Section A6.1.1 Acute Oral Toxicity

Annex Point IIA6.1

Acute Toxic Class Method of Difenacoum in Rats.

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2004) Acute Oral Toxicity Study (Acute Toxic Class Method) of Test Item Difenacoum Technical in Rats. XXXXX. Study Code: 04/904-001P	
1.2	**Data protection**		
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD Guidelines 423 (2001)	
2.2	**GLP**	Yes	
2.3	**Deviations**	None	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	As given in section 2	
3.1.2.2	Purity	99.7%	
3.1.2.3	Stability	Needs to be stored in refrigerator and protected from light.	
3.2	**Test Animals**		
3.2.1	Species	Rats	
3.2.2	Strain	CRL:(WI) BR (Wistar) rats	
3.2.3	Source	Charles River Laboratories Inc.	
3.2.4	Sex	Female	
3.2.5	Age/weight at study initiation	170-194g	
3.2.6	Number of animals per group	3 animals/group	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**	Oral	

Section A6.1.1 Acute Oral Toxicity

Annex Point IIA6.1

Acute Toxic Class Method of Difenacoum in Rats.

3.3.1	Postexposure period	14 days
		Oral
3.3.2	Type	Gavage
3.3.3	Concentration	Gavage 5, 50..... mg/kg bw
3.3.4	Vehicle	1% aqueous methylcellulose containing 2% polysorbate 20.
3.3.5	Concentration in vehicle	0.5 and 5 mg/ml
3.3.6	Total volume applied	10 ml/kg bw
3.3.7	Controls	None

3.4 Examinations

Observations were performed on the skin, fur eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour as well. Particular attention was directed to the observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.
Body weight measurements recorded.
Gross necropsy was performed in every surviving animal and also for dead animals

3.5 Method of determination of LD_{50}

The method used is not intended to allow the calculation of a precise LD_{50} value. The test item is ranked into classes of Globally Harmonised Classification System (GHS) described in OECD Guideline No.423.

3.6 Further remarks

4 RESULTS AND DISCUSSION

4.1 Clinical signs

Animals treated with 5 mg/kg (n=6) were symptom free during the entire observation period.

Decreased activity, squatting position, plameness, piloerection and dyspnoea were observed in 50 mg/kg dose group. The degree of symptoms was slight, moderate and marked. The first symptom appeared 4-6 days after the treatment. Two rats died one day after onset of symptoms; another one died two days after the first symptoms.

See table 6.1.1

4.2 Pathology

Necropsy

In surviving animals (5mg/kg) pulmonary emphysema (4/6) and pin-pricked sizes haemorrhages in the lungs (1/6) were observed as alterations due to the termination process and agony.

In animals found dead (50 mg/kg), sanguineous fur around the nose (2/3), dark red lungs (1/3), haematoma in the thymus (3/3), clay coloured liver (3/3), blood in the thoraic activity (2/3) and haematoma near to the abdominal aorta (2/3) were observed.

4.3 Other

Body Weight

The body weight and body weight gains of surviving animals were normal.

Section A6.1.1 Acute Oral Toxicity

Annex Point IIA6.1

Acute Toxic Class Method of Difenacoum in Rats.

4.4 LD_{50}

Between 5mg/kg body weight and 50 mg/kg body weight. It was ranked into Class 2 of Globally Harmonised Classification System. LD_{50} cut-off dose is 25 mg/kg bw.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

OECD Guideline 423 was followed.

3 female rats were dosed with 5 mg/kg difenacoum technical. A single oral treatment for each animal was carried out by gavage after an overnight food withdrawal. These animals were treated with concentrations of 0.5 mg/ml and 5 mg/ml prepared with 1 % aqueous methylcellulose containing 2 % polysorbate 20. The total treatment volume was 10 ml/kg bw.

For all animals, clinical observations were performed continuously for a half-hour after dosing, then at the first, second, third fourth and fifth hours and daily thereafter. Food was made available again 3 hours after the treatment . Gross necropsy was performed in all animals.

5.2 Results and discussion

Mortality In rats after a single oral dose.

Dose	5 mg/kg	5 mg/kg	50 mg/kg
No. animals tested	3	3	3
Mortality	0/3	0/3	3/3

Body Weight

The body weight and body weight gains of surviving animals were normal.

Necropsy

In surviving animals (5mg/kg) pulmonary emphysema (4/6) and pin-pricked sizes haemorrhages in the lungs (1/6) were observed as alterations due to the termination process and agony.

In animals found dead (50 mg/kg), sanguineous fur around the nose (2/3), dark red lungs (1/3), haematoma in the thymus (3/3), clay coloured liver (3/3), blood in the thoraic activity (2/3) and haematoma near to the abdominal aorta (2/3) were observed.

Clinical Observations

Animals treated with 5 mg/kg (n=6) were symptom free during the entire observation period.

Decreased activity, squatting position, plameness, piloerection and dyspnoea were observed in 50 mg/kg dose group. The degree of symptoms was slight, moderate and marked. The first symptom appeared 4-6 days after the treatment. Two rats died one day after onset of symptoms; another one died two days after the first symptoms.

Between 5mg/kg body weight and 50 mg/kg body weight. It was ranked into Class 2 of Globally Harmonised Classification System. LD_{50} cut-off dose is 25 mg/kg bw.

Section A6.1.1 Acute Oral Toxicity

Annex Point IIA6.1 Acute Toxic Class Method of Difenacoum in Rats.

5.3 Conclusion		The LD_{50} between 5mg/kg body weight and 50 mg/kg body weight. It was ranked into Class 2 of Globally Harmonised Classification System. LD_{50} cut-off dose is 25 mg/kg bw.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	12 April 2006, revised 16 January 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Based on the test result ($5<LD_{50}< 50$) and literature data, difenacoum shall be assigned with the risk phrase R28; Very toxic if swallowed.
Reliability	1
Acceptability	Acceptable
Remarks	Key study
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_1-1. Table for Acute Toxicity

Dose mg/kg	Number of dead / number of investigated	Time of death (range)	Observations (found in all animals that died)
5 mg./kg	0/6	n/a	n/a
50mg/kg	3/3	Days 5-8	haematoma in thymus, clay coloured liver
LD_{50} value	Estimated to be between 5 and 50 mg/kg bw		

Section A6.1.2 Acute Dermal Toxicity

Annex Point IIA6.1 LD_{50} for dermal toxicity to the rat

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2004) Acute Dermal Toxicity Study of Test Item Difenacoum Technical in Rats. XXXXX. Study Code: 04/904-002P	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD Guideline 402	
2.2	**GLP**	Yes	
2.3	**Deviations**	Yes, animals were not prevented from grooming themselves.	X
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Greyish-white powder	
3.1.2.2	Purity	99.7%	
3.1.2.3	Stability	Must be stored in refrigerator, protected from light.	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	CRL:(WI) BR (Wistar) rats	
3.2.3	Source	Charles River (Europe) Laboratories Inc.	
3.2.4	Sex	Female/Male	
3.2.5	Age/weight at study initiation	young healthy adult rats female 202-240 g male 372-407 g	
3.2.6	Number of animals per group	5 animals/sex/group	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**	Dermal	

Section A6.1.2 Acute Dermal Toxicity

Annex Point IIA6.1 LD_{50} for dermal toxicity to the rat

3.3.1	Postexposure period	21 days	
		Dermal	
3.3.2	Area covered	10 % of body surface	
3.3.3	Occlusion	Semiocclusive	X
3.3.4	Vehicle	None	X
3.3.5	Concentration in vehicle	N/A	
3.3.6	Total volume applied	20, 55, 155 mg/kg/day	X
3.3.7	Duration of exposure	24 h	
3.3.8	Removal of test substance	Water	
3.3.9	Controls	N/A	
3.4	**Method of determination of LD_{50}**	Finney Method	
3.5	**Further remarks**	Body weight and necropsy findings were recorded.	
		4 RESULTS AND DISCUSSION	
4.1	**Clinical signs**	No dermal changes were found in either group after 24- hour exposure. Decreased activity, sanguineous fur, lateral position, squatting position, hunched back, decreased righting reflex, decreased grip and limb tone, cyanotic skin, paleness, dyspnoea, piloerection, sanguineous urine, swollen forelimb and lachrymation were observed. Clinical symptoms appeared in four animals of 55 mg/kg group and in all animals of 155 mg/kg group. Onset of symptoms was 7 to 9 days after treatment in 55 mg/kg dose and 5 to 6 days after the treatment in 155 mg/kg dose. Mortality occurred between days 6 and 14.	X
		Three male animals (20mg/kg) were symptom-free during the observation period. In two rats decreased activity, squatting position, paleness, dyspnoea, piloerection were observed for one day (day 7 and 9) and mortality occurred on day 8 and 10 respectively.	
4.2	**Pathology**	<u>Necropsy</u>	
		In animals found dead, bleeding (nasal and oral region, thoracic cavity, urinary bladder, stomach and intestines), haemtoma (subcutaneous, thymus, abdominal cavity, foot) and clay coloured liver were observed.	
		Test item related macroscopic alterations were found in one surviving animal: pale liver and haematoma near to the abdominal aorta were observed. No macroscopic alterations referred to the toxic effect of the test item were seen in the further surviving animals. Pulmonary emphysema, pinpricked-sized haemorrhages in the lungs occurred in several animals, which were caused by the termination process and agony.	
4.3	**Other**	<u>Body Weight and Body Weight Gain</u>	
		The body weight decreased in all animals found dead and in one	

Section A6.1.2 Acute Dermal Toxicity

Annex Point IIA6.1 LD_{50} for dermal toxicity to the rat

	surviving animal dosed with 55 mg/kg. The body weight gain was slightly below that expected in untreated animals of the same age and strain in surviving animals of 55 mg/kg group on weeks 1 and 2. The body weight and body weight gain of male animals, which showed no symptoms were normal. The body weight gain of one dead animal was less than normal, the body weight of the other dead animal decreased.	
4.4 LD_{50}	Value was 51.54 mg/kg in female rats. Male animals proved to be more sensitive than females.	
	5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1 Materials and methods	OECD 402.	
	The objective of the study was to determine the dermal LD_{50} value of the test item. The test item was applied in its undiluted formulation.	
	Animals were shaved at the trunk 24 hours prior to treatment. 10 percent of the total body surface was dosed. The test item was applied in original form by a single dermal route with 24-hour exposure. The observation period after the patch removal was 21 days in female animals since one dosed with 55 mg/kg showed symptoms.	
5.2 Results and discussion	The LD_{50} value was 51.54 mg/kg in female rats. Male animals proved to be more sensitive than females.	
5.3 Conclusion	The removal of the test substance for the skin with warm water is unlikely to have been effective, and since the animals were not prevented from grooming themselves, this study has only demonstrated oral toxicity.	X
5.3.1 Reliability	2	X
5.3.2 Deficiencies	Yes, animals were not prevented from grooming themselves. Additionally, the test material removal was by washing with warm water, and as the test substance has very low water solubility, removal cannot be assured.	X

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date 13 April 2006, revised 16 January 2007

Materials and Methods Points 2.3 and 3.3.3: The entire trunk was wrapped with plastic wrap for 24 h.

Point 3.3.4: According to the test guideline, solids should be moistened with a suitable vehicle to ensure good contact with the skin.

Point 3.3.6: Only the lowest dose (20 mg/kg bw) was given to male rats.

Point 3.6: According to the records (appendix 4) deaths occurred between days 7 and 16.

Results and discussion Point 4.3: In the light of the result of this study, RMS interpretation is that wrapping of the trunk with plastic wrap during exposure and washing (with water) of the exposure site has been enough to guarantee only dermal exposure.

