the 2 500-ppm animals and in the 300-ppm females. Treatment-related **macroscopic findings** were seen in the livers of the 4 500-ppm males and the 2 500-ppm animals. Treatment-related **microscopic findings** found in the livers of the 4 500-ppm males, the 2 500-ppm animals and in the 300-ppm males included necrosis, hypertrophy, fatty change and bile stasis. The incidence of **hepatocellular adenomas and carcinomas** was significantly increased in the 4 500-ppm males and in the 2 500-ppm animals. The NOAEL was considered to be 30 ppm.

B.6.10.6 Reproduction toxicity

Administration of difenoconazole technical to rats at dietary concentrations of 0, 25, 250 and 2500 ppm over two generations, with one mating in each generation, resulted in treatment related effects at 2500 ppm (approximately 178 mg/kg/day). Retarded body weight gain and reduced food consumption were noted in parental animals of both generations. Absolute pup body weights were lower than control body weights at 2500 ppm in both generations. Male and female reproductive organs, mating behaviour, conception, parturition, litter parameters, lactation and weaning were not adversely affected by the administration of difenoconazole at any dose level in either generation. The dose level of 250 ppm was considered the NOAEL for both parental animals and pups in this study.

The teratogenic potential of difenoconazole was investigated in the rabbit at 1, 25 and 75 mg/kg/day. Maternal toxicity, manifest by reduced body weight gain and food consumption was seen during the period of organogenesis at 75 mg/kg/day. Two 75 mg/kg/day animals aborted and a third died of apparent compound related anorexia. A slight increase in resorptions was observed at 75 mg/kg/day, which may have been secondary to maternal toxicity. There were no differences in pregnancy or litter parameters among the treated and control groups. No treatment related external, visceral or skeletal effects were seen. The maternal NOAEL was 25 mg/kg/day and the foetal NOEL/NOAEL was 25 mg/kg/day. There was no evidence of compound related embryotoxic, foetotoxic or teratogenic potential at doses of up to 75 mg/kg/day.

The teratogenic potential of difenoconazole was investigated in the rat at doses of 2, 20, 100 and 200 mg/kg/day. Maternal toxicity, manifested as reduced body weight gain and food consumption was seen during the period of organogenesis at 100 and 200 mg/kg/day. Slight increases in resorptions and reduction in litter size was seen at 200 mg/kg/day but did not reach statistical significance and were attributed to maternal toxicity. Increases in a number of minor skeletal abnormalities at 200 mg/kg/day were considered reversible and/or associated with maternal toxicity. No effects were seen in dams at 1 and 20 mg/kg/day or in foetuses from dams treated at 1, 2 or

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20 mg/kg/day. The maternal NOAEL was 20 mg/kg bw/day and foetal NOAEL was 100 mg/kg/day. There was no evidence of compound related embryotoxic, foetotoxic or teratogenic potential at doses of up to 200 mg/kg/day.

Table: Summary of reproductive toxicity

Study/dose levels	NOAEL	LOAEL target organ/effect	Reference
2-generation reproduction 0, 25, 250, 2500 ppm	250 ppm \equiv 17.3 mg/kg/day	2500 ppm \equiv 178 mg/kg/day body weight, food consumption	Giknis, 1988
rabbit teratology 0, 1, 25, 75 mg/kg/day	25 mg/kg/day (maternal) 25 mg/kg/day (foetal)	75 mg/kg/day (maternal) body weight, food consumption, abortion 75 mg/kg/day (foetal) resorptions	Hummel et al., 1987
rat teratology 0, 20, 20, 100, 200 mg/kg	20 mg/kg/day (maternal) 100 mg/kg/day (foetal)	100 mg/kg/day (maternal) body weight 200 mg/kg/day (foetal) skeletal variations	Lochry, 1987

B.6.10.7 Delayed neurotoxicity

Delayed neurotoxicity studies were not performed because the structure and chemistry of difenoconazole do not resemble chemicals known to induce delayed neurotoxicity. In addition, no effects indicative of nervous system involvement were seen in any of the studies performed with difenoconazole.

B.6.10.8 Further toxicological studies

B.6.10.8.1 Toxicological relevance of the metabolites of difenoconazole

In plants treated with difenoconazole, one difenoconazole specific metabolite and four triazole metabolites were found at levels that exceeded 10% of the TRR (Table B.6.10.8.1-1).

The toxicity of the plant metabolite CGA 205375 that is also formed to a large extent in the mammalian metabolism of difenoconazole is considered to be covered by the toxicity studies performed on the parent compound. In addition, this metabolite was tested individually in an acute oral toxicity study and in a bacterial gene mutation assay. The RMS suggests that the toxicity assessment of the other plant metabolites should include studies of acute oral toxicity and genotoxicity¹ as they are only detected at low levels. Studies of toxicokinetics may indicate if the metabolite accumulate in certain tissues. Since this suggestion is based on the low exposure to metabolites during the representative use, it may be re-evaluated if the use within the EU will expand and include additional applications.

The major metabolites found in the mammalian metabolism of difenoconazole (CGA 205374 and CGA 205375) were further investigated regarding the acute oral toxicity and the ability to induce mutations in bacteria. The

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metabolite CGA-189138 isolated from the liver was investigated regarding the ability to induce mutations in bacteria.

Table B.6.10.8.1-1: Summary of plant residues^{2,3}

Metabolites	Plant metabolism studies	Rotational crops studies
CGA 205375	Oilseed rape straw (14%, 0.69 mg/kg)	
CGA 142856	Wheat grain (20%, 0.28 mg/kg) Mature wheat grain: (≤ 90 %, 0.135 mg/kg^{4,4}) Mature wheat straw: (≤87%, 0.059 mg/kg^{3,5})	Wheat grain (25.9%, 0.087 mg/kg) Wheat stalks (36.2%, 0.042 mg/kg) Wheat husks (39.4%, 0.065 mg/kg)
CGA 71019	Mature wheat grain^{3,4}: (≤ 90 %, 0.135 mg/kg) Mature wheat straw^{3,4}: (≤87%, 0.059 mg/kg)	
CGA 131013	Potato tuber (78.9 %, 0.07 mg/kg)⁶ Tomato fruit (19.3 %, 0.04 mg/kg)⁷ Oilseed rape seeds (56.4 %, 1.27 mg/kg) Oilseed rape pods (12 %, 0.56 mg/kg)	Maize grain (66.2%, 0.138 mg/kg) Wheat grain (44%, 0.148 mg/kg) Wheat stalks (10.4%, 0.012 mg/kg) Wheat husks (19%, 0.031 mg/kg) Lettuce head (30.5%, 0.006 mg/kg) Sugar beet (25.3%, 0.008 mg/kg)
CGA 205369		Maize grain (9.7%, 0.020 mg/kg) Wheat stalks (21.2%, 0.025 mg/kg) Wheat husks (12.1%, 0.019 mg/kg) Lettuce head (42.8%, 0.008 mg/kg) Sugar beet (54.3%, 0.017 mg/kg).

Sections B.6.8.1.2 and B.6.8.1.3 include many toxicological studies of 1, 2, 4-triazole and triazole alanine. As the majority of these studies are old, they do not fulfill the criteria of the current OECD guidelines. However, most of these studies are not necessary for the assessment considering the low exposure to 1, 2, 4-triazole and triazole alanine following the representative use and they are therefore not listed in table B.6.10.8.1-1.

CGA 205369 (Triazole lactic acid) is possibly taken up by the plant from soil. Since the presumed exposure following the representative use considered in this DAR, is assumed to be low, only tests of acute oral toxicity and *in vitro* genotoxicity are required. The notifier refers to a negative result from a DEREK-QSAR studie which was not included in the dossier submission. However, as QSAR analysis is not yet accepted as an alternative method and since the RMS considers that a determination of *in vitro* genotoxicity requires a combination of an Ames test, a gene mutation test on mammalian cells and a chromosome aberration test, the notifier was asked (April 2006) for a justification why further tests were not included in the dossier submission. The notifier has announced that tests of acute oral toxicity and genotoxicity are in progress and the RMS suggests that these will be included in an addendum to this DAR.

¹ Considered to be represented by a combination of an Ames test, a gene mutation test on mammalian cells and a chromosome aberration test.

² plant residues found following the representative use considered in this DAR are shown in bold. Residues found in rotational crops are considered relevant for an assessment of toxicity.

³ See B.7 residue data.

⁴ The highest value obtained in measurements at two geographic areas.

⁵ Identified as 1, 2, 4-triazole (CGA 71019) or triazole acetic acid (CGA-142856) following seed treatment at 4 times higher than the recommended application rate.

⁶ Potato was used to represent carrot.

⁷ Tomato was used to represent pome fruit.

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CGA 71019 (1, 2, 4-triazole) was detected in mature straw and grain of wheat after seed treatment (section B.7.1.13) which is the intended application in the EU regions. The majority of the radioactivity in mature straw and mature grain was aqueous soluble (72 to 90% of the TRR, respectively) and was identified as 1, 2, 4-triazole (CGA-71019) or triazole acetic acid (CGA-142856).

Since CGA 71019 is a minor metabolite that was determined to represent <10% of the urinary metabolites in male rats, it is not considered to have been adequately tested in the mammalian toxicity studies of the parent. 1, 2, 4-triazole was rapidly excreted in rats, mainly in unchanged form and based on its oral LD₅₀, it should be classified as Xn, R22 (harmful if swallowed) according to the Council Directive 67/548/EEG. 1, 2, 4-triazole is currently classified as R63 "Possible risk of harm to the unborn child" (ATP24) and in one of two teratogenicity studies presented in section B.6.8.1.3.6, an increased incidence of cleft palate was observed. Since the exposure to 1, 2, 4-triazole following the conditions of the representative use is far below toxic doses, the teratogenic effects are of no concern in this context.

Genotoxic effects, however, are considered to lack threshold doses and the genotoxicity of 1, 2, 4-triazole must therefore be assessed. Since only Ames tests was presented in the dossier, the notifier was asked (March 2006) for a justification why further tests were not included. The notifier has announced that further tests of genotoxicity are in progress and the RMS suggests that these will be included in an addendum to this DAR.

CGA 131013 (Triazole alanine)

In rats, triazole alanine was rapidly excreted in urine, to a large extent as the unchanged parent compound and to a minor extent as N-acetyl- D,L-triazolylalanine. The acute oral toxicity of triazole alanine was low in rats and mice but a reduced body weight gain was observed in female dogs administered 200 mg/kg bw during 90 days. Triazole alanine was not genotoxic according to the *in vitro/vivo* studies presented. Teratogenic effects of triazole alanine could not be excluded since it was not tested at sufficiently high doses in studies of reproductive toxicity. However, exposure to triazole alanine via plants in doses higher than those tested is unlikely and further studies are thus not required.

The RMS suggests that residues of CGA 131013 in plants are of no concern.

CGA 142856 (Triazole acetic acid)

In rats, triazole acetic acid was rapidly eliminated primarily via urine in unchanged form.

TA is of low acute oral toxicity in rats and it is not mutagenic according to an Ames test, a gene mutation test on mouse lymphoma cells and an *in vitro* chromosome aberration test in human lymphocytes.

The RMS suggests that residues of CGA 142856 in plants are of no concern.

Conclusion:

During the conditions of the representative use, the residues of triazole alanine (CGA 131013) and triazole acetic acid (CGA 142856) in plants are considered to be of no concern. Since the assessment of the toxicological relevance of triazole lactic acid (CGA 205369) and 1, 2, 4-triazole (CGA 71019) residues in plants depends on the results from the *in vitro* genotoxicity tests that are in progress, it cannot be determined at present.

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Table B.6.10.8.1-1: Relevant studies for toxicity assessment of plant metabolites

Toxicological Study	Species; administration	Dose levels	Results	Reference
CGA71019 (Triazole)				
Acute oral toxicity	Rat gavage	250, 500, 1000, 1250, 1500, 1750 1850 (males only), 2000, 2500 mg/kg	LD ₅₀ (males): 1650 mg/kg bw LD ₅₀ (females): 1648 Classification: Xn, R22 (harmful if swallowed)	Thyssen, J. and Kimmerle, G., 1976
Bacterial reverse mutation assay	<i>Salmonella typhimurium</i> strains: TA98, TA100, TA1535 and TA 1537	10.0, 33.3, 100.0, 333.3, 1000 and 5000 µg/plate.	negative	Poth, A., 1989
CGA 131 013 (Triazole alanine)				
Acute oral toxicity	Rat; gavage/intraperitoneal Mouse; gavage	5000 mg/kg	LD ₅₀ > 5000 mg/kg No classification required	Mihail, F., 1982
Acute oral toxicity	Rat; gavage	2000 mg/kg	LD ₅₀ > 2000 mg/kg No classification required	Henderson, C. and Parkinson, G.R., 1980
<i>Salmonella</i> / mammalian-microsome mutagenicity test.	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 and TA 102.	20, 78, 313, 1250 and 5000 µg/plate +/-activation	negative	Deparade, E., 1986
<i>Salmonella</i> /E. coli <i>in vitro</i> / liver-microsome test.	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 <i>Escherichia coli</i> WP2 uvrA	312.5, 625, 1250, 2500 and 5000 /plate +/-activation	negative	Hertner, Th., 1993
<i>Salmonella</i> /microsome test	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 and TA 1538	20, 100, 500, 2500 and 12500 µg/plate +/-activation	negative	Herbold, B., 1983
Unscheduled DNA synthesis	Primary rat hepatocytes	80, 400, 2000 10000 µg/ml	negative	Puri, E., 1986
Mutation test on mammalian cells	Chinese hamster cells	500, 1000, 2000, 4000, 6000, 8000 and 10000 µg/ml +/-activation	negative	Dollenmeier, P., 1986
Micronucleus test <i>in vivo</i>	Chinese hamster (M/F)	5000 mg/kg bw	negative	Strasser, F., 1986
Micronucleus test <i>in vivo</i>	Mouse (M)	2500, 5000 mg/kg bw	negative	Watkins, P.A., 1982
Micronucleus test <i>in vivo</i>	Mouse (M/F)	8000 mg/kg bw	negative	Herbold, B., 1983c
CGA 142856 (Triazole acetic acid)				
Acute oral toxicity	Rat; gavage	5000 mg/kg	LD ₅₀ > 5000 mg/kg No classification required	Thevenaz, P., 1984

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Salmonella / mammalian-microsome mutagenicity test.	<i>Salmonella typhimurium</i> strains: TA98, TA100, TA1535, and TA1537.	20, 80, 320, 1280, and 5120 µg/plate +/-activation	negative	Deparade, E., 1984
In vitro Mammalian Cell Gene Mutation Test	L5178Y mouse lymphoma cells	0.63, 1.25, 2.5, 5, 10 mg/ml +/-activation	negative	Clare, G., 2002
In Vitro Mammalian Chromosome Aberration Test in Human Lymphocytes	Human Lymphocytes	Mitotic index: 0, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 mM Metaphase analysis: 2.5, 5 and 10 mM	negative	Pritchard L., 2002
CGA 205374				
Acute Oral Toxicity	Mouse; gavage	5000 mg/kg bw	LD ₅₀ > 5000mg/kg No classification required	Ohba, K (1991a)
Reverse mutation assay	<i>Salmonella typhimurium</i> strains: TA 98, TA 100, TA 1535, TA 1537 and <i>E. coli</i> WP2uvrA	156, 313, 625, 1250 and 2500 µg/plate +/-activation	negative	Nakajima, M (1991b)
CGA 205375				
Acute Oral Toxicity	Mouse; gavage	0, 1000, 1300, 1600, 2000, 2500 mg/kg	LD ₅₀ = 2309 mg/kg No classification required	Ohba, K (1991b)
Reverse mutation assay	<i>Salmonella typhimurium</i> TA 98, TA 100, TA 1535, TA 1537 and <i>E. coli</i> WP2uvrA	~2.5-320 µg/plate. depending on strain and presence/absence of metabolic activations	Negative	Nakajima, M (1991c)
CGA-189138				
Reverse mutation assay	<i>Salmonella typhimurium</i> strains: TA 98, TA 100, TA 1535 and TA 1537 (histidine-auxotrophic) and <i>E. coli</i> WP2uvrA (tryptophan-auxotrophic)	31.3 (62.5) - 1000 (2000) µg/plate depending on strain and presence/absence of metabolic activations	negative	Nakajima, M (1991a)

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B.6.10.8.2 Supplementary studies on the active substance

The supplementary studies of difenoconazole include an investigation of biochemical and morphological changes in the mouse liver and two cataract studies (performed in chickens and dogs respectively).

The first study was performed in order to further investigate and characterise the increased liver weight, hepatocellular hypertrophy and the adenomas/carcinomas observed in the long term study on mice.

Difenoconazole was administered daily to male mice during two weeks and after termination, the liver weights and the activities of various drug metabolising enzymes were analysed. In order to characterise the hepatotropic effects, the results obtained in mice treated with difenoconazole was compared to those obtained in mice treated with any of three different reference substances. Based on the results of this study, difenoconazole was considered to be a reversible barbiturate-type inducer of metabolising enzymes in the mouse liver and the highest dose of difenoconazole administered that did not induce metabolising enzymes and other parameters in the mouse liver was 10 mg/kg. No peroxisome proliferation was observed.

For a better understanding of the cataract findings observed in the six month study on dogs (O'Connor *et al*, section B.6.3.1.5), the cataractogenicity of difenoconazole was investigated in one study on young chickens and in one study on dogs.

Chickens are known to be sensitive to some cataractogenic substances and in this study they were fed difenoconazole daily at a concentration of 5000 ppm during 56 days. After one month of treatment, cataracts were observed in some animals. Although, the human relevance of these results is difficult to assess, this study is considered to strengthen the suspicion of difenoconazole as being cataractogenic.

In contrast, treatment of dogs with difenoconazole at concentrations between 3000 and 6000 ppm for 18 weeks did not result in the formation of cataracts. However, since only single animals were tested, the results of this study should be interpreted with caution.

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B6.10.9 Acceptable daily intake (ADI)

The acceptable daily intake (ADI) is derived from the NOEL in the most susceptible species in long-term toxicity and multi-generation reproduction studies with the application of an appropriate safety factor.

Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
ORAL:					
2-year combined chronic toxicity/oncogenicity in rat					
Cox, 1989a	0, 0.5/0.6, 1.0/1.3, 24.1/32.8 and 124/170 mg kg ⁻¹ day ⁻¹ (M/F)	Orally, via the diet	NOAEL: M/F: 1.0/1.3 mg kg⁻¹ day⁻¹ 20 ppm	LOAEL: M/F: 24.1/32.8 mg kg⁻¹ day⁻¹ 2500 ppm	2500 ppm: ↓ Body weight ↓ Body weight gain ↓ Food consumption (F) ↓ Carcass weight 500 ppm: ↓ Body weight gain 2500 ppm: ↓ RBC parameters, ↓ WBC parameters Dysproteinemia Altered clinical chemistry parameters 2500 ppm 500 ppm: ↓ Body weight (F) ↓ Hb (F) ↓ Platelet count (M) ↑ ALAT (M) Hepatocellular hypertrophy
Report Supplement: Saunders, 1992	0, 10, 20, 500, 2500 ppm		NOEL: M/F: 1.0/1.3 mg kg⁻¹ day⁻¹ 20 ppm	LOEL: M/F: 24.1/32.8 mg kg⁻¹ day⁻¹ 500 ppm	
18 months oncogenicity study in mice					
Cox, 1989b	0, 1.5/1.9, 4.7/5.6, 46.3/57.8 and 423/513 mg kg ⁻¹ day ⁻¹ (M/F) and 819 mg kg ⁻¹ day ⁻¹ for males at 4500 ppm 0, 10, 30, 300, 2500 (3000 1 st	Orally, via the diet	NOAEL: M/F: <46.3 /57.8 mg kg⁻¹ day⁻¹ 30 ppm	LOAEL: M/F: 46.3 /57.8 mg kg⁻¹ day⁻¹ 300 ppm	4500 ppm: ↑ ALP (M) 2500 ppm: ↑ ALAT ↑ Liver weight Hepatocellular carcinoma ↑ Liver weight (F) ↑ Sorbitoldehydrogenas (SDH) (M) Hepatocellular necrosis (M, F)

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
			NOEL: M/F: 4.7/5.6 mg kg ⁻¹ day ⁻¹ 30 ppm	LOEL: M/F: 46.3 /57.8 mg kg ⁻¹ day ⁻¹ 300 ppm	4500 ppm: ↓ Brain weight (6%) ↓ Testis weight (no dose- response) 2500 ppm: ↑ Carcass weight (not at term.) Altered WBC parameters Macroscopic hepatocellular enlargement Macroscopic hepatocellular masses Bile stasis Hepatocellular fatty change ↓ Body weight ↓ Body weight gain ↑ Liver weight (only females at interim) Hepatocellular hypertrophy Hepatocellular adenoma
1-year in dogs					
Rudzki et al., 1988	0, 0.71/0.63, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg kg ⁻¹ day ⁻¹ (M/F) 0, 20, 100, 500 and 1500 ppm	Orally via the diet	NOAEL: M/F: ≥ 51.2/44.3 mg kg⁻¹ day⁻¹ ≥ 1500 ppm NOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹ 100 ppm	LOAEL: Could not be established LOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹ 500 ppm	1500 ppm: ↓ Food consumption ↓ Body weight gain ↑ ALP (M)
M = male; F = female					

Difenoconazole has a low acute toxicity, is not a selective developmental or reproductive toxicant and does not produce neurotoxic effects. Difenoconazole was considered to be a reversible barbiturate-type inducer of metabolising enzymes in the mouse liver and treatment with Difenoconazole caused an increased incidence of adenomas/carcinomas in mice. In view of the lack of genotoxicity and the finding of tumours only in mice and only at concentrations at which toxicity was observed, the substance is considered not likely to pose a carcinogenic risk to humans.

A **safety factor of 100** is proposed to be sufficient for derivation of the ADI (comprising a factor of 10 for interspecies variations and an additional factor of 10 for intraspecies variations).

It is considered that the ADI is most appropriately derived from the NOEL/NOAEL from the 2 year rat study (since the rat appears to be the most sensitive species) as follows:

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$$ADI_{systemic} = \frac{NOAEL_{2-year, rat}}{SF} = \frac{1.0 \text{ mg / kg bw / day}}{100} = \underline{\underline{0.01 \text{ mg / kg bw / day}}}$$

B.6.10.10 Acceptable operator exposure level (AOEL).

Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
ORAL:					
28-day in rat					
Suter, P., 1986a	27/27, 156/166 and 914/841 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 156/166 mg kg ⁻¹ day ⁻¹ 1500 ppm	LOAEL: M/F: 914/841 mg kg ⁻¹ day ⁻¹ 10 000 ppm	↓ Body weight ↓ Carcass weight ↓ Organ weight
	0, 250, 1 500, 10 000 ppm		NOEL: M/F: <27/27 mg kg ⁻¹ day ⁻¹ <250 ppm	LOEL: M/F: 27/27 mg kg ⁻¹ day ⁻¹ 250 ppm	10 000 ppm: Altered clinical chemical parameters Altered blood parameters ↓ PT time Dysproteinemia
90-day in rat					
Suter, P., 1986b (Wistar rats)	0, 3.3/3.5, 19.9/21.4 and 120.9/ 128.5 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 20/21 mg kg ⁻¹ day ⁻¹ 250 ppm	LOAEL: M/F: 120.9/ 128.5 mg kg ⁻¹ day ⁻¹ 1500 ppm	↓ Body weight ↓ Carcass weight ↓ Heart weight (11%) ↓ Food consumption
	0, 40, 250 and 1500 ppm		NOEL: M/F: 3.3/3.5 mg kg ⁻¹ day ⁻¹ 40 ppm	LOEL: M/F: 20/21 mg kg ⁻¹ day ⁻¹ 250 ppm	1500 ppm: Altered blood parameters Altered clinical chemistry parameters Dysproteinemia ↑ Liver weight ↑ Serum albumin
Cox, R.H., 1987a (Sprague Dawley rats)	0, 1.3/1.7, 13/17, 51/66, 105/131 and 214/275 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 51/66 mg kg ⁻¹ day ⁻¹ 750 ppm NOEL: M/F: 1.3/1.7 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 105/131 mg kg ⁻¹ day ⁻¹ 1500 ppm LOEL: M/F: 13/17 mg kg ⁻¹ day ⁻¹	↓ Body weight ↓ Body weight gain
	0, 20, 200, 750, 1500 and 3000 ppm				1500 ppm: ↓ Carcass weight Altered clinical chemistry parameters Hepatocellular enlargement 750 ppm: ↓ RBC parameters dysproteinemia ↑ Liver weight
			20 ppm	200 ppm	↓ Body weight gain (F)
90-day in mouse					

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
Cox, R.H., 1987b	0, 3.3/4.6, 34.2/45.2 and 440/639 mg kg ⁻¹ day ⁻¹ (M/F) 0, 20, 200, 2500, (7500 and 15000) ppm	Orally via the diet	NOAEL: M/F: 34.2/45.2 mg kg⁻¹ day⁻¹ 200 ppm NOEL: M/F: 3.3/4.6 mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 440/639 mg kg⁻¹ day⁻¹ 2 500 ppm LOEL: M/F: 34.2/45.2 mg kg ⁻¹ day ⁻¹	↓ Ovary weight ↓ Body weight gain 2500 ppm: ↑ Liver weight Macroscopic liver enlargement Hepatocellular vacuolization Hepatocellular coagulative necrosis 20 ppm 200 ppm Hepatocellular enlargement
6 months in dog					
O'Connor et al., 1987	0, 3.6/3.4, 31.3/34.8, 96.6/110.6 and 157.8/203.7 mg kg ⁻¹ day ⁻¹ (M/F) 0, 100, 1000, 3000 and 6000 ppm	Orally via the diet	NOAEL: M/F: 31.3/ 34.8 mg kg⁻¹ day⁻¹ 1000 ppm NOEL: M/F: 3.6/ 3.4mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 96.6/110.6 mg kg⁻¹ day⁻¹ 3000 ppm LOEL: M/F: 96.6/110.6 mg kg ⁻¹ day ⁻¹	6000 ppm: ↓ Body weight ↓ Food consumption ↓ Carcass weight ↓ Prostate weight Cataract 6000 ppm: ↓ Ovary weight (n.s.) ↓ Uterus weight (n.s.) ↑ Platelet count ↓ Calcium Dysproteinemia 3000 ppm: ↑ Liver weight (F) ↑ ALP (F) 100 ppm 1000 ppm ↓ Food consumption (M)
1-year in dog					
Rudzki et al., 1988	0, 0.71/0.63, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg kg ⁻¹ day ⁻¹ (M/F) 0, 20, 100, 500 and 1500 ppm	Orally via the diet	NOAEL: M/F: ≥ 51.2/44.3 mg kg⁻¹ day⁻¹ ≥ 1500 ppm NOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	LOAEL: Could not be established LOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	1500 ppm: ↓ Food consumption ↓ Body weight gain 100 ppm 500 ppm ↑ ALP (M)
DERMAL:					
28-day in rat					
Gerspach, R., 2000	0, 10, 100 or 1000 kg ⁻¹ bw day ⁻¹	Dermal	NOAEL 1 000 kg⁻¹bw day⁻¹	LOAEL >1 000 kg⁻¹bw day⁻¹	

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
			NOEL: 100 kg ⁻¹ bw day ⁻¹		1000 ppm: ↑ Liver weight ↓ Bilirubin levels Hepatocellular hypertrophy ↓ Food consumption Hypertrophy of thyroid gland Hyperkeratosis of application site
M = male; F = female					

According to the guideline of setting the AOEL¹, subchronic toxicity data are appropriate for establishing an AOEL. There are two 90-day studies with rats. The first results in a NOAEL of 250 ppm (20/21 mg/kg bw/day (m/f)) driven by reduced food and water consumptions, reduced body weight, reduced carcass weight, reduced heart weight (Suter, 1986b). The second establishes a NOAEL of 750 ppm (51/66 mg/kg bw/day (m/f)) driven by a reduced body weight gain correlated with lower body weight (>10%) as compared with controls, in females (Cox, 1987a). Body weights were not statistically evaluated in this study by Cox (1987a). Although performed in different strains of rats, both studies result in comparable non-specific changes of non toxicological significance. In order to provide the most conservative estimate for acceptable operator exposure, it is justified to derive the systemic AOEL from the lower of these two NOAEL values, i.e. 250 ppm (equivalent to 20/21 mg/kg bw/day (m/f)). Due to the high bioavailability of difenoconazole (approximately 90% of the dose being absorbed and excreted in bile and urine) there is no need for a correction factor.

$$\text{AOEL} = \frac{\text{NOAEL}_{90 \text{ day rat}}}{\text{Safety factor}} = \frac{20.0 \text{ mg/kg bw/day}}{100} = 0.20 \text{ mg/kg bw/day}$$

B.6.10.11 Acute reference dose (ARfD)

Due to the degree of acute oral toxicity observed with difenoconazole (based on rat acute oral study by Argus *et al.* (1987) and the early deaths observed in the long term toxicity investigation in mice) it is considered necessary to establish an Acute Reference Dose (ARfD) for this compound. The most sensitive species is rat and thus the ARfD is derived from the 90 day toxicity study in rats. The toxicity profile is similar in the short term and long term toxicity studies with body weight as the most sensitive parameter. Body weight reductions occur already at 1st week of treatment (-4%) in the reproduction study at a similar dose as in the 90 day study but is not considered adverse at this time point. However, severe toxicity occurs in the long term toxicity study in mice where deaths occur during the first weeks of study the LOAEL for deaths is 2500 ppm or 423/513 mg/kg bw.

¹ AOEL Guideline for setting of acceptable operator exposure levels (AOELs). Draft. Sanco/xxx/2005 rev.8, 27 January 2005

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Table: Summary of repeated toxicity studies suitable for setting ARfD

Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
ORAL:					
28-day in rat					
Suter, P., 1986a	27/27, 156/166 and 914/841 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 156/166 mg kg⁻¹ day⁻¹ 1500 ppm	LOAEL: M/F: 914/841 mg kg⁻¹ day⁻¹ 10 000 ppm	↓ Body weight ↓ Carcass weight ↓ Organ weight
	0, 250, 1 500, 10 000 ppm		NOEL: M/F: <27/27 mg kg ⁻¹ day ⁻¹ <250 ppm	LOEL: M/F: 27/27 mg kg ⁻¹ day ⁻¹ 250 ppm	10 000 ppm: Altered clinical chemical parameters Altered blood parameters ↓ PT time Dysproteinemia
90-day in rat					
Suter, P., 1986b (Wistar rats)	0, 3.3/3.5, 19.9/21.4 and 120.9/ 128.5 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 20/21 mg kg⁻¹ day⁻¹ 250 ppm	LOAEL: M/F: 120.9/ 128.5 mg kg⁻¹ day⁻¹ 1500 ppm	↓ Body weight ↓ Carcass weight ↓ Heart weight (11%) ↓ Food consumption
	0, 40, 250 and 1500 ppm		NOEL: M/F: 3.3/3.5 mg kg ⁻¹ day ⁻¹ 40 ppm	LOEL: M/F: 20/21 mg kg ⁻¹ day ⁻¹ 250 ppm	1500 ppm: Altered blood parameters Altered clinical chemistry parameters Dysproteinemia ↑ Liver weight ↑ Serum albumin
Cox, R.H., 1987a (Sprague Dawley rats)	0, 1.3/1.7, 13/17, 51/66, 105/131 and 214/275 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 51/66 mg kg⁻¹ day⁻¹ 750 ppm	LOAEL: M/F: 105/131 mg kg⁻¹ day⁻¹ 1500 ppm	↓ Body weight ↓ Body weight gain
	0, 20, 200, 750, 1500 and 3000 ppm		NOEL: M/F: 1.3/1.7 mg kg ⁻¹ day ⁻¹ 20 ppm	LOEL: M/F: 13/17 mg kg ⁻¹ day ⁻¹ 200 ppm	1500 ppm: ↓ Carcass weight Altered clinical chemistry parameters Hepatocellular enlargement 750 ppm: ↓ RBC parameters dysproteinemia ↑ Liver weight ↓ Body weight gain (F)
90-day in mouse					
Cox, R.H., 1987b	0, 3.3/4.6, 34.2/45.2 and 440/639 mg kg ⁻¹ day ⁻¹ (M/F)	Orally via the diet	NOAEL: M/F: 34.2/45.2 mg kg⁻¹ day⁻¹ 200 ppm	LOAEL: M/F: 440/639 mg kg⁻¹ day⁻¹ 2 500 ppm	↓ Ovary weight ↓ Body weight gain
	0, 20, 200, 2500, (7500 and 15000) ppm		NOEL: M/F: 3.3/4.6 mg kg ⁻¹ day ⁻¹	LOEL: M/F: 34.2/45.2 mg kg ⁻¹ day ⁻¹	2500 ppm: ↑ Liver weight Macroscopic liver enlargement Hepatocellular vacuolization

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
			20 ppm	200 ppm	Hepatocellular coagulative necrosis
					Hepatocellular enlargement
6 months in dog					
O'Connor et al., 1987	0, 3.6/3.4, 31.3/34.8, 96.6/110.6 and 157.8/203.7 mg kg ⁻¹ day ⁻¹ (M/F) 0, 100, 1000, 3000 and 6000 ppm	Orally via the diet	NOAEL: M/F: 31.3/ 34.8 mg kg⁻¹ day⁻¹ 1000 ppm NOEL: M/F: 3.6/ 3.4mg kg ⁻¹ day ⁻¹	LOAEL: M/F: 96.6/110.6 mg kg⁻¹ day⁻¹ 3000 ppm LOEL: M/F: 96.6/110.6 mg kg ⁻¹ day ⁻¹	6000 ppm: ↓ Body weight ↓ Food consumption ↓ Carcass weight ↓ Prostate weight Cataract 6000 ppm: ↓ Ovary weight (n.s.) ↓ Uterus weight (n.s.) ↑ Platelet count ↓ Calcium Dysproteinemia 3000 ppm: ↑ Liver weight (F) ↑ ALP (F)
			100 ppm	1000 ppm	↓ Food consumption (M)
1-year in dog					
Rudzki et al., 1988	0, 0.71/0.63, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg kg ⁻¹ day ⁻¹ (M/F) 0, 20, 100, 500 and 1500 ppm	Orally via the diet	NOAEL: M/F: ≥ 51.2/44.3 mg kg⁻¹ day⁻¹ ≥ 1500 ppm NOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	LOAEL: Could not be established LOEL: 51.2/44.3 mg kg ⁻¹ day ⁻¹	1500 ppm: ↓ Food consumption ↓ Body weight gain
			100 ppm	500 ppm	↑ ALP (M)
DERMAL: 28-day in rat					
Gerspach, R., 2000	0, 10, 100 or 1000 kg ⁻¹ bw day ⁻¹	Dermal	NOAEL 1 000 kg⁻¹ bw day⁻¹ NOEL: 100 kg ⁻¹ bw day ⁻¹	LOAEL >1 000 kg⁻¹ bw day⁻¹	1000 ppm: ↑ Liver weight ↓ Bilirubin levels Hepatocellular hypertrophy ↓ Food consumption Hypertrophy of thyroid gland Hyperkeratosis of application site
2-generation reproductive toxicity in rat					
Giknis, MLA., 1988	0, 1.75, 17.3, 178.0	Orally via diet	NOEL/NOAEL (parental animals and pups):	LOAEL: approximately 178 mg/kg	↓ Body weight ↓ Food consumption

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Study	Dose levels	Adminis- tration way	NOAEL/ NOEL	LOAEL/ LOEL	Target organ and effects
	mg/kg/day (overall mean for males and females)		approximately 17.3 mg/kg bw/day	bw/day	
	0, 25, 250, 2500 ppm				
<i>Oncogenicity study in mice</i>					
Cox, 1989b	0, 1.5/1.9, 4.7/5.6, 46.3/57.8 and 423/513 mg kg ⁻¹ day ⁻¹ (M/F) and 819 mg kg ⁻¹ day ⁻¹ for males at 4500 ppm 0, 10, 30, 300, 2500 (3000 1 st two weeks) and 4500 (M) ppm	Orally, via the diet	NOAEL: M/F: <46.3 /57.8 mg kg⁻¹ day⁻¹ 30 ppm	LOAEL: M/F: 46.3 /57.8 mg kg⁻¹ day⁻¹ 300 ppm	4500 ppm: Mortalities (M, F) ↑ ALP (M) 2500 ppm: Mortalities (F) ↑ ALAT ↑ Liver weight Hepatocellular carcinoma ↑ Liver weight (F) ↑ Sorbitaldehydrogenas (SDH) (M) Hepatocellular necrosis (M, F) 4500 ppm: ↓ Brain weight (6%) ↓ Testis weight (no dose- response) 2500 ppm: ↑ Carcass weight (not at term.) Altered WBC parameters Macroscopic hepatocellular enlargement Macroscopic hepatocellular masses Bile stasis Hepatocellular fatty change ↓ Body weight ↓ Body weight gain ↑ Liver weight (only females at interim) Hepatocellular hypertrophy Hepatocellular adenoma
M = male; F = female					

$$\text{ARfD} = \frac{\text{NOAEL}}{\text{Safety factor}} = \frac{20 \text{ mg/kg bw/day}}{100} = 0.20 \text{ mg/kg bw/day}$$

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B.6.10.12 Drinking water limit

The annex VI of Council Directive 91/414/EEC states that the maximum permissible concentration of pesticide substances in ground water is 0.1 µg/L or 10% of the acceptable daily intake (ADI), whichever is lowest.

Assuming a daily average consumption of 2 L water per person and a body weight of 60 kg, the ground water concentration corresponding to 10% of the ADI for difenoconazole would be:

$$0.01 \times 0.1 \times 60 / 2 = 0.03 \text{ mg/l}$$

According to the discussion above, the drinking water limit for difenoconazole is thus 0.1 µg/L.

B.6.11 Acute toxicity including irritancy and skin sensitization of preparations (Annex IIIA 7.1)

B.6.11.1 DIVIDEND® 030 FS

B.6.11.1.1 Acute oral toxicity in rats – Limit Test: Dividend® 030 FS

Reference:	Cantoreggi, S. (1999a): Acute Oral Toxicity in the Rat (Limit Test)
Guideline:	EEC 92/69 B.1
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Dividend® 030 FS
Species / Strain:	Rat / HanIbm:WIST
Doses / No. of animals:	3000 mg/kg / 5/sex/dose
Administration:	Orally, by gavage
Exposure time / Duration:	Single dose, observation period of 14 days post dose

Materials and Methods:

The test material was suspended in distilled water. Animals were acclimatized for 5 days before treatment and 7-11 weeks old at initiation of the study. Body weights were recorded before dosing and on days 7 and 14. All animals were necropsied at termination.

Results:

There were no deaths, effects on body weight development or clinical signs indicative of treatment-related effects in the study. No visible lesions were observed at necropsy.

Conclusion:

The oral LD₅₀ of Dividend® 030 FS in rats was determined to be > 3000 mg/kg. No classification is required according to the Council Directive 67/548/EEC.

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B.6.11.1.2 Acute dermal toxicity in rats – Limit test: Dividend® 030 FS

Reference:	Cantoreggi, S. (1999b): Acute Dermal Toxicity in the Rat (Limit Test)
Guideline:	OECD TG 402, EEC 92/69 B.3
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Dividend® 030 FS
Species / Strain:	Hanlbm:WIST
Doses / No. of animals:	4000 mg/kg / 5 per sex per dose
Administration:	Dermally. The test article was held in place for 24 hours by a semi-occlusive wrapping.
Exposure time / Duration:	24 h / 14 days observation period post exposure

Materials and Methods:

Animals were acclimatized for 5 days before treatment and 8-12 weeks old at initiation of the study. An area of approximately 6 x 6 cm (corresponding to 10% of the body surface) was shaved on the back of each animal 24 h before treatment. After 24 h of exposure, the skin was cleaned with water and skin reaction was evaluated daily during the observation period according to the Draize method. Body weights were recorded before dosing and on days 7 and 14. All animals were necropsied at termination.

Results:

There were no deaths or clinical signs indicative of treatment-related effects in the study. There were no signs of irritation at the application site. Necropsy examinations revealed no visible lesions. **Body weight losses** occurred in two females (4 and 3%, respectively) during the first study week and there was no increase in body weight in two other females during the second week.

Conclusion:

The dermal **LD₅₀** of Dividend® 030 FS in rats was determined to be **> 4000 mg/kg**. **No classification is required** according to the Council Directive 67/548/EEC.