Section A6.1.2 Acute Dermal Toxicity

Annex Point IIA6.1

LD_{50} for dermal toxicity to the rat

Conclusion	The LD_{50} value in female rats was 51.54 mg/kg bw Due to the overall mortality (both sexes) the risk phrase R27; Very to toxic in contact with skin, is warranted.
Reliability	1
Acceptability	Acceptable
Remarks	Key study Hydrometra occurred in two animals. It might be related to inflammation.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_1-1. Table for Acute Toxicity

Dose [20 mg/kg]	Number of dead / number of investigated	Time of death (range)	Observations
20	Females 0/5 Males 2/5	Days 8 and 10	Decreasesd activity, squatting position, paleness, dyspnoea, piloerection
55	Females 3/5	Between days 6-14	Decreased activity, sanguineous fur, lateral position, squatting position, hunched back, decreased righting reflex, decreased grip and limb tone, cyanotic skin, paleness, dyspnoea, piloerection, sanguineous urine, swollen forelimb and lachrymation
155	Females 5/5	Between days 6-14	
LD_{50} value	51.54mg/kg in female rats		

Section 6.1.3 Acute Toxicity

Annex Point IIA6.1 4 Hour Acute Inhalation Toxicity in the Rat

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (1995) Difenacoum: 4 Hour Acute Inhalation Toxicity Study to the Rat. XXXXX. Report No: MLS/9825	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Pelgar Limited	
1.2.2	Companies with access to data	Activa srl (only for use in Annex I listing of difenacoum)	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. / b.p for the purpose of its entry into Annex I authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes – In compliant with the regulatory guidelines of the OECD, US EPA. Specific guideline not stated in report but essentially complies with OECD 403	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Difenacoum	
3.1.1	Lot/Batch number	Sample reference SC7378	X
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Off white solid	
3.1.2.2	Purity	97.7% w/w difenacoum	
3.1.2.3	Stability	Not stated	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	Not stated	
3.2.3	Source	Charles River Laboratories, Wilmington, Mass, USA	
3.2.4	Sex	Male and female	
3.2.5	Age/weight at study initiation	Young adult. Weight of males: 266.4-297.2g. Weight of females: 213.8-225.4g	X
3.2.6	Number of animals per group	5/sex/group	
3.2.7	Control animals	No	

Section 6.1.3 Acute Toxicity

Annex Point IIA6.1 4 Hour Acute Inhalation Toxicity in the Rat

3.3	**Administration/ Exposure**	Inhalation		
3.3.1	Postexposure period	14 days		
		Inhalation		
3.3.2	Concentrations	Nominal concentration	2.76, 6.88, 18.04µg/l	X
		Analytical concentration	3.28, 7.52, 20.33µg/l	X
3.3.3	Particle size	MMAD (mass median aerodynamic diameter) 0.78µm, 0.86 µm and 0.89 µm ± GSD (geometric standard deviation) 2.74µm, 2.41 µm and 3.15µm		
3.3.4	Type or preparation of particles	The aerosol was generated using a glass concentric jet atomiser. The stock solution was pumped to the atomiser using a standard Hamilton Microlab M fitted with a 50µl glass syringe.		
3.3.5	Type of exposure	Nose only		
3.3.6	Vehicle	Acetone		
3.3.7	Concentration in vehicle	5mg/ml		
3.3.8	Duration of exposure	4 h		
3.3.9	Controls	None		X
3.4	**Examinations**	Clinical observations, necropsy		
3.5	**Method of determination of LC_{50}**	The median lethal concentration was estimated by logistic regression. Confidence limits were calculated using a likelihood ratio interval (William 1986)		
3.6	**Further remarks**			
		4 RESULTS AND DISCUSSION		
4.1	**Clinical signs**	Abnormalities generally associated with animals being restrained eg wet fur, were seen in all test groups. At the highest exposure level of 20.33µ/L some animals exhibited slow breathing and chromodacryhorrea. An initial reduction in bodyweight was seen in surviving animals from all exposure groups. In some animals exposed at 20.33µg/L there was significant deterioration in their clinical condition between days 4 and 6 which included subcutaneous haemorrhaging, bleeding from the snout, general decreased activity and reduced respiratory rate. Theses symptoms were consistent with anticoagulant poisoning and the animals were killed *in extemis*		
4.2	**Pathology**	Treatment related changes were seen in those animals killed *in extremis* which included subcutaneous haemorrhaging, haemorrhagic areas in the oral cavity, lung, abdominal fat and nares.		
4.3	**Other**			
4.4	**LC_{50}**	20.74µg/L for males and 16.27 µg/L for females		

Section 6.1.3 Acute Toxicity

Annex Point IIA6.1 4 Hour Acute Inhalation Toxicity in the Rat

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The study essentially follows OECD guideline 403.

Groups of 5 male and 5 female rats were exposed, nose only for a single four hour period to aerosols of difenacoum technical material. The aerosols had concentrations of 3.28, 7.52 and 20.33µg/L.

5.2 Results and discussion

Two males and four females were killed *in extremis* following exposure to 20.33µg/l. Clinical signs, delayed deaths and post mortem findings were consistent with anti-coagulent poisoning. Only slight signs of toxicity were seen in animals exposed to the lower concentrations.

There were no treatment related findings in those animals that went to termination of the study. The absence of significant clinical effects and lack of abnormalities at necropsy in animals that survived to termination indicates a rapid recovery from exposure to a non-lethal concentration of the test material.

5.3 Conclusion

The 4-hour median lethal concentration of difenacoum technical material in the rat was 20.74µg/L for males and 16.27 µg/L for females

5.3.1 Reliability 1

5.3.2 Deficiencies No

Section 6.1.3 Acute Toxicity

Annex Point IIA6.1 4 Hour Acute Inhalation Toxicity in the Rat

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	22 January 2007
Materials and Methods	Point 3.1.1: The certificate of analysis is not included in the test report. Point 3.2.5: The applicant has presented the group mean values. The corresponding individual weights were 261-305 g for males and 204-230 g for females. Point 3.3.2: There is some confusion in the reporting of concentrations (point 4.1.1 of the report). According to table 1 of the test report, 2.76, 6.88 and 18.04 µg/l are difenacoum concentrations in the exposure atmosphere based on liquid chromatography and fluorescence detection rather than nominal concentrations. On the other hand, 3.28, 7.52 and 20.33 µg/l are the particulate concentrations of exposure atmosphere based on gravimetric analysis. Point 3.3.9: A vehicle control group should have been used. However, the result of the study is not seriously compromised without the control group.
Results and discussion	Table 6_1-1: One female (not two) was killed *in extremis* on day 6.
Conclusion	The LC_{50} value is 20.74µg/L/4h (95% confidence limits 12.03-39.76) for males and 16.27 µg/L/4h (95% confidence limits 10.03-26.24) for females. Classification with R26; Very toxic by inhalation is warranted.
Reliability	1
Acceptability	Acceptable
Remarks	Key study
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section 6.1.3 Acute Toxicity

Annex Point IIA6.1 4 Hour Acute Inhalation Toxicity in the Rat

Table A6_1-1. Table for Acute Toxicity (modify if necessary)

Dose µg/L	*Number of dead / number of investigated*	*Time of death*	*Observations*
3.28	Male 0/5 Female 0/5	N/A	
7.52	Male 0/5 Female 0/5	N/A	
20.33	Male 2/5 Female 4/5	Animals were killed *in extremis*. (1M and 2F on day 4, 1F on day 5 and 1M and 2F on day 6)	Subcutaneous haemorrhaging, haemorrhagic areas in the oral cavity, lung, abdominal fat and nares.
LC_{50} value	20.74µg/L for males and 16.27 µg/L for females		

Section A6.1.4 (1) Acute Dermal Irritation

Annex Point IIA6.4

Acute skin irritation of Difenacoum in the rabbit

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2004) Acute skin irritation study of the test item difenacoum technical in rabbits. XXXXX. Study code: 04/904-006N	
1.2	**Data protection**		
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 404	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Difenacoum technical	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	N/A	
3.1.2.2	Purity	99.7 % active ingredient	
3.1.2.3	Stability	Stability if refrigerated and protected from light.	
3.2	**Test Animals**		
3.2.1	Species	Rabbit	
3.2.2	Strain	New Zealand White	
3.2.3	Source	Ferenc Sandor breeder 2173 Kartal, Voros Hadsereg street 131 Hungary	
3.2.4	Sex	Male	
3.2.5	Age/weight at study initiation	10 weeks old, adult albino rabbits. 2846-3009 g.	
3.2.6	Number of animals per group	3	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**	Dermal	
3.3.1	Application		

Section A6.1.4 (1) Acute Dermal Irritation

Annex Point IIA6.4

Acute skin irritation of Difenacoum in the rabbit

3.3.1.1	Preparation of test substance	Test substance was moistened with water.
3.3.1.2	Test site and Preparation of Test Site	Not less than 24 hours prior to the treatment the back of experimental animals was shaved by means of a razor. The test item was applied to a small approximately 6 cm^2 area of skin and covered with a gauze patch, which held loosely in place by adhesive, but not irritating tape.
3.3.2	Occlusion	Semiocclusive
3.3.3	Vehicle	None
3.3.4	Concentration in vehicle	N/A
3.3.5	Total volume applied	0.5g
3.3.6	Removal of test substance	Water
3.3.7	Duration of exposure	4 hours
3.3.8	Postexposure period	72 hours
3.3.9	Controls	N/A
3.4	**Examinations**	
3.4.1	Clinical signs	Yes
3.4.2	Dermal examination	Dermal reactions were scored but no histological examination was performed. (no skin reactions)
3.4.2.1	Scoring system	Evaluated by the Draize (1959) scoring system.
3.4.2.2	Examination time points	Animals were observed for 72 hours. At 1, 24, 48, 72 hours.
3.4.3	Other examinations	Bodyweight measurements were taken.
3.5	**Further remarks**	

4 RESULTS AND DISCUSSION

4.1	**Average score**	
4.1.1	Erythema	Score of 0 for all animals at all times.
4.1.2	Edema	Score of 0 for all animals at all times.
4.2	**Reversibility**	N/A.
4.3	**Other examinations**	None
4.4	**Overall result**	Not classed as irritating for the skin.

5 APPLICANT'S SUMMARY AND CONCLUSION

Section A6.1.4 (1) Acute Dermal Irritation

Annex Point IIA6.4

Acute skin irritation of Difenacoum in the rabbit

5.1	**Materials and methods**	The test item (difenacoum technical) was administered in pure state, in a single dose of 0.5 g to the hairless skins of all experimental animals. After 4 hours the rest of the test item was removed with water of body temperature. The animals were examined at 1, 24, 48 and 72 hours after the patch removal.
5.2	**Results and discussion**	No irritation symptoms (erythema and oedema) or other signs occurred after the patch removal and during the 73-hour observation period. During the study the behaviour and general state of animals were normal.
5.3	**Conclusion**	Difenacoum is not a skin irritant.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	18 April 2006, revised 23 January 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Difenacoum is not irritating to the skin.
Reliability	1
Acceptability	Acceptable
Remarks	Key study The updated version (24th April 2002) of the OECD 404 test guideline stresses the importance of avoiding unnecessary animal testing. *In vivo* testing should be as limited as possible. In this study, sequential application of three patches to one animal was not performed as a first step, but three animals were tested simultaneously.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_1-4S-1. Table for skin irritation study

score (average animals investigated)	time	Erythema	Edema
average score Draize scores (0 to maximum 4)	60 min	0	0
	24 h	0	0
	48 h	0	0
	72 h	0	0
other times	*State time*		
average score	24h, 48h, 72h	0	0

Section 6.1.4 (2) Acute Eye Irritation

Annex Point IIA6.1.4

Acute eye irritation of Difenacoum in rabbits

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2004) Acute eye irritation study of test item Difenacoum technical in rabbits. XXXXX. Study code: 04/904-005N	
1.2	**Data protection**		
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 405. 2002	
2.2	**GLP**	Yes	
2.3	**Deviations**	None	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	N/A	
3.1.2.2	Purity	99.7%	
3.1.2.3	Stability	Stability only affected by light.	
3.2	**Test Animals**		
3.2.1	Species	Rabbit	
3.2.2	Strain	New Zealand White	
3.2.3	Source	Ferenc Sandor breeder, 273 Kartal, Voros Hadsereg street 131, Hungary	
3.2.4	Sex	Male	
3.2.5	Age/weight at study initiation	10 weeks old, adult albino rabbit. 2924-3209g	
3.2.6	Number of animals per group	3	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**		