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B.6.11.1.3 Acute inhalation toxicity in rats – Limit test: Dividend® 030 FS

Reference:	Decker, U., Knappe, C. (1999): 4-Hour Acute Inhalation Toxicity Study in Rats
Guideline:	OECD TG 403; EEC 92/69 B.2; EPA OPPTS 870.1300
GLP:	Yes (certified laboratory), see comments
Acceptability:	See comments
Test substance:	Difenoconazole; formulation Dividend® 030 FS
Species / Strain:	Hanlbm:WIST(SPF) rats
Doses / No. of animals:	Limit test, 6.319 mg/L air / 5 per sex
Administration:	Inhalation, nose-only flow past inhalation
Exposure time / Duration:	4 h / 14 days observation period post exposure

Materials and Methods:

Animals were acclimatized for 6 days before treatment and 9-10 weeks old at exposure. Animals were exposed to an **aerosol** generated with diluted test material (1:1 with bi-distilled water). Body weights were recorded prior to exposure and on days 4, 8 and 15 (day of necropsy). All animals were necropsied at termination. The exposure conditions were as follows:

Table B.6.11.1.3 -1

Parameter	Value determined
Target Concentration mg/l	5
Achieved total Formulation Concentration mg/l	5.17
Mass median aerodynamic diameter (MMAD µm) (geometric standard deviation)	3.70, 3.89 2.33, 2.40
Particulate Gravimetric Concentration mg/l	5.148
Analyzed difenoconazole Concentration mg/l	1.201

Results:

No mortality was observed during the study period. Losses in **body weight** were seen in two males and two females ($\leq 4\%$), and were associated with the clinical sign of rales (wet, crackly lung noises heard on inspiration) in one of the males two and three days after exposure. Stress during restraint in the exposure tubes may have contributed to these findings, but an effect of treatment on the body weight development of the animals was not ruled out by the authors. No other clinical signs were seen during the 15 days observation period. Gross necropsy revealed no observable abnormalities.

Conclusion:

The inhalation **LC₅₀** of Dividend® 030 FS in rats was determined to be **> 6.32 mg/L**. **No classification is required** according to the Council Directive 67/548/EEC.

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Comments:

Technical trials performed before the animal phase of the study were not carried out in compliance with GLP regulations. The particle size of the aerosol could not be measured because of evaporation of some of the test material and consequently condensation inside the impactor used for measuring particle size, and because of adhesive properties of the test material. The chemically determined mean concentration of undiluted formulated test item in the test atmosphere (6.3 mg/l) was lower (41%) than the corresponding nominal concentration (10.8 mg/l), which could be due to adhesive properties of the test item leading to adherence of some of the test aerosol to the inner surface of the aerosol generation system.

B.6.11.1.4 Skin irritation: Dividend® 030 FS

Reference:	Cantoreggi, S. (1999c): Acute Dermal Irritation/Corrosion in the Rabbit.
Guideline:	OECD TG 404, EEC 92/69 B.4
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Dividend® 030 FS
Species / Strain:	Rabbit / New Zealand White
Doses / No. of animals:	0.5 ml undiluted test substance per animal / 3 males
Administration:	Dermally, occluded conditions. See comments.
Exposure time / Duration:	4 h / 7 d observation period post treatment

Materials and Methods:

Animals were acclimatized for 5 days before treatment and 2-4 months old at exposure. Twenty-four hours before treatment an area, approximately 10 cm wide from one flank to the other (across the back) was shaved. The test material was applied to the shaved skin on one flank by means of a 2 x 3 cm gauze patch. A control patch moistened with distilled water was applied to the other flank. The patches were **covered with household aluminium foil** held in place with tape. The dressings were removed after 4 h and the area was washed with water to remove any remaining test substance. The test sites were examined and scored according to the Draize method at 1, 24, 48 and 72 h and at 7 days (sufficient for reversibility) after patch removal.

Results:

The test substance caused skin irritation in all animals beginning 1 hour after application and persisted through 48 or 72 hours. All skin reactions were fully reversed within 7 days after patch removal.

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Table B.6.11.1.4 -1: Individual and mean skin irritation scores according to the Draize scheme

	Erythema and Eschar			Oedema		
Animal no.	186	051	073	186	051	073
after 1 hour	1	1	2	1	1	2
after 24 hours	2	1	2	1	0	2
after 48 hours	2	1	2	1	0	1
after 72 hours	1	0	1	0	0	0
mean score 24-72 h	1.34			0.56		
after 7 days	0	0	0	0	0	0
Additional criteria specified in Directive 1999/45/EC Point 3.2.6.1 fulfilled: Yes/No						

Loss of body weight (0.3-3%) was recorded in all animals between study start and day 3. There were no other clinical signs or mortality during the test period.

Conclusion:

Based on the degree of the skin reactions (mean skin irritation scores 24 to 72 hours after removal of the test material; 1.34 and 0.56) **no classification is required** according to the Council Directive 67/548/EEC.

Comments:

The authors states occluded test conditions in the original report (summary, page 6), whereas the conditions are stated to be semi-occluded by the sponsor (M-III Section 3 (DIVIDEND) ERA5711 (F)-report, page 5). The OECD TG 404 (2002) recommends semi-occlusive test conditions

B.6.11.1.5 Eye irritation: Dividend® 030 FS

Reference:	Cantoreggi, S. (1999d): Acute Eye Irritation/Corrosion in the Rabbit.
Guideline:	OECD TG 405, EEC 92/69 B.5
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Dividend® 030 FS
Species / Strain:	Rabbit / New Zealand White
Doses / No. of animals:	0.1 ml / 3 females
Administration:	Instillation into the everted lower lid of the left eye
Exposure time / Duration:	72 h (sufficient to determine reversibility of the effects)

Materials and Methods:

Animals were acclimatized for 5 days before treatment and 3-5 months old at exposure. The test substance was instilled into the conjunctival sac and the lids were held together for a second. Eyes were examined and scored according to the Draize method at 1, 24, 48 and 72 h after treatment. Body weights were recorded prior to dosing and at termination (72 h).

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Results:

Hyperaemic conjunctival blood vessels were noted in all three animals beginning 1 hour after application and persisting through 24 or 48 hours. All eye reactions were clear within 72 hours after treatment.

Table B.6.11.1.5-1: Eye irritation scores according to the Draize scheme

	Cornea			Iris			Conjunctival redness			Conjunctival chemosis		
Time/Rabbit	158	034	048	158	034	048	158	034	048	158	034	048
after 1 hour	0	0	0	0	0	0	1	1	1	0	0	0
after 24 hours	0	0	0	0	0	0	1	1	1	0	0	0
after 48 hours	0	0	0	0	0	0	1	1	0	0	0	0
after 72 hours	0	0	0	0	0	0	0	0	0	0	0	0
mean scores 24-72h	0			0			0.56			0		
Additional criteria specified in Directive 2001/59/EC Point 3.2.6.2 fulfilled: Yes/No												

There were no other **clinical signs** or **mortality** during the test period. **Body weights** were not affected by treatment.

Conclusion:

Based on the degree of the eye reactions (mean conjunctival redness score 24 to 72 hours after instillation of the test material; 0.56) **no classification is required**, according to the Council Directive 67/548/EEC.

B.6.11.1.6 Skin sensitization in Guinea Pigs - Maximization Test: Dividend® 030 FS

Reference:	Cantoreggi, S (1999e): Skin Sensitization in the Guinea Pig (Maximization Test).
Guideline:	OECD TG 406, 96/54/EC IV. C
GLP:	Yes (certified laboratory)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Dividend® 030 FS
Species / Strain:	Guinea pig / Himalayan Spotted (GOHI)
Doses / No. of animals:	<u>Intradermal Induction Pre-test:</u> 0.5, 1.0, 3.0 and 5.0% in physiological saline, <u>Epidermal Induction Pre-test:</u> 30, 50 and 80% in physiological saline and 100% (undiluted), <u>Intradermal Induction:</u> 5.0% in physiological saline, <u>Epidermal Induction:</u> 100% (undiluted), <u>Epidermal Challenge:</u> 50% in physiological saline / 10 per sex (controls 5 per sex)
Administration:	Intradermal and topical induction; Topical challenge
Exposure time / Duration:	<u>Epidermal induction:</u> 48 hours, <u>Epidermal Challenge:</u> 24 hours / 48 h observation period post challenge

Materials and Methods:

Animals were acclimatized for 5 days before treatment and 1-3 months old at initiation of experiment.

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Intradermal Induction Pre-test:

A 5-cm wide area in the neck region of two animals (1/sex) was shaved 1 h before pair wise intradermal injections of adjuvance and test substance. Injection volume was 0.1 ml and one injection of each pair was injected on each side of the spine. Examinations to determine the highest concentration to produce mild to moderate irritation without systemic toxicity were performed after 24 and 48 h.

Epidermal Induction Pre-test:

A 5-cm wide area in the neck region of two animals (1/sex) was shaved 1 h before pair wise intradermal injections of 1:1 mixture of FCA/physiological saline. Injection volume was 0.1 ml and one injection of each pair was injected on each side of the spine. Seven days later the test substance was applied with Hill Top Chambers™ - two on each flank on each animal. Examinations to determine the highest concentration to produce irritation (for the induction application) and no irritation (for the challenge application) were performed after 24 and 48 h.

Intradermal Induction – day 0:

An area of 5 x 5 cm in the neck region was shaved 1 h before three pairs of injections (0.1 ml) were given – one of each pair on each side of the midline. The injections for the test group consisted of adjuvant/saline mixture (1:1), test substance in saline and finally test substance in adjuvant/saline mixture.

Epidermal Induction – day 8:

A patch with (0.4g) test substance (or saline for controls) was applied to the induced area and held in place for 48h.

Epidermal Challenge – day 21:

One chamber with test substance/vehicle mixture (0.4 ml) was placed on one flank and one chamber with vehicle only was placed on the other flank of the animals of both groups and held in place for 24 h.

Skin reactions after challenge were scored 24 and 48 h after removal of the challenge dressings. The dermal reactions were scored according to the Draize scale. **See comments.**

Results:

Intradermal Induction Pre-test:

The 5.0% concentration was selected for the induction injection for the definitive test.

Epidermal Induction Pre-test:

The 100% (undiluted) concentration was selected for epidermal induction. The 50% concentration was selected for the epidermal challenge application as it produced no skin irritation.

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Epidermal Challenge:

Positive skin reactions were seen on the test flanks in two females at 24 hours and in one of these females at the 48-hour reading, corresponding to a **sensitisation rate of 10%**.

Table B.6.11.1.6-1: Maximization test: Number of animals with signs of allergic skin reactions

	Vehicle flank		Test flank	
Scored after:	24 hours	48 hours	24 hours	48 hours
Vehicle control group	0/10	0/10	0/10	0/10
Test article group	0/20	0/20	2/20	1/20

There were no other **clinical signs** or **mortality** during the test period. **Body weights** were not affected by treatment.

Conclusion:

Based on the degree of the skin reactions (sensitization rate 10%) **no classification is required** according to the Council Directive 67/548/EEC.

Comments:

Because of the staining properties of the test article, the skin application site had to be depilated after the epidermal application (at pre-test) and after challenge - before skin reactions could be evaluated. The OECD TG (No. 406, 1992) states that dermal reactions should be performed according to the Magnusson & Kligman grading scale. However, in this report the Draize scale was used.

B.6.11.1.7 Dividend: Summary of acute toxicity including irritancy and skin sensitization of preparations

The acute toxicity of the test substance A-9142 G is low. It is non-irritating to the skin and eye of rabbits. It does not cause sensitisation by skin contact. According to Council Directive 1999/45/EEC, **no classification for health effects is required**. Since the particle size of Difenoconazole could not be measured during the conditions used in the acute inhalation study, an LC₅₀ value cannot be established. However, according to Annex II of the Council Directive 91/414/EEC, acute inhalation toxicity testing was not necessary as the physico-chemical properties and the approved application scenarios are such that virtually no inhalative exposure will occur during the use of the product.

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Table B.6.11.1.7-1: Acute toxicological data obtained with A-9142 G

Parameter [Reference]	Species Strain; Total No. of animals used	Result mg/kg or effect
Acute oral LD ₅₀ [Cantoreggi 1999a]	Rat Wistar Han; 5 per sex	> 3000 mg/kg
Acute dermal LD ₅₀ [Cantoreggi 1999b]	Rat Wistar Han; 5 per sex	> 4000 mg/kg
Acute inhalation LC ₅₀ [Decker, Knuppe 1999]	Rat Wistar; 5 per sex	n.d.
Skin irritation [Cantoreggi 1999c]	Rabbit New Zealand White (Chbb:NZW); 3 M	non-irritating
Eye irritation [Cantoreggi 1999d]	Rabbit New Zealand White (Chbb:NZW); 3 F	non-irritating
Skin sensitisation – Maximisation Test [Cantoreggi 1999e]	Guinea Pig Himalayan spotted (GOHI); 10 per sex	non-sensitising

B.6.11.2 SCORE® 250 EC**B.6.11.2.1 Acute oral toxicity in rats: Score® 250 EC**

Reference:	Kuhn JO (2003a), CGA-169374 EC (250) (A7402T): Acute Oral Toxicity Study In Rats.
Guideline:	92/69/EEC B.1 (1992)
GLP:	Yes, verified by the US EPA.
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Score® 250 EC (A-7402 T)
Species / Strain:	Rat / Sprague-Dawley
Doses / No. of animals:	<u>Limit test</u> : 5050 mg/kg, <u>Full test</u> : 175, 550, 1750 and 5000 mg/kg bw / 9 females in total
Administration:	Orally, by gavage
Exposure time / Duration:	Single dose, observation period of 14 days post dose

Materials and Methods:

Animals were acclimatized for 5 days before treatment. The initial limit test resulted in the death of the dosed animal and therefore a full test (following the (UDP) up-and-down procedure) was initiated.

Body weights were recorded before dosing and on days 7 and 14. All animals were subjected to gross necropsy. The LD₅₀ value with 95% confidence interval was calculated using the AOT425 Stat program by EPA.

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Results:

No **mortality** occurred at the 175, 550 or 1750 mg/kg levels whereas all three animals dosed at the 5000 mg/kg level died within two days. **Necropsy** of the animals that died revealed crusted/wet/stained fur; discoloured lungs and/or liver; gas and discoloured contents in the gastrointestinal tract, and empty stomach and/or intestines. No abnormalities were noted in animals that survived until the end of the study.

Clinical signs in animals dosed with ≥ 1750 mg/kg included hypothermia (in one animal that died), decreased activity and diarrhoea. Signs were observed on the day of dosing through day 5. No abnormalities were seen in animals dosed with 175 or 550 mg/kg. **Bodyweight** gain in surviving animals was unaffected by treatment.

Conclusion:

The acute oral **LD₅₀** (95% Confidence Interval) in female rats was estimated to be **3129** (1750-5000) mg/kg. **No classification is required** according to the Council Directive 67/548/EEC.

Comments:

The selection of dose and number of animals per dose deviates from the OECD TG Nos. 420 and 423, which are equivalent with the stated guideline (92/69/EEC B.1 (1992)). A total of 5 (1+4) or alternatively 6 (3+3) animals are normally used **for each dose level** investigated, and a dose level that caused death in a limit test (5000 mg/kg) should not be revisited in a main study.

B.6.11.2.2 Acute dermal toxicity in rats –Limit test: Score® 250 EC

Reference:	Johnson I (2004), CGA 169374 EC 250 (A-7402 T): Acute Dermal Toxicity Study In The Rat.
Guideline:	92/69/EEC B.3 (1992)
GLP:	Yes (laboratory certified by the UK authority)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Score® 250 EC (A-7402 T)
Species / Strain:	Rat / Alpk:AP _F SD
Doses / No. of animals:	5000 mg /kg bw / 5 per sex
Administration:	Dermally. The test article was held in place for 24 h by an occlusive wrapping (foil backed gauze patch).
Exposure time / Duration:	24 h / 14 days observation period post dose

Materials and Methods:

Animals were acclimatized for 5 days before treatment and 8-12 weeks old at initiation of the study. An area of approximately 7 x 7 cm was shaved on the back of each animal 24 h before treatment. After 24 h of exposure, the skin was cleaned with water and skin reaction was evaluated daily during the observation period. Body weights were recorded before dosing and on days 8 and 15. All animals were necropsied at termination.

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Results:

There were no **deaths** in this study. **Clinical signs** included vocalisation, aggression or abnormal respiratory noise in five out of ten animals (3 males and 2 females). These signs were considered to be a result of bandaging and not to be compound-related. **Skin irritation** was seen in all animals and desquamation was still apparent in one male and three females at the end of the study. All animals but one female lost weight between study days 1 through 8, but all animals but one female had an overall **gain of body weight** during the study. No treatment-related findings were observed at **necropsy**.

Table B.6.11.2.2-1 : Body weights (g) of rats treated with 5000 mg/kg A-7402 T (Score® 250 EC) dermally

Animal No.	Days after treatment	
	8	15
170 M	300	349
	↓ 3%	
171 M	322	369
	↓ 5%	
172 M	319	374
	↓ 6%	
173 M	287	325
	↓ 6%	
174 M	318	366
	↓ 4%	
226 F	220	236
227 F	229	246
	↓ 2%	
228 F	222	238
	↓ 2%	
229 F	214	233
	↓ 3%	
230 F	218	208
	↓ 0.9%	↓ 5%

Conclusion:

The dermal **LD₅₀** of Score® 250 EC was estimated to be > 5000 mg/kg to male and female rats. **No classification is required** according to the Council Directive 67/548/EEC.

WARNING: This document forms part of an EC evaluation data package and should not be read in isolation. Registration must not be granted on the basis of this document.

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B.6.11.2.3 Acute inhalation toxicity in rats - Limit test: Score® 250 EC

Reference:	Noakes J (2003), CGA 169374 EC 250 (A-7402 T): 4-Hour Acute Inhalation Toxicity Study In Rats.
Guideline:	OECD TG 403, 92/69/EEC B.2 (1992)
GLP:	Yes (laboratory certified by the UK authority)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Score® 250 EC (A-7402 T)
Species / Strain:	Rat / Alpk:APfSD
Doses / No. of animals:	5 mg/L / 5 per sex
Administration:	Inhalation, nose-only
Exposure time / Duration:	4 h / 14 days observation period post exposure

Materials and Methods:

Animals were acclimatized for 5 days before treatment and 7-8 weeks old at exposure to an **aerosol**. The test atmospheres were analysed for particulate concentration and CGA 169374 concentration. Body weights were recorded on days -1, 1, 8 and 15 (day of necropsy). All animals were necropsied at termination.

Table B.6.11.2.3-1: summary of test atmosphere characteristics

Achieved Concentrations (mg/l air) ± SD		
Nominal	Gravimetric	Analytical
10.781	2.985 0.495 (n=4)	6.319 0.356 (n=4)

Results:

The mean total formulation concentration (± SD) was calculated to be 5.17 ± 0.34 . **One male died** on day 3.

Clinical signs during exposure included reduced response to sound, reduced breathing rate and increased breathing depth in all animals. Immediately after exposure decreased activity, reduced foot withdrawal reflex, reduced response to sound were observed in most animals and increased breathing depth reduced breathing rate and abnormal breathing sounds were observed in all animals. Also, salivation and lacrimation was recorded in most animals. All animals had test substance around the snout. During the subsequent observation period clinical signs included gasping (2 males), staining around the nose or mouth (all males and 2 females) and urinary staining for up to four days after exposure. Respiratory noise persisted for both males and females up to day 10. All animals had fully recovered by day 11 of the study. Two females lost body weight during the study.

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Table B.6.11.2.3-2: Individual body weights (g)

Day	Females				
	6	7	8	9	10
1	263	214	263	232	219
8	259 ↓ 2%	248	256 ↓ 3%	253	243
15	252 ↓ 4%	252	251 ↓ 5%	277	247

Necropsy findings for the male found dead on day 3 included staining of the nares and mouth. **These findings were considered to be related to treatment but of uncertain significance.** No necropsy findings were recorded for animals surviving to termination.

Conclusion:

There was one death and clinical signs of distress during the study. The inhalation LC_{50} of Score® 250 EC is **>5.17 mg/L. No classification is required** according to the Council Directive 67/548/EEC.