Section 6.1.4 (2) Acute Eye Irritation

Annex Point IIA6.1.4 Acute eye irritation of Difenacoum in rabbits

3.3.1	Preparation of test substance	None	
3.3.2	Amount of active substance instilled	0.1 g	
3.3.3	Exposure period	The eyes of the test animals were not washed out after the application of the test item.	
3.3.4	Postexposure period	72 hours	
3.4	**Examinations**		
3.4.1	Ophthalmoscopic examination	Yes	
3.4.1.1	Scoring system	Scores were evaluated according to the scoring system by Draize (1959) and OECD 405 (2002)	
3.4.1.2	Examination time points	60min, 24h, 48h, 72h	
3.4.2	Other investigations	None	
3.5	**Further remarks**		
		4 RESULTS AND DISCUSSION	
4.1	**Clinical signs**	One hour after treatment some blood vessels hyperaemic occurred in two animals. In one case the vessels were more diffuse. In two animals the discharge from the eye was "any amount different from normal."	
4.2	**Average score**		
4.2.1	Cornea	See table 6-1-4-E-1	-
4.2.2	Iris	See table 6-1-4-E-1	
4.2.3	Conjunctiva		
4.2.3.1	Redness	See table 6-1-4-E-1	
4.2.3.2	Chemosis	G See table 6-1-4-E-1	
4.3	**Reversibility**	Yes	
4.4	**Other**	None	
4.5	**Overall result**	Classified as not irritating for the eyes. The observed symptoms can be evaluatedas fully reversible alterations.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	

Section 6.1.4 (2) Acute Eye Irritation

Annex Point IIA6.1.4

Acute eye irritation of Difenacoum in rabbits

5.1	**Materials and methods**	Test was carried out to OECD guidelines 405. 0.1 g of difenacoum technical was used in pure state, in a single dose. The test item was not placed into the conjunctival sac of left eye of each animal. The untreated right eye served as control. The treated eyes of the test animals were not washed out following the instillation of 0.1g of test item. The eyes were examined at 1, 24, 48, and 72 hours after the application. The duration of the observation period was sufficient for the statement of reversibility or irreversibility of changes. At the end of the observation period all animals were sacrificed by i.p injection of Nembutal anaesthesia. The eye irritation scores were evaluated according to the scoring system by Draize (1959). Individual reactions of each animal were recorded at each observation time.
5.2	**Results and discussion**	One hour after treatment some blood vessels hyperaemic occurred in two animals. In one case the vessels were more diffuse. In two animals the discharge from the eye was "any amount different from normal." Chemosis, corneal and iris alterations were not found during the study. 24 hours after treatment every animal was symptom free. 48 and 72 hours after treatment every animal was symptom free.
5.3	**Conclusion**	Difenacoum technical has not be classified as irritatinng for the eyes. The observed symtoms can be evaluated as fully reversible alterations.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	21 April 2006, revised 23 January 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Difenacoum technical is not classified for eye irritation according to the Dir. 67/548/EEC.
Reliability	1
Acceptability	Acceptable

Section 6.1.4 (2) Acute Eye Irritation

Annex Point IIA6.1.4

Acute eye irritation of Difenacoum in rabbits

Remarks	Key study The updated version (24th April 2002) of the OECD 405 test guideline stresses the importance of avoiding unnecessary animal testing. *In vivo* testing should be as limited as possible. In this study, an initial test with one animal was not performed, but three animals were tested simultaneously.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_1_4E-1. Results of eye irritation study

	Cornea	Iris	Conjunctiva	
			redness	chemosis
score (average of animals investigated)	0 to 4	0 to 2	0 to 3	0 to4
60 min	0	0	1.3	0
24 h	0	0	0	0
48 h	0	0	0	0
72 h	0	0	0	0
Average 24h, 48h, 72h	0	0	0	0
Area effected	na	na	na	na
Maximum average score (including area affected, max 110)	0	0	1.3	0
Reversibility*	na	na	na	na
average time for reversion	na	na	c	na
Give method of calculation maximum average score. * *c :* *completely reversible* *n c :* *not completely reversible* *n :* *not reversible*				

Section A6.1.5 (1) Skin sensitisation

Annex Point IIA VI.6.1.5

Guinea pig maximisation test (GPMT), Magnusson & Kligman

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (1996) Skin sensitisation test of a 2.5% concentrate in Guinea Pigs, XXXXX, Report number CIT/14302	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar brodifacoum and difenacoum Task Force	
1.2.2		PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 406	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	TCP 0047/94	
3.1.2	Specification	As given in section 2	X
3.1.3	Description	Red liquid	
3.1.4	Purity	2.59% w/w	
3.1.5	Stability	Stable	
3.1.6	Preparation of test substance for application	a) For induction: Intradermal injections: difenacoum 2.5% at 1% (w/w) in sterile isotonic saline solution (0.9% NaCl) Topical application: difenacoum 2.5% used undiluted b) For challenge: Topical application: difenacoum 2.5% used undiluted	
3.1.6.1	Pretest performed on irritant effects	Yes	
3.2	**Test Animals**		
3.2.1	Species	Guinea pigs	
3.2.2	Strain	Dunkin-Hartley	
3.2.3	Source	Centre d'Elevage Lebeau, 78950 Gambais, France	
3.2.4	Sex	Male and female (nulliparous and non-pregnant)	
3.2.5	Age/weight at study initiation	Approximately 3 months Male 324g with standard deviation ± 23g Female 322g with standard deviation ± 14g	X
3.2.6	Number of animals	Control group 10 (5 male, 5 female)	

Section A6.1.5 (1) Skin sensitisation

Annex Point IIA VI.6.1.5

Guinea pig maximisation test (GPMT), Magnusson & Kligman

	per group	Treatment Group 20 (10 male, 10 female) Magnusson & Kligman
3.2.7	Control animals	Yes
3.3	**Administration/ Exposure**	State study type: Adjuvant
3.3.1	Induction schedule	Day 0 – intradermal injection Day 7 – 10% sodium lauryl suphate was applied topically to create a local irritation Day 8 – topical application *(see table in appendix)*
3.3.2	Way of Induction	Intradermal or topical – both Occlusive or semi-occlusive – Occlusive
3.3.3	Concentrations used for induction	Intradermal: difenacoum 2.5% at 1% (w/w)in sterile isotonic saline solution (0.9%NaCl) Topical: difenacoum 2.5% undiluted
3.3.4	Concentration Freunds Complete Adjuvant (FCA)	FCA diluted at 50% (v/v) with 0.9% NaCl
3.3.5	Challenge schedule	Day 22
3.3.6	Concentrations used for challenge	difenacoum 2.5 % undiluted
3.3.7	Rechallenge	No
3.3.8	Scoring schedule	24h, 48h after challenge
3.3.9	Removal of the test substance	After 24 hours with dry or moistened gauze pad
3.3.10	Positive control substance	Separate test with 2,4-dinitro chlorobenzene
3.4	**Examinations**	
3.4.1	Pilot study	Yes
3.5	**Further remarks**	No mortalities

4 RESULTS AND DISCUSSION

4.1 Results of pilot studies

Application by the intradermal route:

Animal number	Concentration of the test substance % (w/w)	Scoring after treatment 24 hours	48 hours	6 days
Male 01	10	irritation	necrosis	crusts
	50	necrosis	necrosis	crusts
Male 02	10	necrosis	necrosis	crusts
	50	necrosis	necrosis	crusts
Female 01	10	necrosis	necrosis	crusts
	50	necrosis	necrosis	crusts
Female 02	10	necrosis	necrosis	crusts
	50	necrosis	necrosis	crusts

Concentration chosen for the main study was therefore 1% (w/w).

Application by cutaneous route:

Section A6.1.5 (1) Skin sensitisation

Annex Point IIA VI.6.1.5

Guinea pig maximisation test (GPMT), Magnusson & Kligman

Animal number	Concentration of the test substance % (w/w)		Scoring after removal of the dressing (1) 24 hours E	O	48 hours E	O
Male 01	100	RF	0	0	-	-
	50	LF	0	0	-	-
Female 01	100	RF	-	-	-	-
	50	LF	-	-	-	-

E : erythema
O : oedema
RF: right flank
LF: left flank
(1): No residual test substance was observed.
- : Examination not performed (male 01 killed on human ground after the first scoring and female 01 found dead).

Concentration chosen for the topical application of the induction phase (day 8) and for the challenge application was 100%.

4.2 Results of test

4.2.1 24h after challenge 0 / 20

4.2.2 48h after challenge 0 / 20

4.2.3 Other findings

4.3 Overall result Reactions seen in 0/20 animals. Classified as not a sensitiser

5 APPLICANT'S SUMMARY AND CONCLUSION

Section A6.1.5 (1) Skin sensitisation

Annex Point IIA VI.6.1.5

Guinea pig maximisation test (GPMT), Magnusson & Kligman

5.1	**Materials and methods**	The study was conducted according to OECD 406 guidelines. Thirty guinea-pigs were allocated to two groups: a control group 1 (5 males and 5 females) and a treated group 2 (10 males and 10 females). On day 1, intradermal injections of Freund's complete adjuvant mixed with the test substance (treated group) or the vehicle (control group) were performed in the dorsal region between the shoulders. On day 7, sodium laurylsulfate in vaseline (10%w/w) was applied on the test site to induce local irritation On day 8, this same test site was treated by topical application of the test substance (treated group) or the vehicle (control group) and was covered by an occlusive dressing for 48 hours Challenge was performed after 14 days. (test day 22) Test substance and vehicle were maintained under an occlusive dressing for 24 hours. Skin reactions were evaluated at 24 and 48 hours. The test concentrations were as follows: Induction (treated group) - intradermal injections: Difenacoum (2.5%) at 1% (w/w) in sterile isotonic saline solution (0.9% NaCl) - topical application: Difenacoum (2.5%) undiluted. Challenge (all groups) - topical applications: difenacoum (2.5%) undiluted. All the animals were killed at the end of the study. No skin samples were taken from the challenge application sites.
5.2	**Results and discussion**	There were no clinical signs or mortalities during the study. No cutaneous reactions were recorded after the challenge application. Reactions seen in 0/20 animals. Classified as not a sensitiser.
5.3	**Conclusion**	Difenacoum (2.5%) was not sensitising to guinea pig skin.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	21 April 2006, revised 23 January 2007
Materials and Methods	Point 3.1.2: The product used in the study is a technical concentrate of the a.s. (2.5% w/v) in solvents. Point 3.2.5: The mean body weight of males was 32**2** g
Results and discussion	Agree with applicant's version.
Conclusion	Under the test conditions difenacoum did not show sensitising potential.

Section A6.1.5 (1) Skin sensitisation

Guinea pig maximisation test (GPMT), Magnusson & Kligman

Annex Point IIA VI.6.1.5

Reliability	2 (see remarks)
Acceptability	Acceptable
Remarks	Key study It is well known that the test substance is very toxic, but disregarding this, SDS was used to irritate the skin. The methodology possibly resulted in low concentrations, and it is not possible to confirm whether optimal concentrations for induction could be used. Table A6_1_5-2: A conclusion of positive response for positive control animals has been made also for three individuals with a remark "scoring masked by marked dryness of the skin" at 48 h.
	COMMENTS FROM …
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_1_5-1. Detailed information including induction/challenge/scoring schedule for skin sensitisation test

Inductions	**GPMT**			**Observations/Remarks** *give information on irritation effects*
	day of treatment	application	day of treatment	
Induction 1	1	Intradermal		No effects noted
Induction 2	8	Topical		Irritation noted in controls and treated on day 10
challenge	22-23	Topical		No effects noted
scoring 1	24			No effects noted
scoring 2	25			No effects noted

Section A6.1.5 (1) Skin sensitisation

Annex Point IIA VI.6.1.5

Guinea pig maximisation test (GPMT), Magnusson & Kligman

Table A6_1_5-2. Result of skin sensitisation test

	Number of animals with signs of allergic reactions / number of animals in group		
	Negative control	**Test group**	**Positive control**
scored after 24h	0 / 10	0 / 20	20/20
scored after 48h	0 / 10	0 / 20	12/20

Section A6.1.5 (2) Skin sensitisation

Buehler test

Annex Point IIA VI.6.1.5

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (1995) Difenacoum, Skin sensitisation to the guinea pig of a 2.5 % concentrate. XXXXX. Report No. MLS/10009.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	PelGar	
1.2.1	Companies with access to data	Activa srl. (only for use in Annex I listing of difenacoum)	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD Guideline	X
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	X
3.1.1	Lot/Batch number	SC7396	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Red liquid	
3.1.2.2	Purity	2.58 % w/w	X
3.1.2.3	Stability	Not stated	
3.1.2.4	Preparation of test substance for application	c) For induction: Topical application: difenacoum 2.5 % at 10 % (w/v) in deionised water. d) For challenge: Topical application: difenacoum 2.5 % at 10 % and 3 % (w/v) in deionised water.	X
3.1.2.5	Pretest performed on irritant effects	Yes	
3.2	**Test Animals**		
3.2.1	Species	Guinea pig	
3.2.2	Strain	Not specified	
3.2.3	Source	Charles River Laboratories, Wilmington, Mass. USA.	
3.2.4	Sex	Female	X
3.2.5	Age/weight at study initiation	Young adult	
3.2.6	Number of animals	Control group 10 Treatment Group 20	

Section A6.1.5 (2) Skin sensitisation
Buehler test

Annex Point IIA VI.6.1.5

	per group		
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Non-Adjuvant	
3.3.1	Induction schedule	Day 0 – The freshly prepared sample was applied on a lint patch and an occlusive dressing used for 6 hours. This was repeated at 7 day intervals to give a total of three 6 hour exposures over 2 weeks. The animals were left untreated for 2 weeks after the final induction prior to challenge. (See table A6 _1_5-1)	
3.3.2	Way of Induction	Topical Occlusive	
3.3.3	Concentrations used for induction	10% w/v preparation in deionised water	
3.3.4	Concentration Freunds Complete Adjuvant (FCA)	N/A	
3.3.5	Challenge schedule	Day 28	
3.3.6	Concentrations used for challenge	3 % and 10 % preparations of the formulation in deionised water.	
3.3.7	Rechallenge	No	
3.3.8	Scoring schedule	24h, 48h after removal of dressings	
3.3.9	Removal of the test substance	No	
3.3.10	Positive control substance	Formaldehyde applied as 12 % solution in deionised water for both the induction and challenge phases.	X
3.4	**Examinations**		
3.4.1	Pilot study	Yes	
3.5	**Further remarks**		
		4 RESULTS AND DISCUSSION	
4.1	**Results of pilot studies**	Challenge: No irritation was observed. Induction: No irritation was observed.	
4.2	**Results of test**		
4.2.1	24h after challenge	0 / 20	
4.2.2	48h after challenge	0 / 20	
4.2.3	Other findings		
4.3	**Overall result**	Reactions seen in 0/20 animals. Classified as not a sensitizer.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	

Section A6.1.5 (2) Skin sensitisation

Buehler test

Annex Point IIA VI.6.1.5

5.1	**Materials and methods**	Thirty guinea-pigs were allocated to two groups: a control group (10 females) and a treated group (20 females). On day 1 the test site was treated by topical application of the test substance (treated group) or the vehicle (control group) and was covered by an occlusive dressing for 6 hours. This was repeated at 7 day intervals to give a total of three 6 hour exposures over 2 weeks. The animals were left untreated for 2 weeks after the final induction prior to challenge. Challenge was performed 14 days after the final induction. Test substance and vehicle were maintained under an occlusive dressing for 6 hours. Skin reactions were evaluated at 24 and 48 hours. The test concentrations were as follows: Induction - Topical application: 10 % w/v preparation of the formulation in deionised water. Challenge - Topical applications: 10 % and 3% w/v preparation of the formulation in deionised water.	
5.2	**Results and discussion**	There were no clinical signs or mortalities during the study. No cutaneous reactions were recorded after the challenge application. Reactions seen in 0/20 animals. Classified as not a sensitizer.	
5.3	**Conclusion**	Difenacoum (2.5 %) was not sensitising to guinea pig skin.	
5.3.1	Reliability	2	
5.3.2	Deficiencies	Specific OECD Guideline not specified	X

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	2 February 2007
Materials and Methods	Point 3.1: The product used in the study is a technical concentrate of the a.s. (2.5% w/v) in solvents. Point 3.1.2.2: The certificate of analysis is not included in the report. Pont 3.1.2.4: Dilution of a liquid sample of presumably low water solubility with deionised water could have been justified. Point 3.2.4: The sex of test animals is not mentioned in the report. Point 3.3.10: According to the tables in the report, the concentration of the positive control substance, formaldehyde was 30%.
Results and discussion	Table 6_1_5-2: At 48 h, 14 out of 20 positive controls showed skin reactions.
Conclusion	Under the test conditions the 2.5% w/v difenacoum formulation concentrate was not sensitising.
Reliability	2 (due to deficincies in reporting)

Section A6.1.5 (2) Skin sensitisation

Buehler test

Annex Point IIA VI.6.1.5

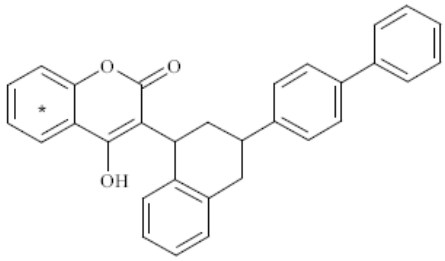
Acceptability	Acceptable
Remarks	Point 2.1 and 5.3.2: The study broadly complies with the OECD 406 test guideline. According to the test guideline, the highest concentration causing mild irritation should be used for induction. According to the test report, a 10% dilution was chosen for induction, because it would be tolerated in repeated applications (based on published information). However, this concentration did not elicit irritation. Key study
	COMMENTS FROM …
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_1_5-1. Detailed information including induction/challenge/scoring schedule for skin sensitisation test

Inductions	**Buehler test**			**Observations/Remarks**
		application	day of treatment	
Induction 1		Topical	1	No effects noted
Induction 2		Topical	7	No effects noted
Induction 3		Topical	14	No effects noted
challenge		Topical	28	No effects noted
scoring 1		Topical	29	No effects noted
scoring 2		Topical	30	No effects noted

Table A6_1_5-2. Result of skin sensitisation test

	Number of animals with signs of allergic reactions / number of animals in group		
	Negative control	**Test group**	**Positive control**
scored after 24h	0 / 10	0 / 20	18/20
scored after 48h	0 / 10	0 / 20	

Section A6.2 (1) Annex Point IIA VI.6.2		Metabolism studies in mammals	
		1 REFERENCE	**Official use only**
1.1	**Reference**	XXXXX (2006) Difenacoum - Metabolism in Rats, XXXXX, Report No. PLG 0005	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa s.r.l. PelGar International Ltd.	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD Guideline No.417	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Difenacoum	
3.1.1	Lot/Batch number	Radiolabelled: CFQ14457 Non-radiolabelled: 03655	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Solid	
3.1.2.2	Purity	Radiolabelled: >97% Non-radiolabelled: 99.3%	
3.1.2.3	Stability	Not stated	
3.1.2.4	Radiolabelling	^{14}C  * denotes position of radiolabel	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	Sprague – Dawley (Crl: CD(SD))	
3.2.3	Source	Charles River UK, Margate, Kent, UK	
3.2.4	Sex	Male and female	
3.2.5	Age/weight at study initiation	Weight: Male: 196-284 g Female: 191-251 g	

Section A6.2 (1) Metabolism studies in mammals
Annex Point IIA VI.6.2

3.2.6 Number of animals per group

Group	Experiment type	Number of animals		
		Male	Female	Total
1	Pilot excretion/balance	1	1	2
2	Main excretion/tissue distribution	4	4	8
3	Main excretion/tissue distribution	4	4	8
4	Plasma/blood cell kinetics	12	12	48
5	Plasma/blood cell kinetics	12	12	48
6	Tissue distribution	5	5	10
7	Repeat dose (pre-test observation)	1	1	2
8	Repeat dose	4	4	8
9	Biliary excretion	3	3	6

3.2.7 Control animals: No

3.3 Administration/ Exposure: Oral (gavage)

3.3.1 Concentration of test substance: X

Low dose: 0.1 mg/kg
High dose: 1 mg/kg

Group	Experiment type	Nominal Dose Level (mg/kg)
1	Pilot excretion/balance	0.1
2	Main excretion/tissue distribution	0.1
3	Main excretion/tissue distribution	1
4	Plasma/blood cell kinetics	0.1
5	Plasma/blood cell kinetics	1
6	Tissue distribution	0.1
7	Repeat dose (pre-test observation)	0.04
8	Repeat dose	0.1
9	Biliary excretion	0.1

3.3.2 Specific activity of test substance: 5.40 MBq/mg, 324000 dpm/µg

3.3.3 Volume applied: N/A

3.3.4 Exposure period: single dose — X

3.3.5 Sampling time: X

Group 1

Urine: 0 - 6 hours, 6 - 24 hours, 24-hour intervals up to 168 hours
Faeces: 24-hour intervals up to 168 hours
Cage-wash: 24-hour intervals
Liver and carcass: at 168 hours

Groups 2 and 3

Urine: 0 - 6 hours, 6 - 24 hours, 24-hour intervals up to 168 hours
Faeces: 24-hour intervals up to 168 hours
Cage-wash: 24-hour intervals
Blood: prior to sacrifice (168 hours)

Section A6.2 (1) Annex Point IIA VI.6.2		Metabolism studies in mammals	
		Organs/tissues[*]: at 168 hours [*] brain, fat (abdominal), gastrointestinal tract (including contents), heart, kidneys, liver, lungs, muscle (skeletal), ovaries (females), residual carcass, spleen, testes (males) and uterus (females)	
		<u>Group 4 and 5</u> *Blood*: Subgroup 1: pre-dose, 1, 4, 24, 96 hours Subgroup 2: 0.25, 2, 6, 48, 120 hours Subgroup 3: 0.5, 3, 12, 72 hours	
		<u>Group 6</u> *Tissues, organs, and carcases* (as in Groups 2 and 3): at 168 hour	X X
		<u>Group 8</u> *Urine*: during 24-hour period after first dose *Faeces*: during 24-hour period after first dose *Excreta, tissues, organs, carcasses, cage-washes* (as in Groups 2 and 3): following final dose *Cage-wash*: 24-hour intervals	
		<u>Group 9</u> *Bile*: 0 - 3, 3 – 6, 6 – 9, 9 – 12, 12- 24, and 24 – 48 hours after dosing *Urine and faeces*: 24-hour intervals up to 48 hours *Cage-wash:* after sacrifice	X
3.3.6	Samples	<u>Group 1</u> Urine, faeces, cage-wash, liver and carcass <u>Groups 2 and 3</u> Urine, faeces, cage-wash, blood, organs and tissues <u>Groups 4 and 5</u> Blood <u>Group 6</u> Tissues, organs, and carcasses <u>Group 8</u> Urine, faeces, excreta, tissues, organs, carcasses, cage-wash <u>Group 9</u> Bile, urine, cage-wash	X
3.3.7	Sample preparation	*Urine:* Receivers cooled in solid carbon dioxide *Faeces*: Receivers cooled in solid carbon dioxide *Blood*: Terminal blood was transferred to a heparinised tube and a subsample retained as whole-blood for measurement of radioactivity and haematocrit, while the remainder was centrifuged to separate the plasma for measurement of radioactivity concentrations. Blood samples were stored unfrozen at ca +4ºC. *Tissues/organs*: Stored at ≤ -15ºC prior to analysis	
		4 RESULTS AND DISCUSSION	
4.1	**Toxic effects, clinical signs**	No effects	