B.6.11.2.4 Skin irritation: Score® 250 EC

Reference:	Johnson IR (2003a), CGA 169374 EC 250 (A-7402 T): Skin Irritation Study In Rabbits.
Guideline:	OECD TG 404 (2002), 92/69/EEC B.4 (1992)
GLP:	Yes (laboratory certified by the UK authority)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Score® 250 EC (A-7402 T)
Species / Strain:	Rabbit / New Zealand White
Doses / No. of animals:	0.5 ml undiluted test substance per animal / 3 (one male and two females)
Administration:	Dermally, semi-occluded conditions.
Exposure time / Duration:	4 h / 10 d observation period post treatment

Materials and Methods:

Animals were acclimatized for 5 days before treatment. Twenty-four hours before treatment an area of 7 x 13 cm was shaved on the left flank of each animal. The test material was applied to shaved skin by a sterile syringe and the treated area was covered with a 2.5 x 2.5 cm gauze patch and tape. The dressings were removed after 4 h and the area was washed with water to remove any remaining test substance. The test sites were examined and scored according to the Draize method at 1, 24, 48 and 72 h and at intervals up to 10 days (sufficient for reversibility) after patch removal. Bodyweights were recorded at the start of the study only.

Results:

The test substance caused erythema and oedema in two out of three animals beginning 24 hours after application and persisting through 4 or 7 days. Additional signs of irritation in these animals included bleeding, cracking and

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scabbing in one and desquamation in both animals. All signs of irritation had completely resolved within 10 days of application.

Table B.6.11.2.4-1: Individual and mean skin irritation scores according to the Draize scheme

Animal ID.	Erythema and Eschar			Oedema		
	39 Male	53 Female	54 Female	39 Male	53 Female	54 Female
after 1 hour	0	0	0	0	0	0
after 24 hours	0	1	1	0	1	1
after 48 hours	0	0	0	0	1	1
after 72 hours	0	1 bl, cr, des	1	0	1	1
mean score 24-72 h	0	0.7	0.7	0	1	1
after 4 days	-	1 cr, sc	2 des	-	3	3
after 7 days	-	1	0	-	0	0
after 10 days	-	0	-	-	0	-

Key: sc: scabs bl: bleeding cr: cracked des: desquamation

Additional criteria specified in Directive 2001/59/EC Point 3.2.6.1 fulfilled: No

Conclusion:

Based on the degree of the skin reactions (mean skin irritation scores 24 to 72 hours after removal of the test material; 0.7 and 1.0) **no classification is required** according to the Council Directive 67/548/EEC.

B.6.11.2.5 Eye irritation: Score® 250 EC

Reference:	Johnson IR (2003b), CGA 169374 EC 250 (A-7402T): Eye Irritation Study In Rabbits.
Guideline:	OECD TG 405 (2002), 92/69/EEC B.5 (1992)
GLP:	Yes (laboratory certified by the UK authority)
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Score® 250 EC (A-7402 T)
Species / Strain:	New Zealand White rabbits
Doses / No. of animals:	0.1 ml / 3 (one female and two males)
Administration:	Instillation into the everted lower lid of the left eye
Exposure time / Duration:	The eyes were examined for up to 21 days

Materials and Methods:

Animals were acclimatized for 5 days before treatment. Undiluted test substance was instilled into the conjunctival sac and the lids were held together for 1-2 seconds. Eyes were examined and scored according to the Draize method at 1, 24, 48 and 72 h after treatment and then at intervals up to 21 days. Immediately after instillation, an assessment of pain reaction was made using a 0-5 scale. Additionally, a modified form of the Kay and Calandra system (1962) and the EPA Label Review Manual (1999) were used to interpret and classify the eye reactions. Body weights were recorded prior to dosing only.

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Results:

Instillation into the eye caused slight initial pain (class 2 on a 0-5 scale). **Conjunctival** effects, beginning 1 hour after application, were seen in all animals and included redness, chemosis, discharge, and haemorrhage.

Corneal opacity and irregular corneal surface were seen in all animals and neovascularisation in two animals.

Iritis was seen in one animal for 2 days. Additional signs of irritation consisted of: lachrymatory or mucoid discharge; erythema, oedema, thickening and convulsion of the eyelids; haemorrhage of the nictitating membrane and dried secretion around the periorbital skin. All signs of irritation had completely resolved within 21 days of instillation.

Table B.6.11.2.5-1: Eye irritation scores according to the Draize scheme

Time	Cornea			Iris			Conjunctiva Redness			Chemosis		
	45 female	54 male	55 male	45 female	54 male	55 male	45 female	54 male	55 male	45 female	54 male	55 male
after 1 hour	0	1	1	0	0	0	2	1	1	2	2	2
after 24 hours	1	1	1	0	0	1	2	1	2	1	2	2
after 48 hours	1	1	1	0	0	1	2	1	2	1	2	2
after 72 hours	1	1	1	0	0	0	2	1	2	1	2	1
Individual mean scores 24-72h	1	1	1	0	0	0.7	2	1	2	1	2	1.7
after 7 days	1	1	0	0	0	0	1	0	0	1	0	0
after 10 days	0	1	-	0	0	-	0	1	-	0	0	-
after 14 days	1	1	-	0	0	-	0	0	-	0	0	-
after 17 days	1	0	-	0	0	-	0	0	-	0	0	-
after 21 days	0	0	-	0	0	-	0	0	-	0	0	-

Additional criteria specified in Directive 2001/59/EC Point 3.2.6.2 fulfilled: No

There were no other **clinical signs** or **mortality** during the test period.

Conclusion:

Based on the degree of the eye reactions (mean scores for cornea, iris and conjunctiva < their respective trigger values, 24 to 72 hours after instillation of the test material) **no classification is required** according to the Council Directive 67/548/EEC.

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B.6.11.2.6 Skin sensitization in Guinea Pigs – BuehlerTest: Score® 250 EC

Reference:	Kuhn JO (2003b), CGA-169374 EC (250) (A7402T): Skin Sensitisation Study In Guinea Pigs.
Guideline:	OECD TG 406 (1992), 96/54/EC B.6 (1996)
GLP:	Yes, verified by the US EPA
Acceptability:	Yes
Test substance:	Difenoconazole; formulation Score® 250 EC (A-7402 T)
Species / Strain:	Guinea pig / Hartley Albino
Doses / No. of animals:	<u>Epidermal Induction</u> : 0.4 ml, 100% undiluted, <u>Epidermal Challenge</u> : 100% undiluted / 10 per sex (controls 5 per sex)
Administration:	Topical induction, topical challenge
Exposure time / Duration:	<u>Induction</u> : 9 x 6-h periods, <u>Challenge</u> : 1 x 6-h period / 48-h observation period post challenge

Materials and Methods:

Body weights were recorded on days 0 and 35.

Epidermal Irritation Screening Pre-test:

An additional four animals were used to determine the maximum dose producing moderate irritation and the maximum non-irritating dose.

Epidermal Induction – days: 0-20:

An area of 8 x 10 cm on the back of the trunk was shaved 24 h before application. Test substance was applied on a gauze patch 2.5 x 2.5 cm, under an occlusive dressing (polyethene film). Control animals were left un-treated during the induction phase.

Epidermal Challenge – day 36:

The test substance was applied, in a manner identical to the induction treatments, to a previously untreated skin site to all animals, including controls. Skin sites were examined 24 and 48 hours after removal of the dressings.

A test substance is considered a sensitizer if the mean scores, the total number of animals with scores and/or the total number of scores for the virgin test site in the tested animals, after challenge, are appreciably greater than those for the control animals.

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Results:*Epidermal Challenge:*

The test substance produced faint irritation (reaction scores 0.5) in one of 10 naïve control animals and in one of 20 test animals after challenge.

Table B.6.11.2.6-1: Number of animals with signs of allergic skin reactions

	Test flank	
Scored after:	24 hours	48 hours
Naïve control group	1/10	0/10
Test item group	1/20	0/20

Conclusion:

Based on the degree of the skin reactions (sensitization rate <15%) **no classification is required**, according to the Council Directive 67/548/EEC.

B.6.11.2.7 Score: Summary of acute toxicity including irritancy and skin sensitization of preparations

According to Council Directive 1999/45/EC, classification of the formulation A-7402 T is not required for its acute oral, dermal or inhalation toxicity.

Table B.6.11.2.7-1: Summary of acute toxicity studies with A-7402 T

Parameter [Reference]	Species Strain; Total No. of animals used	Result mg/kg or effect
Acute Oral LD ₅₀ [Kuhn, J.O. 2003a]	Rat Sprague Dawley; 9 F	3129 mg/kg
Acute Dermal LD ₅₀ [Johnson, I. R. 2004]	Rat Alpk:AP ₁ SD; 5 per sex	> 5000 mg/kg
Acute Inhalation LC ₅₀ [Noakes, J 1988]	Rat Alpk:ApfSD; 5 per sex	> 5.17 mg/L
Skin irritation [Johnson, I. R. 2003a]	Rabbit New Zealand White, 3	non-irritating
Eye irritation [Johnson, I. R. 2003b]	Rabbit New Zealand White, 3	non-irritating
Skin sensitisation – Buehler Test [Kuhn, J.O. 2003b]	Guinea Pig Hartley albino; 15 per sex	non-sensitising

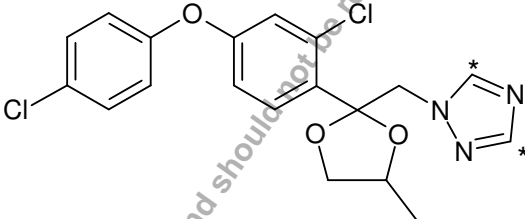
Comments:

The systemic, broad-spectrum **fungicide** A-7402 T (Score[®] 250 EC) is an emulsifiable concentrate containing 250 g/L difenoconazole. It is **intended** for use as a **foliar spray** to control a broad-spectrum of diseases, caused by Ascomycetes, Basidiomycetes and Deuteromycetes, in pome fruit and vegetables. The **maximum** recommended use rate for pome fruit is **75 g a.i./ha**, and the timing of the application is beginning at BBCH 61, with 1 to 4 applications spaced by minimal intervals of 7 to 10 days. The maximum use rate for carrots is **125 g/ha**.

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B.6.12 Dermal absorption (Annex IIIA 7.3)

B.6.12.1 Dermal absorption of Triazole-U-¹⁴C *in vivo* in the rat

Reference:	Hassler S., (2003c). Dermal Absorption of [Triazole-U-¹⁴C] CGA 169374 Formulated as SCORE® 250 EC (A-7402 G) in the Rat (<i>in vivo</i>).	
Guideline:	OECD TG 427 (2000)	
Deviations from TG 427:	The high dose group (2558 µg/cm ² - P3) showed a very high variation of dermal absorption and was therefore repeated (dose group P3a). Results of both high dose groups are given in the original report. The relative humidity exceeded 70% for the middle (50-80%) and the high dose groups (P3), (48-80%), thereby deviating from the recommended relative humidity of 50-60%, never exceeding 70%.	
GLP:	Yes, certified laboratory.	
Acceptability:	Yes	
Test substance / purity:	¹⁴ C-radiolabeled [Triazole-U-14C] Difenconazole, formulated as SCORE® 250 EC (A-7402 G), see comments.	
		
	* = ¹⁴ C position	
	(Non-radiolabelled difenconazole / 99.3% used for the high-dose level)	
Specific activity:	2700 kBq/mg (73 µCi/mg)	2700 kBq/mg (73 µCi/mg)
Radiochemical purity:	98.0%	99.3%
Species / Strain:	Rat / HanBrl: WIST (SPF)	
Doses / No. of animals:	0.0005, 0.0125, 2.5 mg/cm² / 16 animals/dose group (further divided into termination groups at 6, 24, 48, and 72 h after start, with 4 animals/ termination)	
Administration:	Dermally, non-occlusive conditions	
Exposure time / Duration:	6 h (based on anticipated exposure of a plant and field worker) / 72 h observation period post administration	

Materials and Methods:

Test substance was applied to appropriate dorsal areas and confined by a non-absorbing 'O'-ring glued to the skin using an adhesive. The 'O'-ring was covered with a permeable tape and a collar was put around the neck of the rats. After exposure, excess test substance was washed away five times with a soap solution and the skin wash was analysed for radioactivity. A fresh cover tape was applied to the 'O'-ring after the last washing and a

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bandage was applied around the animals in order to prevent detachment of the glued 'O'-ring during the 72 h observation period. The low- and the middle dose levels represent the range of typical concentrations recommended for use in stone fruits and field crops, respectively. The high dose level represents the undiluted formulation (250 g a. i./l).

Urine and faeces were collected from animals individually during the periods of 0-6, 6-24, 24-48, and 48-72 h after application. **Blood** was collected from all animals at sacrifice and in addition at 0.5, 1, 2, 4, 6, 8, 24 and 48 h after application from animals of termination group 72 h. At necropsy, the 'O'-ring and the cover was carefully removed and extracted with solvents.

Results:

General observations

An un-specified number of animals showed symptoms of stress, with chromodacryorrhea during the first hours after administration. There was also **weight loss** between start of exposure and termination throughout all dose groups, generally below 10% but individual rats had weight reductions around or above 10%. The highest reduction in body weight was minus 16%, recorded in a rat of the highest dose group (2.5 mg/cm²). Statistical analysis was not performed on body weights.

Absorption and Excretion

Dermal absorption was calculated as the sum of the systemic absorption and the amount remaining in the treated skin (Table B.6.12.1-1). After 6 h of exposure, these values were approximately 18, 9 and 7% of the dose at the low, middle and high dose, respectively and the corresponding **maximum average dermal systemic absorption** was **38%** (after 24 h), **15%** (after 24 h) and **14%** (after 48 h). A very high degree of variation in absorption values was observed both in the original high dose group (P3) and in the repeated high dose group (P3a). The maximum values obtained in single animals are shown in bold in table Table B.6.12.1-1).

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Table B.6.12.1-1: Summary of dermal absorption and excretion of difenoconazole, formulated as SCORE® (A-7402 G) through rat skin [% of dose]. Maximum individual values are shown in bold.

Dose Level	Low Dose 0.5 µg/cm²				Middle Dose 13 µg/cm²				High Dose 2558 µg/cm²			
Sacrifice Time Point (h)	6	24	48	72	6	24	48	72	6	24	48	72
Urine												
0 - 6 h	0.20	0.15	0.59	0.35	0.11	0.07	0.09	0.12	0.02	0.01	0.01	0.01
6 - 24 h	-	3.51	2.13	2.39	-	1.26	1.20	1.08	-	0.13	0.18	0.11
24 - 48 h	-	-	1.89	2.03	-	-	0.70	0.96	-	-	0.71	0.13
48 - 72 h	-	-	-	0.86	-	-	-	0.44	-	-	-	0.10
Subtotal	0.20	3.66	4.60	5.64	0.11	1.33	1.99	2.59	0.02	0.14	0.90	0.36
Faeces												
0 - 6 h	0.01	0.08	< 0.01	0.06	0.03	< 0.01	< 0.01	0.02	< 0.01	< 0.01	< 0.01	< 0.01
6 - 24 h	-	12.89	4.87	6.72	-	4.52	4.22	2.08	-	0.81	0.56	0.30
24 - 48 h	-	-	7.07	7.65	-	-	3.73	3.43	-	-	1.60	0.81
48 - 72 h	-	-	-	3.63	-	-	-	1.42	-	-	-	0.38
Subtotal	0.01	12.97	11.95	18.07	0.03	4.52	7.96	6.95	< 0.01	0.82	2.16	1.50
Cage Wash	0.02	0.15	0.32	0.23	< 0.01	0.02	0.02	0.02	0.01	0.07	0.65	0.01
Total Excretion	0.22	16.77	16.87	23.94	0.14	5.88	9.96	9.56	0.03	1.02	3.71	1.87
Residues												
Whole Blood*	0.06	0.06	< 0.01	0.02	0.04	0.04	0.01	< 0.01	< 0.01	0.01	0.02	< 0.01
Gastrointestinal Tract	6.13	10.95	6.73	3.00	2.94	5.29	1.90	1.40	0.41	1.09	1.66	0.31
Remaining Carcass	8.88	9.83	4.41	1.47	4.36	3.36	0.97	0.68	6.67	0.69	4.10	1.06
Subtotal	15.08	20.84	11.14	4.49	7.35	8.69	2.88	2.09	7.10	1.81	6.91	1.37
Systemic Absorption	15.30	37.61 42.45	28.01	28.43	7.49	14.57 17.36	12.84	11.65	7.13	2.83	10.62 44.86	3.24
Skin Stripping	9.45	9.77 0.84	10.61	7.70	4.36	4.55 0.22	3.13	2.62	1.34	2.13	1.16 3.53	0.81
Remaining Treated Skin	2.67	0.77	0.50	0.32	1.04	0.22	0.17	0.11	0.23	2.83	3.87	0.85
Application Site	12.13	10.61	11.11	8.02	5.40	4.78	3.31	2.74	1.58	4.96	4.68	1.65
Skin Wash	69.04	49.05	57.72	60.84	75.13	73.41	78.14	75.56	83.62	86.43	76.02	89.21
Cover and O-Ring	6.49	5.70	6.83	2.85	10.07	6.36	3.39	7.67	4.55	3.64	4.11	4.88
Dislodged Dose	75.53	54.75	64.55	63.69	85.21	79.77	81.53	83.22	88.17	90.06	80.13	94.09
Total Recovery	102.96	102.97	103.67	100.14	98.09	99.12	97.68	97.61	96.88	97.85	95.44	98.99

* Residues determined in the part taken of the specimen

Mean **penetration rates** were approximately **0.01, 0.12 and 30 µg·cm⁻²·h⁻¹** for the low- the middle- and the high dose levels, respectively. The penetration rates increased semi-proportionally with the increase of the test substance concentrations: **1:26:5100** (concentration ratio of dose levels) versus **1:12:2400** (ratio of penetration rates). Dermally absorbed test substance was **excreted mainly via feces**. At the end of the experiment (at 72 h), **4.5, 2.1 and 1.4% of the applied low- middle- and high doses**, respectively were still present in the **carcass and gastrointestinal tract**.

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Blood kinetics

Blood residues were **mostly at, or below the detection limits** at all three dose levels. Highest blood residue levels were reached between 6 and 8 h after administration, with 0.01 and 0.26 ppm difenoconazole equivalents for the middle and high dose level, respectively.

Analysis of skin wash

At the end of exposure the **major part** of the applied test substance **could be dislodged from the application site**, consisting of wash-off and recovery in extracts of the cover and 'O'-rings, being altogether 54-76% of the low dose, 79-85% of the middle dose, and 80-94% of the high dose. After the washing procedure, 12, 5 and 5% of the low-, middle- and high dose, respectively remained in or on the treated skin. The major part of the radioactivity was associated with the stratum corneum except for the high dose level (2.5 mg/cm²), which had radioactivity concentrations in the lower skin levels almost equal to that determined in the stratum corneum. Besides the parent compound, an additional poplar fraction (accounting for about 5% of the radioactivity) was present in the skin wash from low-dose animals. In the middle and high dose groups > 96% of the radioactivity was determined as unchanged parent compound.

Conclusion:

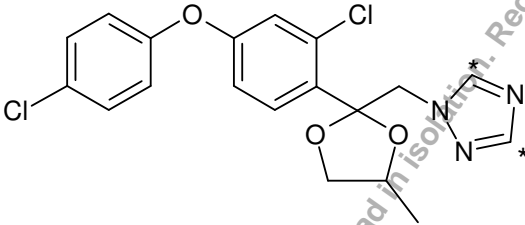
The **maximum average** dermal absorption was approximately 38% (after 24 h), 15% (after 24 h) and 11% (after 48 h) of the low, mid and high dose respectively. However, a very high degree of variation in absorption values was observed in both the original high dose group (P3) and in the repeated high dose group (P3a). In order to cover sensitive individuals, the maximum values obtained in single animals will be used to assess the dermal absorption in humans. These values are **43, 18 and 49%** in low, mid and high dose respectively.

Comments:

The formulation of the test substance in the present study (SCORE[®] 250 EC **A-7402 G**) is not entirely representative. The representative name and formulation is SCORE[®] 250 EC **A-7402 T**. The two formulations share the same composition with the only difference being the grade of **naphthalene** used. For A-7402 G, a higher grade of **naphthalene** is employed than for A-7402 T thus testing with SCORE[®] 250 EC **A-7402 G** formulation may be considered as a worst case situation since a higher concentration of naphthalene most likely increases the dermal absorption of difenoconazole. **Studies performed on SCORE[®] 250 EC A-7402 G are therefore accepted.**

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B.6.12.2 Comparative dermal absorption of Triazole-U-¹⁴C, *in vitro* using rat and human skin

Reference:	Hassler S., (2003b). The Percutaneous Penetration of [Triazole-U-¹⁴C] CGA 169374 Formulated as SCORE® 250 EC (A-7402 G) Through Rat and Human Split-Thickness Skin Membranes (<i>in vitro</i>).
Guideline:	OECD TG 428 (2000)
GLP:	Yes
Acceptability:	Yes
Test substance / purity:	Radiolabelled [Triazole-U- ¹⁴ C] Difenoconazole, formulated as SCORE® 250 EC (A-7402 G), see comments.
	
	* = ¹⁴ C position
	(Non-radiolabelled difenoconazole / 99.3%, used for the high-dose level A3).
Specific activity:	2700 kBq/mg (73 µCi/mg)
Radiochemical purity:	98.0%
Species / Strain:	Rat / HanBrl: WIST (SPF), Human / Caucasian donors
Doses / No. of subjects:	0.5 µg/cm² (typical concentration recommended for use in field for stone fruits), 12 µg/cm² (typical concentration recommended for use in field for field crops), 2345 µg/cm² undiluted formulation SCORE® 250 EC / Dorsal skin from 10 male rats (9 weeks old). Abdominal cadaver skin from 2 human (Caucasian) donors, 1 male (age 66) and 1 female (age 89).
Administration:	Non-occluded condition (donor chamber left open)
Exposure time / Duration:	24 h

Materials and Methods:

Skin membranes were set up in flow-through diffusion cells. Radiolabelled test substance was applied onto skin and perfusates from the receptor chamber were collected hourly (0-6 h) or every second hour (6-24 h). The area of skin exposed to the donor chamber was 3.24 cm². Radioactivity was determined in perfusate, skin rinse (10 ml, ethanol), and skin membranes and in cell wash (ethanol).

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Results:

In rats, **23% of the highest dose** penetrated the skin **within 24 h**, the **total amount** which penetrated the skin during this time was **539 µg/cm²**. The increase of the concentration from the low to the middle and high dose level (1:25:5000) led to an increase of the penetration rate (flux at steady-state conditions) with ratios of 1:23:1300.

Table B.6.12.2-1: *In vitro* percutaneous absorption of difenoconazole formulated as SCORE® (A-7402 G) through rat epidermis

Test System	Rat Skin Membrane					
Applied Dose [µg·cm⁻²]	0.5		12		2345	
Application Area [cm ²]	0.64		0.64		0.64	
Concentration [mg·cm ⁻³]	0.05		1.28		250	
Penetration within	% of dose	µg·cm ⁻²	% of dose	µg·cm ⁻²	% of dose	µg·cm ⁻²
6 h	24.67	0.12	19.70	2.37	2.91	68.26
12 h	47.02	0.23	38.60	4.64	9.53	223.37
24 h	70.80	0.35	63.98	7.69	22.99	539.16
Flux [µg·cm ⁻² ·h ⁻¹]	0.020		0.455		26.155	

For human skin, a **linear correlation between concentration and penetration (flux) rate** was observed at the low and middle dose levels. The penetration rate at the high dose level had an increase ratio of 1:400, whereas the concentration ratio was 1:5000 for the low/high dose level.

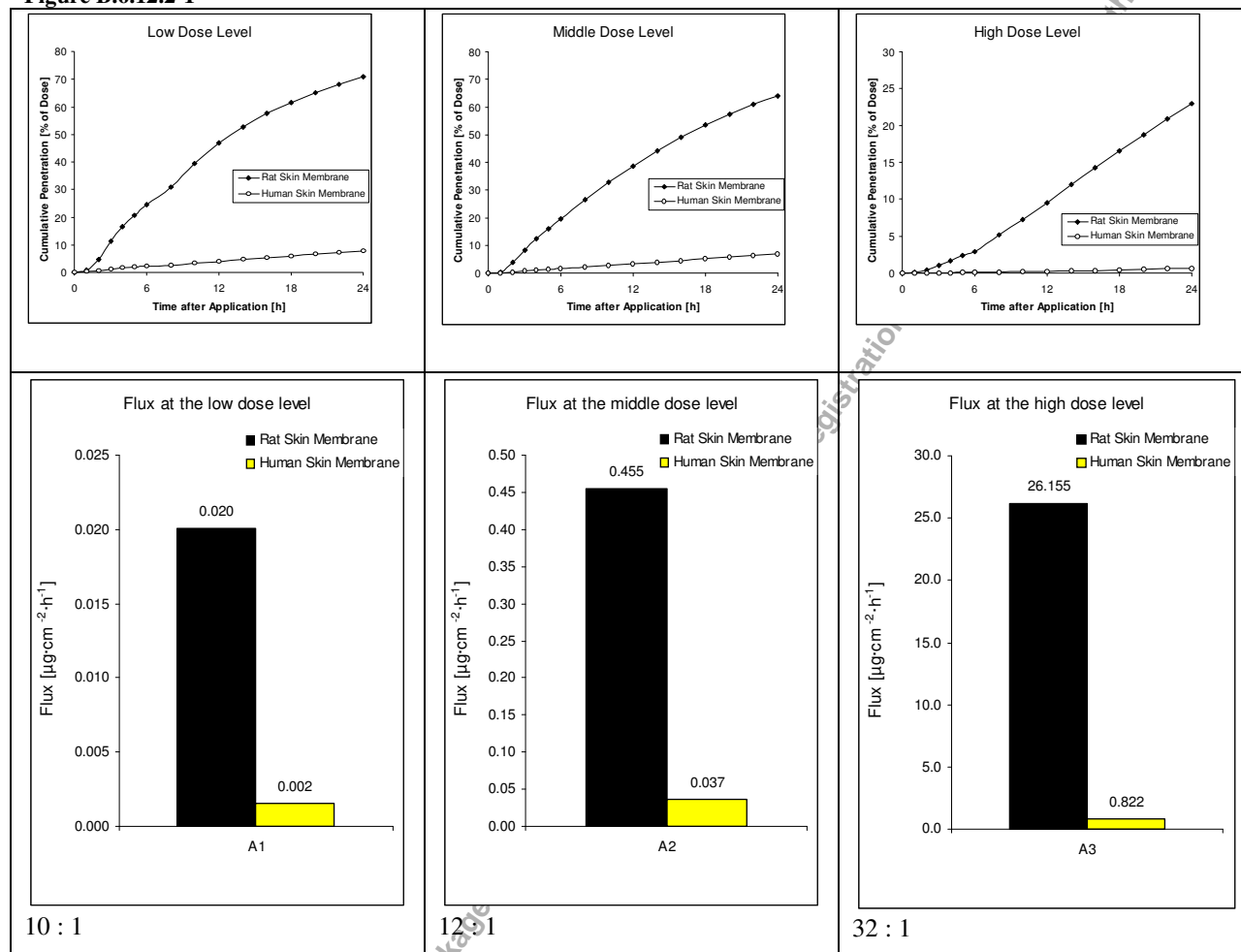
Table B.6.12.2-2: *In vitro* percutaneous absorption of difenoconazole formulated as SCORE® (A-7402 G) through human epidermis

Test System	Human Skin Membrane					
Applied Dose [µg·cm⁻²]	0.5		12		2345	
Application Area [cm ²]	0.64		0.64		0.64	
Concentration [mg·cm ⁻³]	0.05		1.28		250	
Penetration within	% of dose	µg·cm ⁻²	% of dose	µg·cm ⁻²	% of dose	µg·cm ⁻²
6 h	2.22	0.01	1.57	0.19	0.06	1.51
12 h	3.87	0.02	3.27	0.39	0.22	5.17
24 h	7.63	0.04	6.96	0.84	0.66	15.57
Flux [µg·cm ⁻² ·h ⁻¹]	0.002		0.037		0.822	

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At all dose levels **the test substance penetrated faster and to a higher extent through rat skin membranes as compared to human skin membranes.**

Figure B.6.12.2-1



The comparison of the penetration rates at the different dose levels (concentration ratio 1:25:**5000**), revealed an increase of the penetration rates of 1:23:**1300** for rat skin membranes, whereas the penetration rates for human skin membranes revealed a ratio for 1: 19: **400**.

Recovery

Total recovery of applied radioactivity averaged 96-99% for rat skin and 97-100% for human skin.

Conclusion:

Difenconazole, formulated as SCORE® 259 EC penetrated through rat split-thickness skin membrane at a faster rate and to a higher extent than through human split-thickness membranes. At 24 h, **70, 64 and 23% of the applied low- middle- and high doses**, respectively had **penetrated the rat skin**. **Corresponding** penetration for **human skin** was **8, 7 and 0.7%**. The flux ratio for the high dose level ($2345 \mu\text{g}/\text{cm}^2$) was 1:32 human/rat (1:10 and 1:12 at the low and middle dose levels, respectively).

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Comments:

The formulation of the test substance in the present study (SCORE® 250 EC A-7402 G) is not entirely representative. The representative name and formulation is SCORE® 250 EC A-7402 T. The formulation variants A-7402 G and A-7402 T share the same formulation composition but in A-7402 G the normal grade of aromatic hydrocarbon is employed whereas in A-7402 T a naphthalene depleted grade of the same aromatic hydrocarbon is used.

B.6.12.3 Summary and conclusions on dermal absorption

The extent of absorption of radio-labelled difenoconazole formulated as SCORE® 250 EC (A-7402 G) through human skin can be estimated based on the *in vivo* study conducted in rats and the *in vitro* study performed with rat and human skin preparations.

The high dose used in these studies represents the concentrate which is handled in the mixing and loading procedures whereas the low dose represents the diluted spray handled by the operator.

The *in vivo* study in rats showed that during a six hour exposure period to formulated difenoconazole, the mean values of systemically absorbed substance were 15.3%, 7.5%, and 7.1% in the low, middle, and high dose groups respectively. However, the high dose group (P3) showed a very high degree of variation in dermal absorption values and the experiment was therefore repeated (dose group P3a). The relative humidity during the test deviated from the recommendations in OECD 427 which states that the relative humidity should not exceed 70% and preferably be 50-60%. The relative humidity in the middle and high dose (P3) groups were 50-80% and 48-80% respectively. Due to the high variation observed also in the second experiment, a conservative approach was taken and the maximum individual dermal absorption levels reported at each dose level were used for assessing the dermal absorption in humans. Maximum dermal absorption values obtained in the *in vivo* study on rats are reported as the sum of the systemic absorption and the dose remaining in the treated skin (Table B.6.12.3-1). The results from the *in vitro* studies performed on rat and human skin showed a much higher dermal absorption in rats than in human skin (Table B.6.12.3-1). In addition, rats showed a much higher absorption of a low or a mid dose of SCORE® 250 EC (A-7402 G) *in vitro* than *in vivo*. In contrast, the dermal absorption of the high dose was higher *in vivo* than *in vitro*. Since only material that passed the check of skin quality before the start of the study was used, the numbers are considered to be reliable.

The *in vivo* absorption in humans can be estimated from the rat *in vivo* data and the *in vitro* studies using the formula¹

In vivo human absorption = *in vivo* animal absorption x (*in vitro* human absorption / *in vitro* animal absorption)

¹ Sanco/222/2000 rev. 6, 27.November 2002: Guidance Document on Dermal Absorption

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Table B.6.12.3-1: Summary of in vivo and in vitro data on dermal absorption of SCORE® 250 EC (A-7402 G) (conservative approach)

	<i>In vivo</i> (rat)	<i>In vitro</i> (rat)	<i>In vitro</i> (human)
Low (0.5 µg/cm ²)	43	71	7.6
Mid (12.5 µg/cm ²)	18	64	7
High(2500 µg/cm ²)	49	23	0.7
Reference:	Hassler S., (2003c).	Hassler S., (2003b).	Hassler S., (2003b).

Using the equation above and the values reported in table B.6.12.3-1, the calculated human *in vivo* absorption was 4.6%, 2.0%, and 1.4% in the low, middle, and high dose group, respectively.

Conclusion: Accounting for individual variation, the calculated values for maximum absorption rates in humans are 4.6%, 2.0 and 1.4% for the low, middle, and high concentrations, respectively.

B.6.13 Toxicological data on non active substances (Annex IIIA 7.4 and point 4 of the introduction)

Data for all coformulants present in SCORE® 250 EC (A-7402 T) and DIVIDEND®030 FS (A-9142G) are given in Safety Data Sheets. The content of solvent (classified R65) in the preparation (Cn >10%) means that classification for aspiration hazard should be considered. Viscosity (dynamic) is at least 13 mPa.s at 40°C. Dividing by the density (1.059 g/ml) gives the kinematic viscosity = $12.3 \times 10^{-6} \text{ m}^2/\text{s}$. This is above the critical value of $7 \times 10^{-6} \text{ m}^2/\text{s}$ therefore R65 is not required. No additional labelling is considered required according to the safety data sheets of the formulants.

B.6.14.1 Exposure data on SCORE® 250 EC (A-7402 T) (Annex IIIA 7.2)

SCORE® 250 EC (A-7402 T) is an emulsifiable concentrate (EC) containing 250g/litre (23.2% w/w) difenoconazole and is recommended for use in pome fruit and carrots. SCORE® 250 EC (A-7402 T) is applied to pome fruit in southern (SEU) and northern EU (NEU) by vehicle-mounted mist blowers/ hand-held spray equipment and to carrots by vehicle-mounted boom sprayers. A summary of the application methods and the recommended application rates is shown in Table B.6.14.1-1.

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Table B.6.14.1-1: Summary of application methods and rates of SCORE® 250 EC (A-7402 T) relevant for the operator exposure assessment

Field of use	Method of application	Max. application rate g a.i./ha	Spray volume L/ha	Max. application concentration g a.i./L
Pome fruit	NEU: vehicle-mounted mist blowers	18.75-56.25	500-1500	0.1125
	NEU: hand-held spray equipment			
	SEU: vehicle-mounted mist blowers	37.5-75	500-1000	0.15
	SEU: hand-held spray equipment			
Carrot	vehicle-mounted boom sprayers	125	100-500	1.25

B.6.14.1.1 Operator exposure

B.6.14.1.1.1 Estimation of operator exposure

The estimates of total difenoconazole exposure predicted by the UK POEM¹ (Predictive operator exposure model) and the German model² were calculated as a proportion of the proposed AOEL for the active ingredient. Additional assumptions/data utilised in the models are shown in table B.6.14.1.1.1-1.

Table B.6.14.1.1-1: Data utilised in UK POEM and German model

Area Treated in One Day:	50 ha in UK POEM (vehicle mounted boom sprayer) 20 ha in German model (vehicle mounted boom sprayer) 15 ha in UK POEM (vehicle mounted mist blowers) 8 ha in German model (vehicle mounted mist blowers) 1 ha in UK POEM and German model for hand-held application technique for pome fruit
Packaging:	1 litre bottles in the UKPOEM
Inhalation Exposure for Mixer/Loader:	0.01 ml/hr
Inhalation absorption	100%
Dermal absorption from spray (dilution):	4.6%
Dermal absorption from the product (concentrate):	1.4%

¹ Scientific Subcommittee on Pesticides and British Agrochemicals Joint Medical Panel., Estimation of Exposure and Absorption of Pesticides by Spray Operators (UK MAFF) 1986 and the Predictive Operator Exposure Model (POEM – UK MAFF) 1992

² Uniform Principles for Safeguarding the Health of Applicators of Plant Protection Products (Uniform Principles for Operator Protection); Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem, no. 277, 1992

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B.6.14.1.1.1.1 Estimation of operator exposure using the UK POEM Model

Operator exposure to SCORE[®] 250 EC A-7402T was estimated in the UK POEM model using the data in tables B.6.14.1-1 and B.6.14.1.1.1-1. A worst-case situation was modelled by assuming the use of maximum rates and minimum spray volumes and assuming that Personal Protective Equipment (PPE) was not used. The estimated operator exposure using a rotary atomiser is considered to be a worst case situation for hand held applications. A summary of the estimated exposure during the different applications are shown in table B.6.14.1.1.1-1. Full calculations are shown in the appendix.

Table B.6.14.1.1.1-1 Exposure estimates according to the UKPOEM without PPE

Use rate [g a.i./ha]	Water volume [L/ha]	Total systemic exposure [mg/kg bw/day]	Total systemic exposure [% of AOEL] *	Reference in Appendix
Vehicle mounted air blast sprayer, Pome fruit				
NEU (15 ha/day)				
56.25	500	0.013	6.5	1a
SEU (15 ha/day)				
75	500	0.018	8.8	1b
Vehicle mounted boom sprayer, hydraulic nozzles, Carrots				
50 ha/day, standard work-rate				
125	100	0.056	28	1c
Hand-held sprayer, Pome fruit				
NEU				
56.25	500	0.12	60	1d
SEU				
75	500	0.13	65	1e

* AOEL = 0.20 mg/kg bw/day

Conclusion:

The data obtained in the UK POEM model showed that exposure to difenoconazole does not involve a significant risk to the health of the operators concerned for spraying pome fruit using either tractor/vehicle sprayers or handheld applications or for spraying carrots using tractor/vehicle mounted boom sprayers.

Depending on the application method used, the modelling data predicted an operator exposure between 6.5 and 65% of the systemic AOEL for difenoconazole in pome fruit and carrot treatment without the use of PPE.

B.6.14.1.1.1.2 Estimation of operator exposure using the German Model

Operator exposure to SCORE[®] 250 EC A-7402T was estimated in the German model using the data in tables B.6.14.1-1 and B.6.14.1.1.1-1. A worst-case situation was modelled by assuming the use of maximum rates and no use of Personal Protective Equipment (PPE). A summary of the exposure estimates for the different applications are shown in table B.6.14.1.1.1.2-1. Full calculations are shown in the appendix.

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Table B.6.14.1.1.2-1 Exposure estimates according to the German model without PPE

Use rate [g a.i./ha]	Water volume [L/ha]	Total systemic exposure [mg/kg bw/day]	Total systemic exposure [% of AOEL] *	Reference in Appendix 1
Tractor high crops, Pome fruit				
NEU (8 ha/day)				
56.25	Not relevant	0.0044	2.2	2a
SEU (8 ha/day)				
75	Not relevant	0.0058	2.9	2b
Tractor field crops Carrots				
20 ha/day				
125	Not relevant	0.0054	2.7	2c
Hand high, Pome fruit				
NEU (1 ha/day)				
56.25	Not relevant	0.0048	2.4	2d
SEU (1 ha/day)				
75	Not relevant	0.0063	3.2	2e

* AOEL = 0.20 mg/kg bw/day

Conclusion:

The data obtained in the German model showed that exposure to difenoconazole does not involve a significant risk to the health of the operators concerned for spraying pome fruit using tractor or handheld applications or for spraying carrots using tractor. **Depending on the application method used, the modelling data predicted an operator exposure between approximately 2.2 and 3.2% of the systemic AOEL for difenoconazole in pome fruit and carrot treatment without PPE.**

B.6.14.1.1.2 Measure of operator exposure

Measurement of operator exposure was not performed.

B.6.14.1.1.3 Summary of operator exposure

SCORE[®] 250 EC A-7402T will be marketed as an emulsifiable concentrate containing 250 g/l of difenoconazole with an expected smallest package size of 1 litre. The UK POEM model and the German model were used to estimate operator exposure during treatment of crops with the different application methods reported as the intended uses. The estimates of total systemic exposure of difenoconazole predicted by these models were calculated as a proportion of the proposed systemic AOEL (0.2 mg/kg bw).

Worst-case situations were modelled by assuming the use of maximum rates and minimum spray volumes (in UK POEM) and assuming no use of Personal Protective Equipment (Tables B.6.14.1.1.1-1 and B.6.14.1.1.1-2). Since estimates made without the use of PPE resulted in values well below the AOEL, estimates of operator exposure when using PPE were not calculated.

During the conditions studied, the operator exposure to SCORE[®] 250 EC A-7402T using tractor/vehicle mounted airblast sprayers, tractor mounted hydraulic boom sprayers or handheld applications is considered acceptable.

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B.6.14.1.2 Bystander exposure

The estimation of bystander exposure is calculated based on the application data for treatment of pome fruit since it is the intended use that is likely to result in the highest exposure SCORE[®] 250 EC A-7402T for bystanders.