Section A6.2 (1) Annex Point IIA VI.6.2		Metabolism studies in mammals	
4.2	**Recovery of labelled compound**	Excretion/retention of radioactivity (total of urine, cagewash, faeces, G.I.T., kidneys, liver and carcass): 0.1 mg/kg: Male 97.09%, Female 96.32% 1 mg/kg: Male 98.25%, Female 98.03% Distribution of radioactivity into red blood cells was minimal. 41 - 44% (0.1 mg/kg) and 64 – 71% (1 mg/kg) was excreted (mainly via faeces) during the 168 hours after dosing. A substantial proportion of the dose was retained at 168 hours after dosing and distributed through the tissue, most significantly in the liver (28 – 35% dose, 0.1 mg/kg; 12-22% dose, 1 mg/kg) and residual carcass (14 – 24%, 0.1 mg/kg; 9-13%, 1 mg/kg).	X
4.3	**Absorption**	The extent of absorption in bile duct-cannulated rats administered with single oral doses was assessed as the sum of mean values between sexes. Bile was an important route of excretion (19 – 25% dose). A summary of the excretion and retention of radioactivity during 0-48 hours after administration of the doses to bile duct-cannulated rats can be found in table A_6-1	X
4.4	**Kinetic parameters**	At 0.1 mg/kg, no major differences in pharmacokinetic parameters were observed between the sexes apart from T_{max}, which occurred earlier in the plasma of male rats. At single oral doses of 1mg/kg, both the rate and extent of exposure to radioactivity, as reflected by the parameters C_{max} and AUC respectively, increased in comparison to the 0.1 mg/kg dose. However, these increases were higher than the proportionate dose increase and were indicative of dose-dependent i.e. non-linear kinetics. The whole blood to plasma ratios were 0.61 and 0.59 in males and females respectively at the low dose, and 0.59 and 0.57 in males and females at the high dose. These ratios indicated that distribution of radioactivity into red blood cells was minimal. See table A_6-2 for pharmacokinetic parameters derived from mean radioactivity concentrations in plasma and whole-blood of rats.	
4.5	**Distribution**	Following single oral doses at low and high doses, retention of radioactivity at 168 hours was higher at the low dose level than the high dose level. Retention of radioactivity was highest in the liver. A significant proportion was retained in the residual carcass. Of the remaining dose, at both dose levels, 2 -3% was detected in the gastrointestinal tract, and 3 – 6% in the skeletal muscle. Of the remaining tissues, accumulation was low with only a small proportion of the dose retained in tissues at 168 hours (<1.2% dose). Concentrations of radioactivity at 168 hours were highest in the liver for both sexes and dose levels. Concentrations of radioactivity in tissues after repeated dosing were generally in the range of 3-10 fold higher than after single doses and were generally highest at 12 hours (males) and 24 hours (females) after dosing, except in gastrointestinal tract (including contents) (at 6 hours). Concentrations of radioactivity could still be detected in all tissues at 28 days after dosing.	
4.6	**Metabolism**	Difenacoum was extensively metabolised by rats mainly via	X

Section A6.2 (1) Annex Point IIA VI.6.2		Metabolism studies in mammals	
		hydroxylation and conjugation. Unchanged difenacoum represented a maximum of 12.8 dose (liver) and 2.9% dose (faeces). Four major metabolites (F5, F6, F7, and F8) were observed in faecal extracts. F5 and F6 formed glucuronide conjugates, had a similar polarity to F7 and F8 and were considered probable isomers, based on the difenacoum structure. F7 and F8 were identified as isomers of hydroxylated difenacoum. Four significant metabolites (B5, B6, B7 and B8) were also observed in enzyme treated bile. These were glucuronide conjugates. The aglycones of the metabolites corresponded to faecal metabolites F5, F6, F7 and F8 respectively. Two major metabolites, L7 and L8 were observed in liver extracts. These were identified as isomers of hydroxylated difenacoum and corresponded to faecal metabolites F7 and F8	
4.7	**Excretion**	Following single oral doses of ^{14}C-difenacoum, 41 - 44% dose (0.1 mg/kg) and 64 – 71% dose (1 mg/kg) was excreted during 0 – 168 hours. Excretion was mainly via the faeces, with less than 1% of dose (0.1 mg/kg) and less than 3% dose (1 mg/kg) excreted in the urine. There were no substantial differences in patterns of excretion between sexes. The excretion and retention of radioactivity during 0 – 168 hours after the final dose following administration of seven consecutive daily oral doses of was investigated. There were no substantial differences in excretion patterns between single and repeat level oral doses.	X
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The absorption, distribution, metabolism and excretion of ^{14}C-difenacoum were studied after single oral doses at 0.1 mg/kg and 1 mg/kg and after seven consecutive daily doses at 0.1 mg/kg. Biliary excretion in bile duct-cannulated rats was also evaluated after single oral doses of ^{14}C-difenacoum at 0.1 mg/kg. The study was performed according to OECD Guideline No.417.	
5.2	**Results and discussion**	Following single oral doses of ^{14}C-difenacoum at 0.1 mg/kg and 1 mg/kg, 41 - 44% dose and 64 – 71% dose was excreted respectively during 0 – 168 hours. Excretion was mainly via the faeces, with less than 1% of dose (0.1 mg/kg) and less than 3% dose (1 mg/kg) excreted in the urine. A substantial proportion of the dose was retained at 168 hours after dosing and was distributed throughout the tissues, most significantly in the liver (28 -35% dose at 0.1 mg/kg and 12 – 22% dose at 1 mg/kg) and residual carcass (14 – 24% dose at 0.1 mg/kg and 9 - 13% dose at 1 mg/kg), but also detected in other tissues. Pharmacokinetic parameters indicated that increasing the dose level from 0.1 mg/kg to 1 mg/kg resulted in an increase in the plasma T_{max} in males from 6 to 12 hours. The whole blood T_{max} in males and the whole blood and plasma T_{max} in females remained unchanged at 12 hours. Ratios of whole to plasma ratios indicated that a distribution of radioactivity into red blood cells was minimal. Concentrations of radioactivity in tissues at 168 hours were highest in the liver for both sexes and dose levels. After repeat dosing, they were generally in the range of 3 – 10 fold higher than after single doses. Concentrations in tissues were generally highest at 12 and 24 hours after dosing, in males and females respectively, except in gthe gastrointestinal tract. Radioactivity could still be detected in all tissues 28 days after dosing.	X

Section A6.2 (1) Annex Point IIA VI.6.2		Metabolism studies in mammals	
		Difenacoum was extensively metabolised by rats mainly via hydroxylation and conjugation. Unchanged difenacoum represented a maximum of 12.8 dose (liver) and 2.9% dose (faeces). Four major metabolites (F5, F6, F7,and F8) were observed in faecal extracts. Four significant metabolites (B5, B6, B7 and B8) were also observed in enzyme treated bile. Two major metabolites, L7 and L8 were observed in liver extracts.	
5.3	**Conclusion**		
5.3.1	Reliability	1	
5.3.2	Deficiencies	No	

Section A6.2 (1) Annex Point IIA VI.6.2	Metabolism studies in mammals	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	13 March 2007	
Materials and Methods	Point 3.3.4: In addition to single dose groups, the animals in groups 7 (pre-test group) and 8 (main study) received difenacoum for 7 days (144 h). Point 3.3.5: For Group 1 animals, also expired air was monitored for radioactivity. In Group 6 (tissue distribution), one male and one female was sacrificed at 6, 12, 24 h, and on Day 14 and Day 28 Group 7 was a pre-test group for the repeated dose test, dosed with non-radiolabelled test substance. The GIT (including contents), liver and the remaining carcass were analysed for radioactivity in the bile cannulated animals (Group 9). Point 3.3.6: GIT, liver and carcass shall be added as analysed samples.	
Results and discussion	Point 4.2: Recovery of labelled compound: recovery for high dose males should be 98.27% and for females 97.9%. RMS added the right figures (in parenthesis) to Table 6_2-1. Point 4.3: The text should be as follows: Following the single oral doses of 0.1 or 1.0 mg/kg bw, the absorption appeared to be slow, with maximum concentrations in blood reached until at least 6 and usually 12 h post-dose. Pharmacokinetic profile was relatively similar in both sexes. Oral absorption in bile duct-cannulated rats was extensive since 68.39% (males) and 66.77% (females) of the administered dose was absorbed after single low dose (0.1 mg/kg bw). The extent of absorption was estimated by summing the values for bile, urine, liver and carcass. Bile was an important route of excretion, accounting 19 – 25% of the dose. A summary of the excretion and retention of radioactivity during 0-48 hours after administration of the doses to bile duct-cannulated rats can be found in table A_6-3 added by RMS. 4.6: The following amendment is suggested: Difenacoum was extensively metabolised by rats mainly via hydroxylation and conjugation. The metabolite profiles (faeces and liver) were similar in both sexes and with both dose levels. Unchanged difenacoum represented a maximum of 12.8% dose in liver (low dose females) and 2.9% dose in faeces (high dose males). Point 4.7: In the pilot study no radioactivity was detected in the expired air during 24 h post-dose. Thus, this route of excretion was not monitored in the main study. Point 5.2: The following sentence should be added: Absorption of difenacoum after oral intake is extensive since 68.39 % and 66.77 % of the administered dose was absorbed after single low dose.	
Conclusion	Agree with applicant’s version (added by oral absorption percentages).	

Section A6.2 (1) **Annex Point IIA VI.6.2**	**Metabolism studies in mammals**	
Reliability	1	
Acceptability	Acceptable	
Remarks	Key study Point 3.3.1: The doses were quite high. Even higher oral absorption percentages might have been possible, if the doses would have been, e.g., an order of magnitude lower. RMS added tables A6_2-3, A6_2-4 and A6_2-5 to clarify data on oral absorption and metabolite profile of difenacoum.	
	COMMENTS FROM ...	
Date	*Give date of comments submitted*	
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*	
Results and discussion	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Reliability	*Discuss if deviating from view of rapporteur member state*	
Acceptability	**Discuss if deviating from view of rapporteur member state**	
Remarks		

Table A6_2-1. Excretion and retention of radioactivity during 0-48 (0-168) hours after administration

Sample	0.1 mg/kg		1 mg/kg	
	Male	Female	Male	Female
Urine	0.92	0.88	2.37	1.00
Cagewash	nd	nd	0.10	0.05
Faeces	39.98	34.26 (43.26)	68.59	63.16
G.I.T.	3.09	2.39	1.96	2.58
Kidneys	0.69 (0.67)	0.66	0.29	0.27
Liver	28.44	35.01	13.03 (12.03)	21.75
Carcass	23.99	14.12	12.93	9.09
Total	**97.09**	**96.32**	**98.25 (98.27)**	**98.03 (97.9)**

Table A6_2-2. Pharmacokinetic parameters from mean radioactivity concentrations

Sample	0.1 mg/kg		1 mg/kg	
	Male	Female	Male	Female
Plasma				
C_{max} (µg equiv/g)	0.020	0.019	0.578	0.662
T_{max} (hours)	6	12	12	12
AUC (µg equiv/g)	1.18	1.55[a]	31.1	44.4
AUC_t (µg equiv/g)	0.982	1.20	28.9	37.9
$T_{1/2}$ (hours)	45.1	54.7[a]	31.0	42.5
Whole-blood				
C_{max} (µg equiv/g)	0.014	0.013	0.344	0.365
T_{max} (hours)	12	12	12	12
AUC (µg equiv/g)	0.660[a]	0.884[a]	18.2	25.1
AUC_t (µg equiv/g)	0.599	0.711	17.1	21.5
$T_{1/2}$ (hours)	35.2[a]	50.7[a]	29.0	41.8

[a] data did not meet the acceptance criteria and therefore this value should be treated with caution

Tables added by the CA

Table 6_2-3. Excretion and retention of radioactivity (% of dose) during 0-48 hours after single low dose (0.1 mg/kg bw) of C^{14}-difenacoum to bile duct-cannulated rats.

	Bile	Urine	Faeces	Liver	Carcass	Absorption
♂	24.78	1.14	23.64	25.10	17.37	68.39
♀	18.93	0.67	26.67	35.83	11.34	66.77

Table 6_2-4. Mean recoveries of difenacoum in liver and carcass at 168 hours after repeated (0.1 mg/kg), and single oral doses of C^{14}-difenacoum at low (0.1 mg/kg bw) and high (1.0 mg/kg bw) doses.