Assuming a maximum application rate for SCORE[®] 250 EC A-7402T of 75 g a.i./ha and an even distribution over the area sprayed (10,000 m²), the concentration would be 7.5 mg/m². Further, assuming a maximal drift of 3.60%¹ and a bystander located 10 meters from the field, the deposition on the bystander could reach 0.27 mg/m². If one-half of the body surface (totalling approximately 1 m²) is exposed, the skin deposition would be 0.27 mg SCORE[®] 250 EC A-7402T.

Using a skin absorption assumption of 4.6%, the absorbed dose would be 0.01242 mg of difenoconazole.

Assuming a 60 kg body weight, the exposure would be 0.000207 mg/kg bw and day.

Compared to the AOEL for difenoconazole, 0.2 mg/kg bw and day, the potential exposure of bystanders is less than 1%. Therefore, bystander exposure to difenoconazole during the agricultural use of SCORE[®] 250 is considered acceptable.

Given the estimate above, no measurement of bystander exposure is required.

B.6.14.1.3 Worker exposure

B.6.14.1.3.1 Estimation of worker exposure

Worker exposure to difenoconazole in orchards was estimated using the formula:

$$(AR/LAI) * TF * T * (DA/Bw)$$

Where

AR (application rate): 56.25-75g a.s/ha

LAI (leaf area index): 1

TF: 4500 cm²/h

T: 8h

DA (dermal absorption): 4.6%

Bw: 60 kg

As proposed by the re-entry working group in the Europoem II project, an indicative TC of 4500 cm² was used in the calculations to cover the dermal exposure of bare hands and the body exposure assuming (protective) clothing. In the documentation submitted, the notifier presented an estimated worker exposure based on the German re-entry model using a worst case TC of 30'000 cm²/person/h and a FDR of (1 µg/cm²)/(kg a.i/ha). Since the estimated exposure is approximately 10% of the proposed AOEL in both calculations, the worker exposure to difenoconazole in orchards is considered acceptable. However, as a basic rule, treated areas should

¹Rautmann, D., Strelake, M., Winkler, R. (2001) New basic drift values in the authorisation procedure for plant protection products. In: Workshop on risk assessment and risk mitigation measures in the context of the authorisation of plant protection products (WORMM; Forster, R., Strelake, M. Eds.), 27-29 September, 1999, Heft 383, Biologischen Bundesanstalt für Land - und Fortwirtschaft, Berlin and Braunschweig, Germany.

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not be re-entered before spray deposit on leaf surfaces has completely dried. Worker exposure to difenoconazole following treatment of carrots is of similar magnitude to the exposure in orchards thus calculations are not presented.

Table B.6.14.1.3.1-1: Exposure estimates

Use rate [g a.i./ha]	Water volume [L/ha]	Total systemic exposure [mg/kg bw/day]	Total systemic exposure [% of AOEL] *	Reference in Appendix 1
Pome fruit				
NEU				
56.25	Not relevant	0.016	8	
SEU				
75	Not relevant	0.021	10	-
Carrot				
125	Not relevant	0.019	10	-
* AOEL = 0.20 mg/kg bw/day				

B.6.14.1.3.2 Measurement of worker exposure

Worker exposure studies were not performed.

B.6.14.1.3.3 Summary of worker exposure

During the conditions of the intended use of SCORE® 250 EC A-7402T, the worker exposure is ≤10% of AOEL. Worker exposure to SCORE® 250 EC A-7402T is considered acceptable.

B.6.14.2 Exposure data on DIVIDEND®030 FS (A-9142 G)

DIVIDEND®030 FS (A-9142G) is a liquid formulation (flowable concentrate) containing 30g/litre (2.86% w/w) difenoconazole and is recommended for seed treatment in cereals. All estimates of exposure data in this section were calculated by the notifier using the SEEDTROPEX model¹ and there were no field studies included in the dossier submission. Dermal absorption studies of DIVIDEND®030 FS (A-9142G) were not performed. The notifier has provided a justification why the results from the dermal absorption tests with SCORE® 250 EC (A-7402T) can be used. The dermal absorption value (1.4 %) for undiluted SCORE® 250 EC (A-7402T) can be considered as a worst case since it contains almost a ten fold higher concentration of the active ingredient than DIVIDEND®030 FS (A-9142G). Furthermore the organic solvent component of the SCORE® formulation is likely to give a higher bioavailability than aqueous DIVIDEND®030 FS (A-9142G). The undiluted concentration of SCORE® 250 EC (A-7402T) (250 g/L) is therefore considered comparable to the undiluted A-9142G (30 g/L) and thus a dermal absorption of 1.4% in humans can be used for the exposure calculations of A-9142G as a seed

¹ Worker exposure during seed treatment and sowing of treated seed in the UK and France: An Overview, Report N° TMF 4896, G Chester, JM Wiseman, Zeneca Agrochemicals: PG Pontal, Rhône-Poulenc Agro, 2 May 1996. (Data property of the SEEDTROPEX Group to which SYNGENTA is a member).

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treatment in cereals. The recommended application rate of DIVIDEND®030 FS (A-9142G) is 1 to 2 litres product per tonne seed. The product is applied diluted or undiluted.

Package types and sizes are:

High density polyethylene container	20 L
Steel drum with inner varnish	200 L
High density polyethylene tank container	1000 L

Table B.6.14.2-1: Recommended use rates of A-9142 G

Active ingredient	Seed	g a.i./ton seed	Slurry volume/ton seed		Maximum in-use concentration of a.i. (g/L)
			Minimum	Maximum	
CGA 169374	Cereals (barley, wheat, rye, oat, triticale)	30 to 60	1	2	30 (product used undiluted)

B.6.14.2.1 Operator exposure

B.6.14.2.1.1 Estimation of operator exposure

The estimated total difenoconazole exposure during seed treatment was assessed using the SEEDTROPEX model and was reported as a proportion of the proposed AOEL for the active ingredient. The SEEDTROPEX model has been developed by the SEEDTROPEX Group to which the notifier is a member. The model is based on two exposure studies (performed in the UK and France respectively) measuring operator exposure during calibration of the equipment, mixing/loading of the slurry (pre-mix and fast-couple system), bagging of treated seeds and cleaning of the equipment.

The assumptions/data utilised in the calculation of operator exposure during seed treatment are summarised in Table B.6.14.2.1.1-1.

Table B.6.14.2.1.1-1:

Seed to be treated:	Cereals
Application rate:	2 L/ton seed
Spray volume:	product used undiluted
Size of product containers:	20 L polyethylene canister (smallest pack size as worst case)
Mixing/loading:	pre-mix and fast-couple
Treatment technique:	continuous flow seed treaters with closed mixing chamber
Treatment capacity:	10 tons per hour, 90 tons per day
Normal length of working day:	10 hours, of these 1 hour for loading, calibration and cleaning
Seed bagging*:	9 hours per day
Dermal absorption of CGA 169374:	1.4% (undiluted formulation)
Standard operator body weight:	60 kg

* in the SEEDTROPEX Model, the exposure during bagging is only related to the working time and the bag size, but not related to the amount of seed handled per operator.

Results of the model calculations and comparison with the established AOEL

The results of the model calculations are summarized in Table B.6.14.2.1.1-2. A full calculation is shown in the appendix (3).

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Table B.6.14.2.1.1-2: Estimated operator exposure to difenoconazole during seed treatment operations with undiluted A-9142 G and % account of AOEL (SeedTropex Model)

Activity	Exposure [mg/person/day]			Systemic Exposure [mg/kg bw/day]		% of AOEL (AOEL: 0.2 mg/kg bw/day)	
	Dermal		Inhalative	Potential (no PPE)	Estimated actual (PPE)	Potential (no PPE)	Estimated actual (PPE)
	Potential (no PPE)	Estimated actual (PPE)					
Calibration	1.0	0.43	0.041	0.0009	0.0008	0.45	0.4
Mixing/loading							
- Pre-mix	1.3	0.31	0.028	0.0008	0.0005	0.4	0.25
- Fast-couple	1.4	1.40	0.028	0.0008	0.0008	0.4	0.4
Bagging:							
- 25 kg bags	24.0	7.76	1.176	0.0252	0.0214	12.6	10.7
- 50 kg bags	10.7	4.25	0.135	0.0047	0.0032	2.35	1.6
- 500 kg bags	14.5	6.64	0.655	0.0143	0.0125	7.2	6.25
Cleaning	26.2	2.50	0.476	0.0140	0.0085	7	4.25

PPE (Personal Protective Equipment): cotton protective clothing (work trousers, long sleeved work jackets) and nitrile gloves

In all seed treatment related activities the model calculations indicate that the systemic operator exposure to difenoconazole is below the established AOEL of 0.2 mg/kg bw/day independent of the use or non-use of personal protective equipment.

B.6.14.2.1.2 Measurement of operator exposure

No measurement of operator exposure was performed with A-9142 G.

B.6.14.2.2 Bystander exposure

Bystander exposure in stationary seed treatment facilities is considered to be rare. If an incidental presence of bystanders would occur at a seed treatment facility, it is assumed to be a short duration of exposure and normally lower than that of seed treatment operators who are occupationally exposed longer.

Therefore, it is assumed that there will be no risk to persons being incidentally exposed during seed treatment operations with A-9142 G.

B.6.14.2.2 Measurement of bystander exposure

No measurement of bystander exposure was performed with A-9142 G.

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B.6.14.2.3 Worker exposure

B.6.14.2.3.1 Estimation of worker exposure

The potential exposure of workers handling treated seed (i.e. during loading of the drill and sowing) was assessed by the SEEDTROPEX model¹ and reported as a proportion of the proposed AOEL for the active ingredient.

The notifier states that according to the generic database used to develop the model, it appears that the highest level of potential exposure is on hands from loading the drill and that the model parameter for normalisation of the worker exposure during sowing (and drill loading) is the working time.

In the model, a 10-hour working day and a worker body weight of 60 kg was assumed. The estimated exposure is given in the Table B.6.14.2.3.1-1 and a full calculation is given in the appendix (4b).

Table B.6.14.2.3.1-1: Worker exposure during sowing treated seed (loading of the drill and sowing)

Active ingredient	Total potential dermal exposure [mg/kg bw/day]	Estimated actual dermal exposure [mg/kg bw/day]	Inhalation exposure [mg/kg bw/day]	Estimated systemic exposure [mg/kg bw/day] ¹⁾	% of AOEL (AOEL = 0.20mg/kg bw/day)
CGA 169374	0.246	0.122	0.003	0.0050	2.5

¹⁾ based on 1.4% dermal absorption

Estimates of systemic exposure of workers who are handling seed previously treated with A-9142 G is below the established AOEL of 0.2 mg/kg bw/day.

B.6.14.2.3.2 Measurement of worker exposure

No worker exposure studies were performed.

B.6.14.2.4 Summary of exposure data on DIVIDEND®030 FS (A-9142G)

All estimates of human exposure to DIVIDEND®030 FS (A-9142G) were calculated by the notifier using the SEEDTROPEX model². The RMS considers that this model requires more extensive data than the two existing studies in order to be accepted as a general model for estimation of exposure during seed treatment. Therefore, results obtained using the SEEDTROPEX model should be interpreted with caution. However, DIVIDEND®030 FS (A-9142G) is of low acute toxicity and the values obtained using the SEEDTROPEX model are well below the AOEL for difenoconazole. Therefore, the risk of harmful effects in operators handling treated seed is presumed to be low if appropriate protective clothing is worn and basic hygienic rules are observed.

¹ Worker exposure during seed treatment and sowing of treated seed in the UK and France: An Overview, Report N° TMF 4896, G Chester, M Wiseman, Zeneca Agrochemicals: PG Pontal, Rhône-Poulenc Agro, 2 May 1996. (Data property of the SEEDTROPEX Group to which SYNGENTA is a member).

² An exception is the parameter %AOEL which has been re-calculated in the table of section B.6.14.2 since the RMS chose a different AOEL than the notifier.

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B.6.15 References relied on

The dossier submitted contains many studies on plant metabolites of difenoconazole. However, the toxicity assessment of plant metabolites relies only on studies on toxicokinetics, acute oral toxicity and genotoxicity¹. All additional studies on plant metabolites presented in this DAR are not listed in this section, in accordance with the Guidance document SANCO/10435/2004 15 April 2005-rev 7, as they are not considered relevant to the assessment.

Annex No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	EU Data Protection Claimed (Y/N)*	Owner **
IIA 5.1/01	Esumi, Y	1992	Absorption, distribution and excretion of CGA 169374 in rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Co., [REDACTED] Report No ABR1488 Not GLP Not Published Syngenta File N° CGA169374/0905	N	SYN
IIA 5.1/02	Hassler, S.	2003a	Disposition of [4-Chloro-phenyl-U-14C] CGA 169374 in the Rat after Multiple Oral Administrations Syngenta Crop Protection AG, Basel, Switzerland [REDACTED] [REDACTED] No 051AM03 GLP Not Published Syngenta File N° CGA169374/2361	Y	SYN
IIA 5.1/03	Craine, EM	1987a	Metabolism of triazole 14C-CGA 169374 in the rat. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0726	N	SYN
IIA 5.1/04	Craine, EM	1987b	Metabolism of phenyl 14C-CGA 169374 in the rat. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0727	N	SYN
IIA 5.1/05	Capps, TM, McFarland, JE, Cassidy, JE	1988	Metabolism of triazole-14C and phenyl-14C-CGA 169374 in the rat: Distribution of radioactivity Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No ABR-88043 Not GLP Not Published Syngenta File N° CGA169374/0264	N	SYN

¹ Considered to be represented by a combination of at least an Ames test, a gene mutation test on mammalian cells and a chromosome aberration test.

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IIA 5.1/06	Capps, TM, Barr, HP, Carlin, TJ	1990	Characterization and identification of major triazole-14C and phenyl-14C-CGA 169374 metabolites in rats. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No ABR-90019 GLP Not Published Syngenta File N° CGA169374/0363	N	SYN
IIA 5.2.1/01	Argus, MA, Ricci, JM, Huber, KR, Schiavo, DM, Hazelette, JR, Green, JD	1987	CGA 169374 tech.: Acute oral toxicity study in rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0247	N	SYN
IIA 5.2.1/02	Hartmann, HR	1990	CGA 169374 tech.: Acute oral toxicity in the mouse Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 891514 GLP Not Published Syngenta File N° CGA169374/024	N	SYN
IIA 5.2.2/01	Mastrocco, F, Ricci, JM, Huber, KR., Schiavo, DM, Hazelette, JR, Green, JD	1987a	CGA 169374 tech.: Acute dermal toxicity study in rabbits Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0250	N	SYN
IIA 5.2.3/01	Hartmann, HR	1991	CGA 169374 tech.: Acute inhalation toxicity in the rat Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 901436 GLP Not Published Syngenta File N° CGA169374/0437	N	SYN
IIA 5.2.4/01	Glaza, SM	1991a	Primary dermal irritation study of CGA 169374 technical in rabbits. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0475	N	SYN
IIA 5.2.4/01	Tisdell, M	1992	Supplemental information for primary dermal irritation study of CGA 169374 technical in rabbits Brian Christensen Companies, Inc., Minnetonka, United States [REDACTED] Report No HWI 10503687 GLP Not Published Syngenta File N° CGA169374/2103	N	SYN

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IIA 5.2.5/01	Glaza, SM	1991b	Primary eye irritation study of CGA 169374 technical in rabbits. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0474	N	SYN
IIA 5.2.6/01	Mastrocco, F, Ricci, JM, Huber, KR, Schiavo, DM, Hazelette, JR, Green, JD	1987b	CGA 169374 tech.: Dermal sensitization study in female Guinea pigs Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0255	N	SYN
IIA 5.3.1/01	Suter, P	1986a	28-Day cumulative oral toxicity (feeding) study with CGA 169374 in the rat Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 040770 Not GLP Not Published Syngenta File N° CGA169374/0281	N	SYN
IIA 5.3.2/01	Suter, P	1986b	13-Week oral toxicity (feeding) study with CGA 169374 in the rat Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No RCC 040757 GLP Not Published Syngenta File N° CGA169374/0256	N	SYN
IIA 5.3.2/02	Cox, RH	1987a	CGA 169374 tech.: Subchronic toxicity/metabolism study in rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 483-242 GLP Not Published Syngenta File N° CGA169374/0257	N	SYN
IIA 5.3.2/03	Cox, RH	1987b	CGA 169374 tech.: Subchronic toxicity/metabolism study in mice Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 483-241 GLP Not Published Syngenta File N° CGA169374/0288	N	SYN
IIA 5.3.2/04	O'Connor, DJ, McCormick, GC, Green, JD	1987	CGA 169374 tech.: 26-Week oral toxicity study in dogs Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0258	N	SYN
IIA 5.3.2/05	Rudzki, MW, McCormick, GC, Arthur, AT	1988	CGA 169374 tech.: Chronic toxicity study in dogs Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0262	N	SYN

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IIA 5.3.3/01	Gerspach, R	2000	CGA 169374 tech.: 28-day repeated dose dermal toxicity study in rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 993072 GLP Not Published Syngenta File N° CGA169374/2046	N	SYN
IIA 5.4.1/01	Dollenmeier, P	1986a	CGA 169374 tech.: L5178Y/TK+/- mouse lymphoma mutagenicity test Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 850570 GLP Not Published Syngenta File N° CGA169374/0276	N	SYN
IIA 5.4.1/02	Ogorek, B	1990	CGA 169374 tech.: Salmonella and escherichia/liver-microsome test Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 901061 GLP Not Published Syngenta File N° CGA169374/0359	N	SYN
IIA 5.4.1/03	Hertner, T	1992	CGA 169374 tech.: Autoradiographic DNA repair test on rat hepatocytes in vitro Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 923124 GLP Not Published Syngenta File N° CGA169374/0697	N	SYN
IIA 5.4.1/04	Lloyd, M	2001	CGA 169374 tech.: Induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells Syngenta Crop Protection AG, Basel, Switzerland Gowance Laboratories, North Yorkshire, United Kingdom, Report No 252/293-D6172 GLP Not Published Syngenta File N° CGA169374/2194	Y	SYN
IIA 5.4.1/05	Fox, V	2001	CGA 169374 tech.: In vitro cytogenetic assay in human lymphocytes Syngenta Crop Protection AG, Basel, Switzerland Central Toxicology Laboratory (CTL), Cheshire, United Kingdom, Report No SV1090 GLP Not Published Syngenta File N° CGA169374/2153	Y	SYN
IIA 5.4.1/06	Ogorek, B	2001	CGA 169374 tech.: Cytogenetic test on Chinese hamster cells in vitro Syngenta Crop Protection AG, Basel, Switzerland Syngenta Crop Protection AG, Stein, Switzerland, Report No 20013013 GLP Not Published Syngenta File N° CGA169374/2191	Y	SYN

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IIA 5.4.2/01	Ogorek, B	1991	CGA 169374 tech.: Micronucleus test, mouse Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 911041 GLP Not Published Syngenta File N° CGA169374/0455	N	SYN
IIA 5.5/01	Cox, RH	1989a	Combined chronic toxicity and oncogenicity study of CGA 169374 technical in rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 483-249 GLP Not Published Syngenta File N° CGA169374/0260	N	SYN
IIA 5.5/01	Saunders, S	1992	Historical control data [SD rats, 104-week studies, adrenal and pancreas] Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No N/A Not GLP Not Published Syngenta File N° CGA169374/0705	N	SYN
IIA 5.5/02	Cox, RH	1989b	CGA 169374 tech.: Oncogenicity study in mice Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 483-250 GLP Not Published Syngenta File N° CGA169374/0261	N	SYN
IIA 5.6.1/01	Giknis, MLA	1988	CGA 169374 tech.: A two generation reproductive study in albino rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0279	N	SYN
IIA 5.6.2/01	Hummel, HE, Youreneff, MA, Giknis, MLA, Yau, ET	1987	CGA 169374 tech.: Teratology study in rabbits. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] GLP Not Published Syngenta File N° CGA169374/0277	N	SYN
IIA 5.6.2/02	Lochry, EA	1987	Developmental toxicity study of CGA 169374 technical (FL-851406) administered orally via gavage to CrI:COBS CD (SD)BR presumed pregnant rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 203-005 GLP Not Published Syngenta File N° CGA169374/0278	N	SYN
IIA 5.8.1/01	Nakajima, M	1991a	Reverse mutation assay of CGA-189138 Novartis Crop Protection AG, Basel, Switzerland Biosafety Research Center of Japan, -, Japan, Report No 1809 GLP Not Published Syngenta File N° CGA189138/0001	N	SYN