Dose level	Concentration in liver (% of dose)		Concentration in carcass (% of dose)	
	♂	♀	♂	♀
low	28	35	24	14
high	12	22	13	9
repeated	79	127	88	69

Table 6_2-4. Proportions of radioactive components in liver, bile and faecal extracts.

Dose level	Liver: 2 major metabolites (sum of metabolites, % of dose)			Faeces: 4 major metabolites (sum of metabolites,% of dose)			Bile: 4 major metabolites (sum of metabolites,% of dose)		
	♂	♀	u.d.	♂	♀	u.d.	♂	♀	u.d.
low	10	16	12	21	26	1.2	19	13	0.35
high	5	14	3.5	39	38	2.6	-	-	-
repeated	42[a]	36[a]	26[a]	70[b]	78[b]	3.3[b]	-	-	-

u.d. = unchanged difenacoum
[a] % of liver, [b] % of chromatogram values
Liver and faecal samples were collected 7 days after dosing, and bile sample after 48 hours.

Section A6.3.1 **Annex Point IIA VI.6.3**	**Short-term repeated dose toxicity, oral (28 days)**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	Not required as Subchronic oral toxicity test (90 day repeated dose) available in Section 6.4.1	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	5 February 2007	
Evaluation of applicant's justification	The applicant's justification is acceptable. A short-term repeated dose toxicity study is not required when an adequate subchronic toxicity study is available in a rodent. Although there are some limitations in the subchronic oral study in rats, there is enough data for risk assessment.	
Conclusion	An oral short-term toxicity study is not needed.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.3.2 **Annex Point IIA VI.6.3**	**Short-term repeated dose toxicity (dermal) 28 days**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	The toxicological properties of anticoagulants are already well researched and fully elucidated, for this reason it is deemed to be scientifically unjustified to conduct a study for which the end points have been reasonable determined by previous studies conducted on analogous substances (bromadiolone and brodifacoum). These analogous substances have similar physico-chemical and toxicological properties to Difenacoum, hence subchronic data can be read across to Difenacoum from the analogous substances. Hence, all endpoints have been reasonably assessed by the analogous substances. Difenacoum is a well-known compound which has been used extensively for many years. The properties of Difenacoum are understood as is its mode of action. Anticoagulant rodenticides such as Difenacoum are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is thought to be the inhibition of K_1 epoxide reductase. The anticoagulants accumulate and are stored in the liver until broken down. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. The technical active ingredient has a high level of purity and there are no other substances that are of concern included as impurities or additives. There are also no other known significant toxic effects. In addition, based on animal welfare grounds a dermal 28-day study is considered to be of no value as this additional animal testing would not provide any additional relevant data than is not already available from the analogous substances.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	5 February 2007	

Section A6.3.2 **Annex Point IIA VI.6.3**	**Short-term repeated dose toxicity (dermal) 28 days**
Evaluation of applicant's justification	Although not agreeing on the applicant's justification as a whole, some relevant aspects have been brought up and the overall conclusion is acceptable. Generally, short term repeated dose toxicity studies are not required when an adequate subchronic toxicity study is available in a rodent.. Because an acceptable (with limitations) subchronic toxicity study in a rodent by oral route (A6.4.1) is available, a short term repeated dose toxicity study by dermal route is not needed. Route-to-route extrapolation is considered possible and the mode of action of difenacoum is such that no specific effects are likely to be discovered after administration by dermal route. Due to the nature and use pattern of the product, human exposure through skin is the main route of exposure, but it is not quantitatively so significant that it would necessitate toxicity studies by dermal route. Furthermore, due to the mode of action and the toxicity test animals being the target organisms, it is technically difficult to perform a good repeated dose toxicity test.
Conclusion	The repeated dose toxicity (28 days) test by dermal route with difenacoum is not needed.
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.3.3 **Annex Point IIA VI.6.3**	**Short-term repeated dose toxicity (inhalation) 28 days**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	The toxicological properties of anticoagulants are already well researched and fully elucidated, for this reason it is deemed to be scientifically unjustified to conduct a study for which the end points have been reasonable determined by previous studies conducted on analogous substances (bromadiolone and brodifacoum). These analogous substances have similar physico-chemical and toxicological properties to Difenacoum, hence subchronic data can be read across to Difenacoum from the analogous substances. Hence, all endpoints have been reasonably assessed by the analogous substances. Difenacoum is a well-known compound which has been used extensively for many years. The properties of Difenacoum are understood as is its mode of action. Anticoagulant rodenticides such as Difenacoum are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is thought to be the inhibition of K_1 epoxide reductase. The anticoagulants accumulate and are stored in the liver until broken down. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. The technical active ingredient has a high level of purity and there are no other substances that are of concern included as impurities or additives. There are also no other known significant toxic effects. Inhalation is not considered to be a significant potential route of entry due to low v.p and solid bait nature of product. In addition, based on animal welfare grounds an inhalation 28-day study is considered to be of no value as this additional animal testing would not provide any additional relevant data than is not already available from the analogous substances.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	5 February 2007	

Section A6.3.3 **Annex Point IIA VI.6.3**	**Short-term repeated dose toxicity (inhalation) 28 days**
Evaluation of applicant's justification	Although not agreeing on the applicant's justification as a whole, some relevant aspects have been brought up and the overall conclusion is acceptable. Generally, short term repeated dose toxicity studies are not required when an adequate subchronic toxicity study is available in a rodent. Because an acceptable (with limitations) subchronic toxicity study in a rodent by oral route (A6.4.1) is available, and route-to-route extrapolation is considered possible, a short term repeated dose toxicity study by inhalation is not needed. The mode of action of difenacoum is such that no specific effects are likely to be discovered after administration by inhalation. Due to the low vapour pressure of the active substance and the physical nature of the representative product (wax block), exposure from the gas phase is unlikely. Furthermore, due to the mode of action and the toxicity test animals being the target organisms, it is technically difficult to perform a good repeated dose toxicity test.
Conclusion	The repeated dose toxicity (28 days) test by inhalation with difenacoum is not needed.
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.4.1 **Annex Point IIA VI.6.4**	**Subchronic oral test second species**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	The toxicological properties of anticoagulants are already well researched and fully elucidated, for this reason it is deemed to be scientifically unjustified to conduct a study for which the end points have been reasonably determined by previous studies conducted on analogous substances (bromadiolone and brodifacoum). These analogous substances have similar physico-chemical and toxicological properties to Difenacoum, hence subchronic data can be read across to Difenacoum from the analogous substances. Hence, all endpoints have been reasonably assessed by the analogous substances. Difenacoum is a well-known compound which has been used extensively for many years. The properties of Difenacoum are understood as is its mode of action. Anticoagulant rodenticides such as Difenacoum are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is thought to be the inhibition of K_1 epoxide reductase. The anticoagulants accumulate and are stored in the liver until broken down. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. The technical active ingredient has a high level of purity and there are no other substances that are of concern included as impurities or additives. There are also no other known significant toxic effects. In addition, based on animal welfare grounds a second species 90-day study is considered to be of no value as this additional animal testing would not provide any additional relevant data than is not already available from the analogous substances. Under these circumstances, a second species 90-day study is considered to be of no value, and an unnecessary waste of experimental animals.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	5 February 2007	

Section A6.4.1 **Annex Point IIA VI.6.4**	**Subchronic oral test second species**
Evaluation of applicant's justification	Waiving of a subchronic study with a second (non-rodent) species is justified. The mode of action of difenacoum is well characterised and no other effects are expected to be seen in studies with other species compared to effects in rodents, which are also the most sensitive species for rodenticides. A subchronic study in dogs is not scientifically or ethically justified.
Conclusion	Waiving of a subchronic study in a non-rodent species is acceptable.
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.4.1 Subchronic toxicity

Annex Point IIA VI.6.4

90-day feeding study in rats

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (1995) DIFENACOUM: 90-day Feeding Study in the Rat, XXXXX, Report MLS/10016	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with Access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 408	
2.2	**GLP**	Yes	X
2.3	**Deviations**	No	X
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	TCP 0012	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Off-white powder	
3.1.2.2	Purity	Difenacoum 98.3%	X
3.1.2.3	Stability	Stable	
3.2	**Test Animals**		
3.2.1	Species	Rats	
3.2.2	Strain	Wistar	
3.2.3	Source	Charles River Laboratories, Wilmington, Mass., U.S.A.	
3.2.4	Sex	Male and female	
3.2.5	Age/weight at study initiation	Age not stated Male 173-225 g Female 152-192 g	X
3.2.6	Number of animals per group	8 animals/sex/group	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of treatment	90 days	

Section A6.4.1 Subchronic toxicity

Annex Point IIA VI.6.4 90-day feeding study in rats

3.3.2	Frequency of exposure	Daily	
3.3.3	Postexposure period	None	
3.3.4	**Oral**		
3.3.4.1	Type	Gavage	
3.3.4.2	Concentration	1, 2, 3, and 6 µg/ml	
3.3.4.3	Vehicle	Polyethylene glycol 300	
3.3.4.4	Concentration in vehicle	0, 0.01, 0.02 0.03 and 0.06 mg/kg	
3.3.4.5	Total volume applied	1ml/100gm	
3.3.4.6	Controls	Vehicle	
3.4	**Examinations**		
3.4.1	Observations		
3.4.1.1	Clinical signs	Yes - twice daily	X
3.4.1.2	Mortality	Yes - Daily	
3.4.2	Body weight	Yes – Prior to dosing and then daily	X
3.4.3	Food consumption	Yes	
3.4.4	Water consumption	No	
3.4.5	Ophthalmoscopic examination	No	
3.4.6	Haematology	Yes 1 ml of blood from tail vein from all animals on days 1, 15, 30, 50, 80 and 90 prior to termination. Parameters: Haematocrit, haemoglobin concentration, total white cell count, red cell count, mean cell volume, mean cell haemoglobin and concentration. A differential white cell count on a Romanowsky-stained blood film and the appearance of red cells examined in all groups.	X
3.4.7	Clinical Chemistry	Yes All animals at end of study after termination. By cardiac puncture and collected in tubes containing lithium heparin. Parameters: sodium, potassium, glucose, cholesterol, plasma urea, bilirubin, creatinine, creatinine kinase, total protein, albumin, alanine transaminase, aspartate transaminase, plasma alkaline phosphatase, gamma glutamyl transferase, gamma glutamyl transpeptidase, triglycerides, calcium, chloride, phosphorus (as phosphate).	X
3.4.8	Urinalysis	No	
3.5	**Sacrifice and**		

Section A6.4.1 Subchronic toxicity

Annex Point IIA VI.6.4 90-day feeding study in rats

	pathology		
3.5.1	Organ Weights	Yes organs: liver, kidneys, adrenal glands, testes, brain	
3.5.2	Gross and histopathology	Yes; all dose groups Organs removed and not processed: Aorta, bladder, bone (femur), cervix, eye, harderian gland, mammary gland, mesenteric lymph node, nasal passages, oesophagus, oral cavity, ovary, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skin, spinal cord, sternum, thymus, thyroid/parathyroid gland, trachea, uterus, voluntary muscle, macroscopically abnormal tissue Organs for histology: Adrenal gland, brain, caecum, cervical lymph node, colon, duodenum, epididymis, heart, ileum, jejunum, kidney, liver, lung, pancreas, rectum, salivary gland, spleen, stomach, testis All tissues were fixed in a 10% neutral buffered formol saline except for epididymis and testis which were fixed in bouin's solution and embedded in paraffin wax. 5µm sections were cut and stained with haematoxylin and eosin for examination.	X
3.5.3	Other examinations	None	
3.5.4	Statistics	Initial bodyweights were considered by analysis of variance and subsequent bodyweights were considered by analysis of covariance on the initial bodyweight. Food consumption, haematology, blood chemistry and organ weights were considered by analysis of variance or covariance. Least-square means for each group using LSMEAN option in SAS PROC GLM. All statistical tests were two-sided.	
3.6	**Further remarks**		
		4 RESULTS AND DISCUSSION	
4.1	**Observations**		
4.1.1	Clinical signs	There were no clinical symptoms that were considered to be of toxicological significance. Piloerection, salivation and signs of diarrhoea were seen in all groups and the control and was therefore considered unrelated to the treatment with difenacoum.	X
4.1.2	Mortality	No mortalities at any dose	
4.2	**Body weight gain**	No significant differences in weight gain across the treatment groups and control.	
4.3	**Food consumption and compound intake**	No significant effects were seen	
4.4	**Ophtalmoscopic examination**	Not investigated	
4.5	**Blood analysis**		
4.5.1	Haematology	All animals other than the highest dose group of 0.06mg/kg/day revealed normal coagulation and haematology compared to the control group. At this dose, there was a slight elevation of the KCT time.	X
4.5.2	Clinical chemistry	No effects were seen in any of the treatment groups compared to the control.	X
4.5.3	Urinalysis	Not investigated	