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IIA 5.8.1/02	Lai, K., Simoneaux, B.	1986a	Balance study of ¹⁴ C-triazole in orally dosed rats. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No ABR-86021 Not GLP Not Published Syngenta File N° CGA71019/0001	N	SYN
IIA 5.8.1/03	Weber, H., Patzschke, K., Wegner, L.A.	1978	1,2,4-Triazole- ¹⁴ C - Biokinetic studies on rats. [REDACTED] Report No 7920 Not GLP Not Published Syngenta File N° CGA71019/0027	N	TDMG
IIA 5.8.1/04	Ecker, W.	1980	Biotransformation of 1,2,4-(3(5)- ¹⁴ C) Triazole in rats. Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No PF 1471 Not GLP Not Published Syngenta File N° CGA71019/0028	N	SYN
IIA 5.8.1/06	Thyssen, J., Kimmerli, G.	1976	1,2,4-Triazole occupational toxicology report. Bayer AG, Leverkusen, Germany Bayer AG Toxicological Institute, Wuppertal- Elberfeld, Germany, Report No 5926 Not GLP Not Published Syngenta File N° CGA71019/0025	N	TDMG
IIA 5.8.1/16	Poth, A.	1989	Salmonella typhimurium reverse mutation assay with 1H-1,2,4-Triazole. Novartis Crop Protection AG, Basel, Switzerland Brian Christensen Companies, Inc., Minnetonka, United States, Report No 158400 GLP Not Published Syngenta File N° CGA71019/0026	N	SYN
IIA 5.8.1/23	Hamboeck, H	1983a	CGA 131013 tech.: Distribution, degradation and excretion of D,L-2-amino-3-(1-H-1,2,4-triazol-1-yl)- propanoic acid (D,L-triazolylalanine) in the rat Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 1/83 GLP Not Published Syngenta File N° CGA131013/0005	N	TDMG
IIA 5.8.1/24	Lai, K., Simoneaux, B.	1986b	Balance study of ¹⁴ C-triazole alanine in orally dosed rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No ABR-86023 Not GLP Not Published Syngenta File N° CGA131013/0003	N	TDMG
IIA 5.8.1/25	Hamboeck, H	1983b	CGA 131013 tech.: The metabolism of D,L-2- amino-3-(1H-1,2,4-triazol-1-yl)-propanoic acid (D,L-triazolylalanine) in the rat Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 11/83 Not GLP Not Published Syngenta File N° CGA131013/0004	N	TDMG

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IIA 5.8.1/26	Lai, K, Simoneaux, B	1986c	The metabolism of triazole alanine in the rat Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No ABR-86041 Not GLP Not Published Syngenta File N° CGA131013/0001	N	TDMG
IIA 5.8.1/27	Mihail, F	1982	Triazolylalanine (THS 2212): Expanded version of Bayer report No. 11229, dated October 19, 1982 [Acute toxicity studies] Novartis Crop Protection AG, Basel, Switzerland [REDACTED] [REDACTED] Report No 11229 A Not GLP Not Published Syngenta File N° CGA131013/0030	N	TDMG
IIA 5.8.1/28	Henderson, C, Parkinson, GR	1980	R152056: Acute oral toxicity to rats Novartis Crop Protection AG, Basel, Switzerland [REDACTED] [REDACTED] GLP (according to the policies and procedures of Imperial Chemical Industries). Not Published Syngenta File N° CGA131013/0029	N	TDMG
IIA 5.8.1/34	Hertner, Th.	1993	CGA 131013 tech.: Salmonella and escherichia/liver-microsome test Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 933002 GLP Not Published Syngenta File N° CGA131013/0031	N	TDMG
IIA 5.8.1/35	Herbold, B	1983a	THS 2212 triazolylalanine: Salmonella/microsome test for point mutagenic effect Novartis Crop Protection AG, Basel, Switzerland Bayer AG Toxicological Institute, Wuppertal- Elberfeld, Germany, Report No 11388 Not GLP Not Published Syngenta File N° CGA131013/0012	N	TDMG
IIA 5.8.1/36	Dollenmeier, P	1986b	CGA 131013 tech.: Point mutation test with Chinese hamster cells V79 Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 860258 GLP Not Published Syngenta File N° CGA131013/0015	N	TDMG
IIA 5.8.1/39	Puri, E	1986	CGA 131013 tech.: Autoradiographic DNA repair test on rat hepatocytes Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 860184 GLP Not Published Syngenta File N° CGA131013/0008	N	TDMG
IIA 5.8.1/41	Richold, M, Allen, JA, Williams, A, Ransome, SJ	1981	Cell transformation test for potential carcinogenicity of R152056 Novartis Crop Protection AG, Basel, Switzerland Huntingdon Research Centre Ltd., Huntingdon, United Kingdom, Report No ICI 394A/81153 GLP Not Published Syngenta File N° CGA131013/0011	N	TDMG

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IIA 5.8.1/42	Strasser, F	1986	CGA 131013 tech.: Micronucleus test (Chinese hamster) Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 860185 GLP Not Published Syngenta File N° CGA131013/0009	N	TDMG
IIA 5.8.1/43	Watkins, PA	1982	R152056: 3-(1,2,4-triazol-1-yl) alanine (ICI 156,342) - Micronucleus test in CBC F1 mice Novartis Crop Protection AG, Basel, Switzerland Central Toxicology Laboratory (CTL), Cheshire, United Kingdom, Report No CTL/C/1164 GLP Not Published Syngenta File N° CGA131013/0016	N	TDMG
IIA 5.8.1/44	Herbold, B	1983c	THS 2212 triazolylalanine: Micronucleus test for mutagenic effect on mice Novartis Crop Protection AG, Basel, Switzerland Bayer AG Toxicological Institute, Wuppertal-Elberfeld, Germany, Report No 11054 Not GLP Not Published Syngenta File N° CGA131013/0013	N	TDMG
IIA 5.8.1/48	Lai, K, Simoneaux, B	1986d	Balance study of 14C-triazole acetic acid in orally dosed rats Novartis Crop Protection AG, Basel, Switzerland Report No ABR-86022 Not GLP Not Published Syngenta File N° CGA142856/0004	N	SYN
IIA 5.8.1/49	Lai, K, Simoneaux, B	1986e	The metabolism of 14C-triazole acetic acid in the rat Novartis Crop Protection AG, Basel, Switzerland Report No ABR-86028 Not GLP Not Published Syngenta File N° CGA142856/0006	N	SYN
IIA 5.8.1/50	Thevenaz, P	1984	CGA 142856: Acute oral LD 50 in the rat Novartis Crop Protection AG, Basel, Switzerland Report No 840887 Not GLP Not Published Syngenta File N° CGA142856/0001	N	SYN
IIA 5.8.1/52	Deparade, E	1984	CGA 142856; Salmonella/mammalian-microsome mutagenicity test Novartis Crop Protection AG, Basel, Switzerland Ciba-Geigy Ltd., Basel, Switzerland, Report No 840864 Not GLP Not Published Syngenta File N° CGA142856/0003	N	SYN
IIA 5.8.1/53	Clare, G	2002	Triazole Acetic Acid: Mammalian Cell Mutation Assay Huntingdon Life Sciences Ltd. UK Report No. TM88 Not published GLP Syngenta File No. CGA142856/0018	Y	SYN

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IIA 5.8.1/54	Pritchard L	2002	Triazole Acetic Acid: In Vitro Mammalian Chromosome Aberration Test Huntingdon Life Sciences Ltd. UK Report No. TM83 Not published GLP Syngenta File No. CGA142856/0017	Y	SYN
IIA 5.8.1/55	Ohba, K.	1991a	Acute oral toxicity study of CGA-205374 in mice. Ciba-Geigy Japan Ltd., Tokyo, Japan Report No CG-1B250 GLP Not Published Syngenta File N° CGA205374/0001	N	SYN
IIA 5.8.1/56	Nakajima, M	1991b	Reverse mutation assay of CGA-205374 Novartis Crop Protection AG, Basel, Switzerland Biosafety Research Center of Japan, -, Japan, Report No 1746 GLP Not Published Syngenta File N° CGA205374/0002	N	SYN
IIA 5.8.1/57	Ohba, K	1991b	Acute oral toxicity study of CGA-205375 in mice Ciba-Geigy Japan Ltd., Tokyo, Japan Report No CG-1B260 GLP Not Published Syngenta File N° CGA205375/0001	N	SYN
IIA 5.8.1/58	Nakajima, M.	1991c	Reverse mutation assay of CGA-205375 Novartis Crop Protection AG, Basel, Switzerland Biosafety Research Center of Japan, -, Japan, Report No 1747 GLP Not Published Syngenta File N° CGA205375/0002	N	SYN
IIA 5.8.2/01	Thomas, H	1992	The effect of CGA 169374 tech. on selected biochemical and morphological liver parameters following subchronic administration to male mice. Novartis Crop Protection AG, Basel, Switzerland Report No CB 91/15 Not GLP Not Published Syngenta File N° CGA169374/0520	N	SYN
IIA 5.8.2/02	Schoch, M, Schneider, M	1987	CGA 169374 tech.: 56-Day feeding cataractogenicity in young chicken Novartis Crop Protection AG, Basel, Switzerland 871210 Not GLP Not Published Syngenta File N° CGA169374/0282	N	SYN
IIA 5.8.2/03	Janick, T, Frei, H, Vogel, O, Pappritz, G, Mladenovic, P	1989	CGA 169374 tech.: Oral toxicity (feeding) study in dogs - assessment of cataractogenic potential. Novartis Crop Protection AG, Basel, Switzerland 097132A GLP Not Published Syngenta File N° CGA169374/0284	N	SYN
Annex III data and information – DIVIDEND 030FS					

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IIIA 7.1.1/01	Cantoreggi, S.	1999a	CGA 169374 FS 030, (A-9142 G) - Acute oral toxicity in the rat (Limit test) Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 993058 GLP Not Published Syngenta File N° CGA169374/1760	N	SYN
IIIA 7.1.2/01	Cantoreggi, S.	1999b	CGA 169374 FS 030, (A-9142 G) - Acute dermal toxicity in the rat (Limit test) Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 993059 GLP Not Published Syngenta File N° CGA169374/1761	N	SYN
IIIA 7.1.4/01	Cantoreggi, S.	1999c	CGA 169374 FS 030, (A-9142 G) - Acute dermal irritation/corrosion in the rabbit Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 993060 GLP Not Published Syngenta File N° CGA169374/176	N	SYN
IIIA 7.1.5/01	Cantoreggi, S.	1999d	CGA 169374 FS 030, (A-9142 G) - Acute eye irritation/corrosion in the rabbit Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 993061 GLP Not Published Syngenta File N° CGA169374/1763	N	SYN
IIIA 7.1.6/01	Cantoreggi, S.	1999e	CGA 169374 FS 030, (A-9142 G) - Skin sensitization in the Guinea Pig (Maximization test) Novartis Crop Protection AG, Basel, Switzerland [REDACTED] Report No 993062 GLP Not Published Syngenta File N° CGA169374/1776	N	SYN
Annex III data and information – SCORE 250EC					
IIIA 7.1.1/01	Kuhn, J.	2003a	CGA-1169374 EC (250) (A7402T): Acute Oral Toxicity Study in Rats Syngenta Crop Protection AG, Basel, Switzerland [REDACTED] Report No 1497-03 GLP Not Published Syngenta File N° CGA169374/2369	Y	SYN
IIIA 7.1.2/01	Johnson, I.R.	2004	CGA 169374 EC 250 (A-7402 T): Acute Dermal Toxicity Study in the Rat Syngenta Limited, Cheshire, United Kingdom [REDACTED] Report No CR3622 GLP Not Published Syngenta File N° CGA169374/2426	Y	SYN

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IIIA 7.1.3/01	Noakes, J.	2003a	CGA169374 EC250 (A7402T): 4 Hour Acute Inhalation Toxicity Study in Rats Syngenta Limited, Cheshire, United Kingdom [REDACTED] Report No HR2437 GLP Not Published Syngenta File N° CGA169374/2410	Y	SYN
IIIA 7.1.4/01	Johnson, I.	2003a	CGA 169374 EC (A-7402T): Skin Irritation Study, in the Rabbit Syngenta Crop Protection AG, Basel, Switzerland [REDACTED] Report No EB5027 GLP Not Published Syngenta File N° CGA169374/2382	Y	SYN
IIIA 7.1.5/01	Johnson, I.	2003b	CGA 169374 EC 250 (A-7402T): Eye Irritation Study in the Rabbit Syngenta Crop Protection AG, Basel, Switzerland [REDACTED] Report No FB6025 GLP Not Published Syngenta File N° CGA169374/2408	Y	SYN
IIIA 7.1.6/01	Kuhn, J.	2003b	CGA-169374 EC (250) (A7402T): Skin Sensitisation Study in Guinea Pigs Syngenta Crop Protection AG, Basel, Switzerland [REDACTED] Number 7645-03 GLP Not Published Syngenta File N° CGA169374/2371	Y	SYN
IIIA 7.3/01	Hassler, S.	2003c	Dermal absorption of [Triazole-U-14C] CGA 169374 formulated as SCORE R 250 EC (A-7402 G) in the rat (in vivo) [REDACTED] Report No 051AM01 GLP Not Published Syngenta File N° CGA169374/2354	Y	SYN
IIIA 7.3/02	Hassler, S.	2003b	The percutaneous penetration of [Triazole-U-14C] CGA 169374 formulated as SCORE R 250 EC (A-7402 G) through rat and human split-thickness skin membranes (in vitro) [REDACTED] Report No 051AM02 GLP Not Published Syngenta File N° CGA169374/2353	Y	SYN

*: Protection for 5 years claimed from date of decision concerning listing in Annex I - the study report has not been submitted any of the Member States in support of an application for authorization, or (though the study report has been submitted) has not been used any of the Member States as the basis for decision on the initial authorization, or to maintain a given authorization, of a plant protection product before the date of submission of the dossier to Rapporteur Member State.

**: Owners' code identifications and names (SYN = Syngenta, TDMG = Triazole Derivative Metabolite Group)

The notifier has performed a literature search using a number of databases (Doc. M-II). References deemed worthy of consideration were included in the dossier submission. All test and study reports owned by the notifier were included in the dossier submission (Doc L-II, section 3).

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Annex B.6: Toxicology and Metabolism

APPENDIX Exposure calculations

1a. UK-POEM: PREDICTED OPERATOR EXPOSURE POME FRUIT NEU (VEHICLE MOUNTED AIR BLAST SPRAYER)

EXPOSURE DURING MIXING AND LOADING

Container size	1	litres
Hand contamination/operation	0,01	ml
Application dose	0,225	litres product/ha
Work rate	15	ha/day
Number of operations	4	/day
Hand contamination	0,04	ml/day
Protective clothing	None	
Transmission to skin	100	%
Dermal exposure to formulation	0,04	ml/day

DERMAL EXPOSURE DURING SPRAY APPLICATION

Application technique	Tractor-mounted/trailed broadcast air-assisted sprayer: 500 l/ha		
Application volume	500	spray/ha	
Volume of surface contamination	400	ml/h	
Distribution	Hands	Trunk	Legs
	10%	65%	25%
Clothing	None	Permeable	Permeable
Penetration	100%	2%	5%
Dermal exposure	10	5,2	5
			ml/h
Duration of exposure	6	h	
Total dermal exposure to spray	121,2	ml/day	

ABSORBED DERMAL DOSE

	Mix/load		Application	
Dermal exposure	0,04	ml/day	121,2	ml/day
Concen. of a.s. product or spray	250	mg/ml	0,1125	mg/ml
Dermal exposure to a.s.	10	mg/day	13,635	mg/day
Percent absorbed	1,4	%	4,6	%
Absorbed dose	0,14	mg/day	0,62721	mg/day

INHALATION EXPOSURE DURING SPRAYING

Inhalation exposure	0,05	ml/h
Duration of exposure	6	h
Concentration of a.s. in spray	0,1125	mg/ml
Inhalation exposure to a.s.	0,03375	mg/day
Percent absorbed	100	%
Absorbed dose	0,03375	mg/day

PREDICTED EXPOSURE

Total absorbed dose	0,80096	mg/day
Operator body weight	60	kg
Operator exposure	0,013349333	mg/kg bw/day

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**1b. UK-POEM: PREDICTED OPERATOR EXPOSURE POME FRUIT SEU
(VEHICLE MOUNTED AIR BLAST SPRAYER)**

EXPOSURE DURING MIXING AND LOADING			
Container size	1	litres	
Hand contamination/operation	0,01	ml	
Application dose	0,3	litres product/ha	
Work rate	15	ha/day	
Number of operations	5	/day	
Hand contamination	0,05	ml/day	
Protective clothing	None		
Transmission to skin	100	%	
Dermal exposure to formulation	0,05	ml/day	
DERMAL EXPOSURE DURING SPRAY APPLICATION			
Application technique	Tractor-mounted/trailed broadcast air-assisted sprayer: 500 l/ha		
Application volume	500	spray/ha	
Volume of surface contamination	400	ml/h	
Distribution	Hands	Trunk	Legs
	10%	65%	25%
Clothing	None	Permeable	Permeable
Penetration	100%	2%	5%
Dermal exposure	10	5,2	5 ml/h
Duration of exposure	6	h	
Total dermal exposure to spray	121,2	ml/day	
ABSORBED DERMAL DOSE			
	Mix/load		Application
Dermal exposure	0,05	ml/day	121,2 ml/day
Concen. of a.s. product or spray	250	mg/ml	0,15 mg/ml
Dermal exposure to a.s.	12,5	mg/day	18,18 mg/day
Percent absorbed	1,4	%	4,6 %
Absorbed dose	0,175	mg/day	0,83628 mg/day
INHALATION EXPOSURE DURING SPRAYING			
Inhalation exposure	0,05	ml/h	
Duration of exposure	6	h	
Concentration of a.s. in spray	0,15	mg/ml	
Inhalation exposure to a.s.	0,045	mg/day	
Percent absorbed	100	%	
Absorbed dose	0,045	mg/day	
PREDICTED EXPOSURE			
Total absorbed dose	1,05628	mg/day	
Operator body weight	60	kg	
Operator exposure	0,017604667	mg/kg bw/day	

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**1c. UK-POEM: PREDICTED OPERATOR EXPOSURE CARROT
(VEHICLE MOUNTED BOOM SPRAYER)**

EXPOSURE DURING MIXING AND LOADING			
Container size	1	litres	
Hand contamination/operation	0,01	ml	
Application dose	0,5	litres product/ha	
Work rate	50	ha/day	
Number of operations	25	/day	
Hand contamination	0,25	ml/day	
Protective clothing	None		
Transmission to skin	100	%	
Dermal exposure to formulation	0,25	ml/day	
DERMAL EXPOSURE DURING SPRAY APPLICATION			
Application technique	Tractor-mounted/trailed boom sprayer: hydraulic nozzles		
Application volume	100	spray/ha	
Volume of surface contamination	10	ml/h	
Distribution	Hands	Trunk	Legs
	65%	10%	25%
Clothing	None	Permeable	Permeable
Penetration	100%	5%	15%
Dermal exposure	6,5	0,05	0,375 ml/h
Duration of exposure	6	h	
Total dermal exposure to spray	41,55	ml/day	
ABSORBED DERMAL DOSE			
	Mix/load	Application	
Dermal exposure	0,25	ml/day	41,55 ml/day
Concen. of a.s. product or spray	250	mg/ml	1,25 mg/ml
Dermal exposure to a.s.	62,5	mg/day	51,9375 mg/day
Percent absorbed	1,4	%	4,6 %
Absorbed dose	0,875	mg/day	2,389125 mg/day
INHALATION EXPOSURE DURING SPRAYING			
Inhalation exposure	0,01	ml/h	
Duration of exposure	6	h	
Concentration of a.s. in spray	1,25	mg/ml	
Inhalation exposure to a.s.	0,075	mg/day	
Percent absorbed	100	%	
Absorbed dose	0,075	mg/day	
PREDICTED EXPOSURE			
Total absorbed dose	3,339125	mg/day	
Operator body weight	60	kg	
Operator exposure	0,055652083	mg/kg bw/day	

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**1d. UK-POEM: PREDICTED OPERATOR EXPOSURE POME FRUIT NEU
(HAND-HELD EQUIPMENT)**