Section A6.4.1 Subchronic toxicity

Annex Point IIA VI.6.4

90-day feeding study in rats

4.6	**Sacrifice and pathology**		
4.6.1	Organ weights	No effects were seen in any of the treatment groups compared to the control.	
4.6.2	Gross and histopathology	Macroscopic post mortem showed male rats that received 0.06 mg/kg/day had pale areas in the lung, testis and liver in a single individual. There were no other treatment related findings in animals at other dose groups.	X
		Increased incidence of haemorrhaging in males that received the 0.06 mg/kg/day dose rate.	
4.7	**Other**		
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The study was conducted according to OECD 408. The study consisted of one control group and three treatment groups with 16 rats in each group. Each rat was dosed daily with the dose levels: 0, 0.01, 0.02, 0.03, 0.06 mg/kg/day of active substance in the vehicle polyethylene glycol 300. Difenacoum was administered in PEG 300 and the concentration was adjusted to give a constant volume of 1ml/100gm of bw for each dose level. One preparation was made for each concentration (1, 2, 3 and 6µg/ml). The control received PEG 300.	
		Animals were dosed once daily from day 1 until the experiment was terminated on day 90 with 1 ml of dosng formulation per 100 gm body weight by oral gavage. The actual volume given to each animal was adjusted daily according to its bodyweight.	
		Animals were observed twice daily. Any changes in behaviour or clinical condition were recorded daily during the study. The bodyweight of each animal was recorded daily, immediately before dosing. Haematology, blood chemistry, necropsy with histopathology was performed.	
5.2	**Results and discussion**	The concentration of difenacoum in Peg 300 was determined to be within 5% of the target concentration at each dose level.	
		There were no deaths during the study. Treatment with difenacoum did not cause any clinical symptoms to be of toxicological significance. Piloerection, salivation and signs of diarrhoea were seen in all groups and the control and therefore considered unrelated to the treatment with difenacoum.	X
		No toxicologically important effects were noted on clinical condition, bodyweight or organ weights in any of the treatment groups.	
		At the highest dose rate of 0.06 mg/kg/day two out of the eight male animals exhibited an increased tendency of haemorrhaging in various tissues. This group did exhibit a slight increase in clotting time indices.	X
5.3	**Conclusion**		
5.3.1	LO(A)EL	Not stated	
5.3.2	NO(A)EL	Male 0.03 mg/kg/day (cumulative dose over 90 days – 2.7 mg/kg)	X
		Female 0.06 mg/kg/day (cumulative dose over 90 days – 5.4 mg/kg)	
5.3.3	Other	None	

Section A6.4.1 Subchronic toxicity

Annex Point IIA VI.6.4

90-day feeding study in rats

5.3.4	Reliability	1
5.3.5	Deficiencies	No

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date 8 February 2007

Materials and Methods

Point 3.1.2.2: Reference to Section 2 of the dossier is made for specification, but the test is performed with a substance of little lower purity.

Point 3.2.5: Male weight was 173-226 g at study initiation.

Points 3.3.4.2 and 3.3.4.4: The concentrations presented under these points shall be exchanged. 0, 0.01, 0.02, 0.03 or 0.06 mg/kg bw/day was administered to the animals.

Point 3.3.4.5: The volume administered was not constant but adjusted to daily body weights.

Point 3.4.1.1: The number of daily observations is not specified in the report.

Point: 3.4.2: Records for body weights (individual) are presented in ten days intervals.

Point 3.4.6: In addition, platelet count was analysed. Haematology parameters were measured at the end of the study, except for prothrombin (PT) and kaolin-cephalin (KCT) times, which were measured on days 1, 15, 30, 50, 80 and 90.

Point 3.4.7: Gamma glutamyl transpeptidase was not measured.

Point 3.5.2: According to the table no. 11, histopathological analysis was not performed on testis, stomach, salivary glands and intestine. Instead, mesentric lymph node, nasal passage, larynx, thymus, bladder and prostate gland were analysed.

Section A6.4.1 Subchronic toxicity

Annex Point IIA VI.6.4

90-day feeding study in rats

Results and discussion	Point 4.1.1 and 5.2: According to table 4 of the report, piloerection was not observed in controls. Pont 4.3: There are no records on food consumption. Point 4.5.1 and 5.2: It is not absolutely clear from table 6 of the study report whether the PT and KCT measurements imply to both sexes because the number of measured individuals is not stated. However, the text says "All animals other than the highest dose group revealed normal coagulation". For clarity it should be added that the slightly elevated PTs in the high dose group returned within the normal range of the laboratory when measured on day 90 of the study. Point 4.5.2: There were no treatment-related effects in clinical chemistry parameters. Point 4.6.2: The following text shall be added to the applicant's version: There were also single observations of red area(s) in the cervical lymphnode, pancreas and thymus (individual(s) not known) receiving 0.03 mg/kg bw/day, but these were not considered treatment-related. In histopathology, one or two observations of slight haemorrhage in epididymis, heart, mesenteric lymph node, spleen , kidney, lung and bladder (individual(s) not known) at 0.06 mg/kg bw/day, and in some other organs (including those showing red areas at 0.03 mg/kg bw/day) as well, scattered in all lower dose groups with no dose-response. In females, macroscopically there was only one observation of red area(s) in cervical lymph node at 0.06 mg/kg bw/day and in thymus at 0.02 mg/kg bw/day and at 0.06 mg/kg bw/day, respectively, but they were not considered treatment-related. In histopathology, there were single observations of slight haemorrhage in a variety of organs, scattered in all exposed groups, but this was considered normal pathology. Clear dose response was not evident. A separate statement of a pathologist is not available. The possible coincidence of macroscopic and microscopic findings can not be verified due to lack of individual data.
Conclusion	A suggestive NOAEL is 0.03 mg/kg bw/day for both sexes, on the basis of change in blood coagulation parameter KCT and supported by histological findings.
Reliability	3 (due to deficiencies in performance and reporting of the study)
Acceptability	Acceptable as a supportive study to reveal the effect of difenacoum on blood coagulation. The overall data in the dossier is adequate for risk assessment, and for animal welfare reasons no further repeated dose toxicity studies are necessary.
Remarks	Point 2.2: Although originally performed in compliance with GLP requirements, the GLP status of the study is no longer complete because the missing individual data can not be presented because the testing facility is no longer in operation. Point 2.3: 10 animals/sex should have been at each dose level, and somewhat higher doses should have been used. Ophthalmological examination and recording of food consumption should have been performed. No historical control data is presented. In the result section of the study report, the dose is incorrect (0-6µg/kg/day) in the text for haematology (subheading 4.5) and in some of the tables. RMS has added information to Table 6_3-1.

Section A6.4.1 Subchronic toxicity

Annex Point IIA VI.6.4

90-day feeding study in rats

	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_4_1-1. Results of haematology and clinical chemistry

Parameter changed	**Unit**	**0mg/kg**	**0.01mg/kg**	**0.02mg/kg**	**0.03mg/kg**	**0.06mg/kg**
	Days					
PT (secs) mean (m+f)	50	16.34	16.98	15.34	16.87	18.74 (↑)
PT (secs) mean (m+f)	80	16.87	16.74	15.98	16.12	18.69 (↑)
KCT (secs) mean (m+f)	80	25.24	24.27	23.14	25.21	30.47 (↑)
KCT (secs) mean (m+f)	90	24.98	23.18	24.13	25.12	34.26 (↑)
Haematology (all figures are mean)						
Haemoglobin g/dl	90	15.4 (m), 15.8(f)	15.3 (m), 14.9(f)	15.1 (m), 14.5(f)	15.3 (m), 15.1(f)	15.4 (m), 14.7(f)
Haemocrit	90	0.531(m), 0.528(f)	0.480(m), 0.494(f)	0.482(m), 0.509(f)	0.494(m), 0.508(f)	0.482(m), 0.499(f)
Red blood cell count (x10**12/1)	90	7.87(m), 7.88(f)	7.88(m), 7.27(f)	7.51(m), 6.84(f)	7.71(m), 7.58(f)	6.23(m), 6.95(f)
Platelet count (x10**9/1)	90	935(m), 714(f)	820(m), 878(f)*	784(m), 845(f)	784(m), 864(f)	790(m), 968(f)**
White blood cell count (x10**9/1)	90	10.44(m), 5.36(f)	9.72(m), 4.44(f)	11.00(m), 7.22(f)	7.05(m), 6.85(f)	10.68(m), 6.78(f)
Clinical chemistry (all figures are mean)						

Plasma albumin (g/100 ml)	90	3.58(m), 4.34(f)	3.28, 3.94(f)	3.30, 3.74(f)	3.28, 3.68(f)	3.09(m)*, 4.12(f)
Plasma alanine transaminase (IU/l)	90	39.4(m), 40.2(f)	35.6(m), 38.4(f)	43.0(m), 55.8(f)	44.6(m), 43.8(f)	48.0(m)*, 48.7(f)
Plasma aspartate transaminase (IU/l)	90	43.8(m), 40.6(f)	39.4(m), 40.8(f)	50.4(m), 41.2(f)	45.8(m), 44.8(f)	47.3(m), 51.0(f)*
Plasma cholesterol (mg/100 ml)	90	78.4(m), 85,4(f)	72.5(m), 78.8(f)	78.3(m), 85.6(f)	60.5(m), 91.8(f)	65.3(m), 98.7(f)*

M=male, f=female
Number of animals = 80 in total , 8 males and 8 females in each dose group.
PT = Prothrombin time (normal range at the laboratory 14.0-18.0)
KCT=Kaolin-Cephalin times (normal range at the laboratory 20.0-30.0)
Only effects that show a slight increase or decrease have been presented in the tables.
* Significantly different from the control at the 5% level (Students t-test, two-sided)
** Significantly different from the control at the 1% level (Students t-test, two-sided)

Table A6_4-1-2. Results of repeated dose of difenacoum in relation to clinical signs and mortality(in mg/kg/day) toxicity study for male and female rats.