EXPOSURE DURING MIXING AND LOADING

Container size	1	litres
Hand contamination/operation	0,01	ml
Application dose	0,225	litres product/ha
Work rate	1	ha/day
Number of operations	200	/day
Hand contamination	2	ml/day
Protective clothing	None	
Transmission to skin	100	%
Dermal exposure to formulation	2	ml/day

DERMAL EXPOSURE DURING SPRAY APPLICATION

Application technique	Hand-held rotary atomiser equipment (2.5 l tank). Outdoor, high level target		
Application volume	500	spray/ha	
Volume of surface contamination	50	ml/h	
Distribution	Hands	Trunk	Legs
	10%	65%	25%
Clothing	None	Permeable	Permeable
Penetration	100%	15%	20%
Dermal exposure	5	4,875	2,5
			ml/h
Duration of exposure	6	h	
Total dermal exposure to spray	74,25	ml/day	

ABSORBED DERMAL DOSE

	Mix/load		Application	
Dermal exposure	2	ml/day	74,25	ml/day
Concen. of a.s. product or spray	250	mg/ml	0,1125	mg/ml
Dermal exposure to a.s.	500	mg/day	8,353125	mg/day
Percent absorbed	1,4	%	4,6	%
Absorbed dose	7	mg/day	0,38424375	mg/day

INHALATION EXPOSURE DURING SPRAYING

Inhalation exposure	0,01	ml/h
Duration of exposure	6	h
Concentration of a.s. in spray	0,1125	mg/ml
Inhalation exposure to a.s.	0,00675	mg/day
Percent absorbed	100	%
Absorbed dose	0,00675	mg/day

PREDICTED EXPOSURE

Total absorbed dose	7,39099375	mg/day
Operator body weight	60	kg
Operator exposure	0,123183229	mg/kg bw/day

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**1e. UK-POEM: PREDICTED OPERATOR EXPOSURE POME FRUIT SEU
(HAND-HELD EQUIPMENT)**

<i>EXPOSURE DURING MIXING AND LOADING</i>			
Container size	1	litres	
Hand contamination/operation	0,01	ml	
Application dose	0,3	litres product/ha	
Work rate	1	ha/day	
Number of operations	200	/day	
Hand contamination	2	ml/day	
Protective clothing	None		
Transmission to skin	100	%	
Dermal exposure to formulation	2	ml/day	
DERMAL EXPOSURE DURING SPRAY APPLICATION			
Application technique	Hand-held rotary atomiser equipment (2,5 l tank). Outdoor, high level target		
Application volume	500	spray/ha	
Volume of surface contamination	50	ml/h	
Distribution	Hands	Trunk	Legs
	10%	65%	25%
Clothing	None	Permeable	Permeable
Penetration	100%	15%	20%
Dermal exposure	5	4,875	2,5 ml/h
Duration of exposure	6	h	
Total dermal exposure to spray	74,25	ml/day	
ABSORBED DERMAL DOSE			
	Mix/load		Application
Dermal exposure	2	ml/day	74,25 ml/day
Concen. of a.s. product or spray	250	mg/ml	0,15 mg/ml
Dermal exposure to a.s.	500	mg/day	11,1375 mg/day
Percent absorbed	1,4	%	4,6 %
Absorbed dose	7	mg/day	0,512325 mg/day
INHALATION EXPOSURE DURING SPRAYING			
Inhalation exposure	0,01	ml/h	
Duration of exposure	6	h	
Concentration of a.s. in spray	0,15	mg/ml	
Inhalation exposure to a.s.	0,009	mg/day	
Percent absorbed	100	%	
Absorbed dose	0,009	mg/day	
PREDICTED EXPOSURE			
Total absorbed dose	7,521325	mg/day	
Operator body weight	60	kg	
Operator exposure	0,125355417	mg/kg bw/day	

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**2a. GERMAN MODEL: PREDICTED OPERATOR EXPOSURE POME FRUIT NEU
(VEHICLE MOUNTED AIR BLAST SPRAYER)**

<i>Equipment/crops</i>	<i>Tractor field crops</i>		<i>Tractor high crops</i>	
Dose		kg ai/ha	0,05625	kg ai/ha
Work rate		ha/day	8	ha/day
Amount handled	0	kg/day	0,45	kg/day
			Dermal	Inhalation
Gloves			0,01	
Coverall + sturdy footwear			0,05	
Broad-brimmed headwear			0,5	
Hood and visor			0,05	
Particle filtering half-mask (FF2 SL or P2)			0,8	0,05
Half-mask with combination filter (A1P2)			0,8	0,02
<u>MIXING/LOADING</u>				
	Tractor field crops		Tractor high crops	
Liquid	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,0006	0	0,0006	0,00027
RPE (FF2SL or P2)		0		0,0000135
RPE (A1P2)		0		0,0000054
Hands mix	2,4	0	2,4	1,08
Gloves		0		0,0108
WP	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,07	0	0,07	0,0315
RPE (FF2SL or P2)		0		0,001575
RPE (A1P2)		0		0,00063
Hands mix	6	0	6	2,7
Gloves		0		0,027
WG	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,008	0	0,008	0,0036
RPE (FF2SL or P2)		0		0,00018
RPE (A1P2)		0		0,000072
Hands mix	2	0	2	0,9
Gloves		0		0,009
<u>APPLICATION</u>				
	Tractor field crops		Tractor high crops	
	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation	0,001	0	0,018	0,0081
RPE (FF2SL or P2)		0		0,000405

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RPE (A1P2)		0		0,000162
Head	0,06	0	1,2	0,54
Broad-brimmed headwear		0		0,27
Hood and visor		0		0,027
RPE (FF2SL or P2)		0		0,432
RPE (A1P2)		0		0,432
Hands	0,38	0	0,7	0,315
Gloves		0		0,00315
Body	1,6	0	9,6	4,32
Coverall + sturdy footwear		0		0,216
Body weigh		60		Total exposure= 0,004359
Inhalation absorption (%) (Ai)		100		(mg/kg bw * dag)
Dermal abs mix/loading (%) (Ad)		1,4		
Dermal abs spraying (%) (Ad)		4,6		
Im = inhalation mix		0,00027		
Ia = inhalation application		0,0081		
D ham= dermal exposure hands		1,08		
	during mix			
D he= dermal exposure head		0,54		
D ha= dermal exposure hands		0,315		
D b= dermal exposure body		4,32		

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**2b. GERMAN MODEL: PREDICTED OPERATOR EXPOSURE POME FRUIT SEU
(VEHICLE MOUNTED AIR BLAST SPRAYER)**

Equipment/crops	Tractor field crops		Tractor high crops	
Dose		kg ai/ha	0,075	kg ai/ha
Work rate		ha/day	8	ha/day
Amount handled	0	kg/day	0,6	kg/day
			Dermal	Inhalation
Gloves			0,01	
Coverall + sturdy footwear			0,05	
Broad-brimmed headwear			0,5	
Hood and visor			0,05	
Particle filtering half-mask (FF2 SL or P2)			0,8	0,05
Half-mask with combination filter (A1P2)			0,8	0,02
<u>MIXING/LOADING</u>				
	Tractor field crops		Tractor high crops	
Liquid	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,0006	0	0,0006	0,00036
RPE (FF2SL or P2)		0		0,000018
RPE (A1P2)		0		0,0000072
Hands mix	2,4	0	2,4	1,44
Gloves		0		0,0144
WP	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,07	0	0,07	0,042
RPE (FF2SL or P2)		0		0,0021
RPE (A1P2)		0		0,00084
Hands mix	6	0	6	3,6
Gloves		0		0,036
WG	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,008	0	0,008	0,0048
RPE (FF2SL or P2)		0		0,00024
RPE (A1P2)		0		0,000096
Hands mix	2	0	2	1,2
Gloves		0		0,012
<u>APPLICATION</u>				
	Tractor field crops		Tractor high crops	
	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day

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Inhalation	0,001	0	0,018	0,0108
RPE (FF2SL or P2)		0		0,00054
RPE (A1P2)		0		0,000216
Head	0,06	0	1,2	0,72
Broad-brimmed headwear		0		0,36
Hood and visor		0		0,036
RPE (FF2SL or P2)		0		0,576
RPE (A1P2)		0		0,576
Hands	0,38	0	0,7	0,42
Gloves		0		0,0042
Body	1,6	0	9,6	5,76
Coverall + sturdy footwear		0		0,288
Body weigh	60		Total exposure=	0,005812
Inhalation absorption (%) (Ai)	100		(mg/kg bw * dag)	
Dermal abs mix/loading (%) (Ad)	1,4			
Dermal abs spraying (%) (Ad)	4,6			
Im = inhalation mix	0,00036			
Ia = inhalation application	0,0108			
D ham= dermal exposure hands	1,44			
during mix				
D he= dermal exposure head	0,72			
D ha= dermal exposure hands	0,42			

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**2c. GERMAN MODEL: PREDICTED OPERATOR EXPOSURE CARROT
(VEHICLE MOUNTED BOOM SPRAYER)**

Equipment/crops	Tractor field crops	
Dose	0,125	kg ai/ha
Work rate	20	ha/day
Amount handled	2,5	kg/day

Gloves

Coverall + sturdy footwear

Broad-brimmed headwear

Hood and visor

Particle filtering half-mask (FF2 SL or P2)

Half-mask with combination filter (A1P2)

MIXING/LOADING

	Tractor field crops	
Liquid	mg/kg ai	mg ai/day
Inhalation mix	0,0006	0,0015
RPE (FF2SL or P2)		0,000075
RPE (A1P2)		0,00003
Hands mix	2,4	6
Gloves		0,06

WP	mg/kg ai	mg ai/day
Inhalation mix	0,07	0,175
RPE (FF2SL or P2)		0,00875
RPE (A1P2)		0,0035
Hands mix	6	15
Gloves		0,15

WG	mg/kg ai	mg ai/day
Inhalation mix	0,008	0,02
RPE (FF2SL or P2)		0,001
RPE (A1P2)		0,0004
Hands mix	2	5
Gloves		0,05

APPLICATION

	Tractor field crops	
	mg/kg ai	mg ai/day
Inhalation	0,001	0,0025
RPE (FF2SL or P2)		0,000125

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RPE (A1P2)		0,00005	
Head	0,06	0,15	
Broad-brimmed headwear		0,075	
Hood and visor		0,0075	
RPE (FF2SL or P2)		0,12	
RPE (A1P2)		0,12	
Hands	0,38	0,95	
Gloves		0,0095	
Body	1,6	4	
Coverall + sturdy footwear		0,2	
Body weigh	60	Total exposure=	0,0053767
Inhalation absorption (%) (Ai)	100	(mg/kg bw * dag)	
Dermal abs mix/loading (%) (Ad)	1,4		
Dermal abs spraying (%) (Ad)	4,6		
Im = inhalation mix	0,0015		
Ia = inhalation application	0,0025		
D ham= dermal exposure hands	6		
during mix			
D he= dermal exposure head	0,15		
D ha= dermal exposure hands	0,95		
D b= dermal exposure body	4		

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2d. GERMAN MODEL: PREDICTED OPERATOR EXPOSURE POME FRUIT NEU (HAND-HELD EQUIPMENT)

Equipment/crops	Tractor field crops		Tractor high crops		Hand high crops		Hand field crops	
Dose		kg ai/ha		kg ai/ha	0,05625	kg ai/ha		kg ai/ha
Work rate		ha/day		ha/day	1	ha/day		ha/day
Amount handled	0	kg/day	0	kg/day	0,05625	kg/day	0	kg/day

	Dermal	Inhalation
Gloves	0,01	
Coverall + sturdy footwear	0,05	
Broad-brimmed headwear	0,5	
Hood and visor	0,05	
Particle filtering half-mask (FF2 SL or P2)	0,8	0,05
Half-mask with combination filter (A1P2)	0,8	0,02

MIXING/LOADING

	Tractor field crops		Tractor high crops		Hand high crops		Hand field crops	
Liquid	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,0006	0	0,0006	0	0,05	0,0028125	0,05	0
RPE (FF2SL or P2)		0		0		0,0001406		0
RPE (A1P2)		0		0		5,625E-05		0
Hands mix	2,4	0	2,4	0	205	11,53125	205	0
Gloves		0		0		0,1153125		0

WP	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,07	0	0,07	0	0,8	0,045	0,8	0
RPE (FF2SL or P2)		0		0		0,00225		0
RPE (A1P2)		0		0		0,0009		0
Hands mix	6	0	6	0	50	2,8125	50	0
Gloves		0		0		0,028125		0

WG	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,008	0	0,008	0	0,02	0,001125	0,02	0
RPE (FF2SL or P2)		0		0		5,625E-05		0
RPE (A1P2)		0		0		0,0000225		0
Hands mix	2	0	2	0	21	1,18125	21	0
Gloves		0		0		0,0118125		0

APPLICATION

	Tractor field crops		Tractor high crops		Hand high crops		Hand field crops	
	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation	0,001	0	0,018	0	0,3	0,016875	no data	0
RPE (FF2SL or P2)		0		0		0,0008438		0

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RPE (A1P2)	0	0	0,0003375	0
Head	0,06	0	1,2	0
Broad-brimmed headwear	0	0	0,135	0
Hood and visor	0	0	0,0135	0
RPE (FF2SL or P2)	0	0	0,216	0
RPE (A1P2)	0	0	0,216	0
Hands	0,38	0	0,7	0
Gloves	0	0	0,0059625	0
Body	1,6	0	9,6	0
Coverall + sturdy footwear	0	0	0,0703125	0
Body weigh	60	Total exposure=	0,004761	
Inhalation absorption (%) (Ai)	100	(mg/kg bw * dag)		
Dermal abs mix/loading (%) (Ad)	1,4			
Dermal abs spraying (%) (Ad)	4,6			
Im = inhalation mix	0,0028125			
Ia = inhalation application	0,016875			
D ham= dermal exposure hands	11,53125			
during mix				
D he= dermal exposure head	0,27			
D ha= dermal exposure hands	0,59625			
D b= dermal exposure body	1,40625			

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2e. GERMAN MODEL: PREDICTED OPERATOR EXPOSURE POME FRUIT SEU (HAND-HELD EQUIPMENT)

Equipment/crops	Tractor field crops		Tractor high crops		Hand high crops		Hand field crops	
Dose		kg ai/ha		kg ai/ha	0,075	kg ai/ha		kg ai/ha
Work rate		ha/day		ha/day	1	ha/day		ha/day
Amount handled	0	kg/day	0	kg/day	0,075	kg/day	0	kg/day

	Dermal	Inhalation
Gloves	0,01	
Coverall + sturdy footwear	0,05	
Broad-brimmed headwear	0,5	
Hood and visor	0,05	
Particle filtering half-mask (FF2 SL or P2)	0,8	0,05
Half-mask with combination filter (A1P2)	0,8	0,02

MIXING/LOADING

	Tractor field crops		Tractor high crops		Hand high crops		Hand field crops	
Liquid	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,0006	0	0,0006	0	0,05	0,00375	0,05	0
RPE (FF2SL or P2)		0		0		0,0001875		0
RPE (A1P2)		0		0		0,000075		0
Hands mix	2,4	0	2,4	0	205	15,375	205	0
Gloves		0		0		0,15375		0

WP	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,07	0	0,07	0	0,8	0,06	0,8	0
RPE (FF2SL or P2)		0		0		0,003		0
RPE (A1P2)		0		0		0,0012		0
Hands mix	6	0	6	0	50	3,75	50	0
Gloves		0		0		0,0375		0

WG	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation mix	0,008	0	0,008	0	0,02	0,0015	0,02	0
RPE (FF2SL or P2)		0		0		0,000075		0
RPE (A1P2)		0		0		0,00003		0
Hands mix	2	0	2	0	21	1,575	21	0
Gloves		0		0		0,01575		0

APPLICATION

	Tractor field crops		Tractor high crops		Hand high crops		Hand field crops	
	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day	mg/kg ai	mg ai/day
Inhalation	0,001	0	0,018	0	0,3	0,0225	no data	0
RPE (FF2SL or P2)		0		0		0,001125		0
RPE (A1P2)		0		0		0,00045		0

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Head	0,06	0	1,2	0	4,8	0,36	no data	0
Broad-brimmed headwear		0		0		0,18		0
Hood and visor		0		0		0,018		0
RPE (FF2SL or P2)		0		0		0,288		0
RPE (A1P2)		0		0		0,288		0
Hands	0,38	0	0,7	0	10,6	0,795	no data	0
Gloves		0		0		0,00795		0
Body	1,6	0	9,6	0	25	1,875	no data	0
Coverall + sturdy footwear		0		0		0,09375		0

Body weigh	60	Total exposure=	0,006348
Inhalation absorption (%) (Ai)	100	(mg/kg bw * dag)	
Dermal abs mix/loading (%) (Ad)	1,4		
Dermal abs spraying (%) (Ad)	4,6		
Im = inhalation mix	0,00375		
Ia = inhalation application	0,0225		
D ham= dermal exposure hands	15,375		
during mix			
D he= dermal exposure head	0,36		
D ha= dermal exposure hands	0,795		
D b= dermal exposure body	1,875		

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DIVIDEND®030 FS (A-9142 G): Model calculations**3a. Calculated exposure to difenoconazole (CGA 169374) during seed treatment (includes calibration, mixing/loading, bagging, and cleaning)**

SCENARIO VARIABLES		
Seed to be treated		Cereals
Concentration of a.i. in the slurry		30 g/L
Dilution factor		product used undiluted
Pack size		20 L canister
Application rate		2 L product/ton seed
Amount seed treated		10 tons / hour
Throughput		90 tons / day
Amount a.i. handled		5.4 kg / day
Worker body weight		60 kg
Dermal absorption		1.4%
Mixing/loading		Pre-mix and fast-couple

WORKER EXPOSURE SUMMARY TABLE (Geometric mean values)							
TASK	Total potential dermal exposure	Estimated actual dermal exposure	Inhalation exposure	Frequency of operations per day (bagging: h/day)	Total potential dermal exposure	Estimated actual dermal exposure	Inhalation exposure
	no PPE	PPE			no PPE	PPE	
	mg/person per operation or hour				mg/person/day		
Calibration (mg/operation)	0.98	0.43	0.041	1	0.98	0.43	0.041
Mixing/loading (mg/operation)							
- Pre-mix	0.14	0.03	0.003	9	1.27	0.31	0.028
- Fast-couple	0.16	0.16	0.003	9	1.40	1.40	0.028
Bagging (mg/hour)							
- 25 kg bags	2.67	0.862	0.131		24.0	7.76	1.18
- 50 kg bags	1.19	0.472	0.015		10.7	4.25	0.135
- 500 kg bags	1.62	0.737	0.073		14.5	6.64	0.655
Cleaning (mg/operation)	26.2	2.50	0.476	1	26.2	2.50	0.476
Systemic exposure* (mg/kg bw/day)							
	dermal no PPE	dermal PPE	inhalation				
Calibration	0.0002	0.0001	0.0007				
Mixing/loading							
- Pre-mix	0.0003	0.0001	0.0005				
- Fast-couple	0.0003	0.0003	0.0005				
Bagging							
- 25 kg bags	0.0056	0.0018	0.0196				
- 50 kg bags	0.0025	0.0010	0.0023				
- 500 kg bags	0.0034	0.0015	0.0109				
Cleaning	0.0061	0.0006	0.0079				

* calculated according to the following formula:

$$[(\text{Dermal exposure} \times 1.4\% \text{ dermal absorption}) + (\text{inhalatory exposure} \times 100\%)] / 60 \text{ kg}$$
 (protective clothing includes cotton clothing and nitrile gloves)

DIFENOCONAZOLE
Annex B.6: Toxicology and Metabolism

3b. Estimated exposure to difenoconazole (CGA 169374) during sowing treated seed

VARIABLES		
Working time / day		10 hours
Worker body weight		60 kg
Dermal absorption		1.4%

Exposure calculations for loading and sowing of seeds (mg/h basis) (Geometric mean values)					
		Total potential dermal exposure	Estimated actual dermal Exposure	Inhalation exposure	Estimated systemic exposure * (mg/kg bw/day)
Model data	mg/person per hour	1.48	0.733	0.020	
	mg/person per day	14.8	7.33	0.200	
CGA 169374	mg/kg bw/day	0.246	0.122	0.003	0.005
* calculated according to the following formula: [(Estimated actual dermal exposure 1.4% dermal absorption) + (inhalatory exposure x 100% absorption)] / 60 kg.					