Parameter	**Control**		**0.01mg/kg/day**		**0.02mg/kg**		**0.03mg/kg**		**0.06mg/kg**		**dose-response +/-**	
	m[a]	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**
number of animals examined	8	8	8	8	8	8	8	8	8	8		
Mortality	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8		
Clinical signs												
Activity decreased	-	-	-	1/8	-	-	2/8	3/8	5/8	2/8		
Salivation	6/8	4/8	5/8	3/8	7/8	6/8	2/8	7/8	7/8	8/8		
Diarrhoea	7/8	2/8	6/8	-	7/8	-	5/8	2/8	6/8	3/8		
Stains around nose	2/8	3/8	3/8	4/8	3/8	-	4/8	3/8	7/8	5/8		
Piloerection	-	-	-	1/8	-	-	2/8	-	5/8	3/8		

Section A6.4.2 Annex Point IIA VI.6.4	Subchronic dermal toxicity test	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	The toxicological properties of anticoagulants are already well researched and fully elucidated, for this reason it is deemed to be scientifically unjustified to conduct a study for which the end points have been reasonable determined by previous studies conducted on analogous substances (bromadiolone and brodifacoum). These analogous substances have similar physico-chemical and toxicological properties to Difenacoum, hence subchronic data can be read across to Difenacoum from the analogous substances. Hence, all endpoints have been reasonably assessed by the analogous substances. Difenacoum is a well-known compound which has been used extensively for many years. The properties of Difenacoum are understood as is its mode of action. Anticoagulant rodenticides such as Difenacoum are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is thought to be the inhibition of K_1 epoxide reductase. The anticoagulants accumulate and are stored in the liver until broken down. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. The technical active ingredient has a high level of purity and there are no other substances that are of concern included as impurities or additives. There are also no other known significant toxic effects. In addition, based on animal welfare grounds a dermal 90-day study is considered to be of no value as this additional animal testing would not provide any additional relevant data than is not already available from the analogous substances.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	5 February 2007	

Section A6.4.2 **Annex Point IIA VI.6.4**	**Subchronic dermal toxicity test**
Evaluation of applicant's justification	Although not agreeing on the applicant's justification as a whole, some relevant aspects have been brought up and the overall conclusion is acceptable. Generally, an acceptable subchronic toxicity study in one species by oral route is considered necessary (A6.4.1 available) for risk assessment, and because route-route extrapolation is considered possible, waiving of a subchronic study by dermal route is justified. The mode of action of difenacoum is such that no specific effects are likely to be discovered after administration through dermal route. Due to the nature and use pattern of the product, human exposure through skin is the main route of exposure, but it is not quantitatively so significant that it would necessitate toxicity studies by dermal route. Furthermore, due to the mode of action and the toxicity test animals being the target organisms, it is technically difficult to perform a good subchronic toxicity test.
Conclusion	Subchronic toxicity tests by dermal route with difenacoum are not needed in either rodents or non-rodents.
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.4.3 Annex Point IIA VI.6.4	Subchronic inhalation toxicity test	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	The toxicological properties of anticoagulants are already well researched and fully elucidated, for this reason it is deemed to be scientifically unjustified to conduct a study for which the end points have been reasonable determined by previous studies conducted on analogous substances (bromadiolone and brodifacoum). These analogous substances have similar physico-chemical and toxicological properties to Difenacoum, hence subchronic data can be read across to Difenacoum from the analogous substances. Hence, all endpoints have been reasonably assessed by the analogous substances. Difenacoum is a well-known compound which has been used extensively for many years. The properties of Difenacoum are understood as is its mode of action. Anticoagulant rodenticides such as Difenacoum are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is thought to be the inhibition of K_1 epoxide reductase. The anticoagulants accumulate and are stored in the liver until broken down. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. The technical active ingredient has a high level of purity and there are no other substances that are of concern included as impurities or additives. There are also no other known significant toxic effects. Under these circumstances, a inhalation 90-day study is considered to be of no value, and an unnecessary waste of experimental animals.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	5 February 2007	
Evaluation of applicant's justification	Although not agreeing on the applicant's justification as a whole, some relevant aspects have been brought up and the overall conclusion is acceptable. An acceptable subchronic toxicity study in one species by oral route is considered necessary (A6.4.1 available) for risk assessment and route-to-route extrapolation is considered possible. The mode of action of difenacoum is such that no specific effects are likely to be discovered after repeated administration by inhalation. Due to the low vapour pressure of the active substance and the physical nature of the representative product (wax block), exposure from the gas phase is unlikely Furthermore, due to the mode of action and the toxicity test animals being the target organisms, it is technically difficult to perform a good subchronic toxicity test.	
Conclusion	The subchronic inhalation toxicity test with difenacoum is not needed.	

Section A6.4.3 **Annex Point IIA VI.6.4**	**Subchronic inhalation toxicity test**
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.5 **Annex Point IIA I.E.**	**Chronic toxicity**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	The toxicological properties of anticoagulants are already well researched and fully elucidated, for this reason it is deemed to be scientifically unjustified to conduct a study for which the end points have been reasonable determined by previous studies conducted on analogous substances (bromadiolone and brodifacoum). These analogous substances have similar physico-chemical and toxicological properties to Difenacoum, hence chronic data can be read across to Difenacoum from the analogous substances. Hence, all endpoints have been reasonably assessed by the analogous substances. Difenacoum is a well-known compound which has been used extensively for many years. The properties of Difenacoum are understood as is its mode of action. Anticoagulant rodenticides such as Difenacoum are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is the inhibition of K_1 epoxide reductase. The anticoagulants accumulate in the liver. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. All studies on vertebrates show the same effects, primarily loss of blood coagulation, and these are shown clearly in acute studies. To avoid acute effects, doses in repeat dose studies must be kept very low, and the potential for exposure to rodenticides is limited by the nature of their use. A second species repeated dose study is therefore also considered unjustified. In addition, based on animal welfare grounds a chronic toxicity study is considered to be of no value as this additional animal testing would not provide any additional relevant data than is not already available from the analogous substances.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	7 February 2007	

Section A6.5 **Annex Point IIA I.E.**	**Chronic toxicity**
Evaluation of applicant's justification	Although not agreeing in every detail of the applicant's justification RMS can agree on the conclusion that waiving of chronic toxicity studies is scientifically justified. MSs have accepted a refined waiving concept for rodenticides due to the unique nature of these substances as being used to kill the animals in nature, which should also be used to test the human toxicity of the compound. This fact together with the known mode of action (anticoagulation due to vitamin K_1 deficiency) of difenacoum, leads inevitably to major technical difficulties in performing valid long-term (or even studies with much shorter duration) toxicity tests in mammals. Based on available toxicity data there is no indication of other toxic effects than anticoagulation (teratogenicity is not considered here). Although the frequency and duration of exposure of professional users is above the level of lower concern and secondary exposure of small children does not meet the criteria of "negligible", RMS considers that scientific and ethical reasons dominate to support waiving of chronic toxicity studies with difenacoum.
Conclusion	Waiving of chronic toxicity studies in rodents is accepted due to ethical reasons and known mode of action.
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.6.1 Genotoxicity in vitro

Annex Point IIA VI.6.1 *In vitro* gene mutation study in *Salmonella typhimurium*

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2002) Difenacoum: Reverse mutation assay, Ames Test, using *Salmonella typhimurium*, XXXXX report 1558/005	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with Access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 471	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Off white powder	
3.1.2.2	Purity	99.7% (difenacoum)	
3.1.2.3	Stability	Stable under test conditions	
3.2	**Study Type**	Bacterial reverse mutation test	
3.2.1	Organism/cell type	S. typhimurium: TA 1535, TA 1537, TA 98, TA 100, TA 102	
3.2.2	Deficiencies / Proficiencies	Not applicable	
3.2.3	Metabolic activation system	S9 mix S9 was prepared in-house from the livers of male Sprague-Dawley rats weighing ~250g. The S9 was stored at at -196°C.	

Section A6.6.1 Genotoxicity in vitro

Annex Point IIA VI.6.1 *In vitro* gene mutation study in *Salmonella typhimurium*

3.2.4	Positive control	+S9: 2-aminoanthracene (TA100, TA1535, TA1537) benzo-(a)-pyrene (TA 98) Danthron (TA 102) -S9: N-ethyl-N'-nitro-N-nitrosoguanidine (EENG) – 3 µg/plate for T100 and 5 µg/plate for TA1535 9-Aminoacridine (9AA) – 80 µg/plate for TA1537 Mitomycin C (MMC) – 0.5 µg/plate for TA102 4-Nitroquinoline-1-oxide (4NQO) – 0.2 µg/plate for TA98
3.3	**Administration / Exposure; Application of test substance**	
3.3.1	Concentrations	Preliminary experiment: 0, 0.15, 0.5, 1.5, 5, 15, 50, 150, 500, 1500 and 5000µg/plate Expt. 1: - S9: 50, 150, 500, 1500 and 5000 µg/plate +S9: 50, 150, 500, 1500 and 5000 µg/plate Expt. 2: - S9: 50, 150, 500, 1500 and 5000 µg/plate +S9: 50, 150, 500, 1500 and 5000 µg/plate
3.3.2	Way of application	Dissolved in dimethyl sulphoxide
3.3.3	Pre-incubation time	0 hours
3.3.4	Other modifications	None
3.4	**Examinations**	*See tables in appendix for examinations and results*
3.4.1	Number of cells evaluated	Not applicable
		4 RESULTS AND DISCUSSION
4.1	**Genotoxicity**	
4.1.1	without metabolic activation	No
4.1.2	with metabolic activation	No
4.2	**Cytotoxicity**	In the preliminary study, although not affecting the background lawn, there was a reduction in the frequency of revertant colonies (cytotoxicity) at several upper dose levels tested with the strain TA100. Also in the main study, the number of revertant colonies was reduced in many cases before precipitation occurred.

Section A6.6.1 Genotoxicity in vitro

Annex Point IIA VI.6.1 *In vitro* gene mutation study in *Salmonella typhimurium*

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The study was conducted according to OECD 471.

The test material was assayed for mutation on five histidine requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537, TA102) both in the absence and presence of metabolic activation by an phenobarbitone/beta-naphthoflavone-induced rat liver post-mitochondrial fraction (S9) in two separate experiments.

For all assays bacteria were cultured overnight in nutrient broth. Bacteria were taken from master cultures checked for strain characteristics according to literature references of Maron and Ames and De Serres and Shelby.

Rat liver enzyme induction (S9) was prepared from male Sprague-Dawley rats by oral administration of a mixture of phenobarbitone and beta-naphthoflavone.

Range-finder:

Cytotoxicity was tested at 0-5000 µg/plate dose range

Main experiments:

The substance was tested in all five strains using triplicate plates with and without S9. Negative (solvent) controls were included, both with and without S9. Positive controls in were included for each tester strain both with and without S9.

Measured aliquots (0.1ml) of one of the bacterial cultures were dispensed into sets of test tubes followed by 2.0ml of molten, trace histidine supplemented, top agar, 0.1ml of the test material formulation, vehicle or positive control and either 0.5ml of S9-mix or phosphate buffer. The contents of each test tube were mixed and equally distributed onto the surface of Vogel-Bonner Minimal agar plates. This procedure was repeated, in triplicate, for each bacterial strain and for each concentration of test material both with and without S9-mix.

All pf the plates were incubated at 37°C for ~ 48 hours and the frequency of revertant colonies assessed using Domino colony counter. Manual counts were performed at 5000µg/plate because of an opaque film and excessive test material precipitation.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment determined.

Positive control data was within acceptable historical ranges and induced clear increases in revertant numbers confirming discrimination between strains and an active S9 preparation.

Statistical analysis:

A reproducible, dose-related and statistically (Dunnett's method of linear regression) significant increase in the revertant count in at least one strain of bacteria.

Section A6.6.1 Genotoxicity in vitro

Annex Point IIA VI.6.1 *In vitro* gene mutation study in *Salmonella typhimurium*

5.2	**Results and discussion**	Positive control data were within acceptable historical ranges and induced clear increases in revertant numbers confirming discrimination between strains and an active S9 preparation. The test material caused no visible reduction in the growth of the bacterial lawn at any dose level although reductions in the frequency of revertants colonies were observed in the majority of the tester strains at the upper dose levels both with and without S9-mix. The test material was tested up too the maximum recommended dose level of 5000µg/plate. A whitish, opaque film was noted at and above 1500µg/plate and a white powdery precipitate was observed at 5000µg/plate. No significant increases in the frequency of revertants colonies were recorded of any of the bacterial strains, with any dose of the test material, either with or without metabolic activation.
5.3	**Conclusion**	The test material difenacoum is considered to be non-mutagenic.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	3 May 2006, revised 7 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Difenacoum is not mutagenic under the test conditions.
Reliability	1
Acceptability	Acceptable
Remarks	Key study The spontaneous mutation rates (=results of concurrent negative controls) could have been presented separately for cases "with S9" and "without S9".
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_6_1-1. Mean revertants/plate (±SD) in the absence of S-9

Treatment (µg/plate)	Strain				
	TA100	TA1535	TA102	TA98	TA1537
Experiment 1					
0	81 (5.7)	20 (5.5)	341 (8.4)	19 (4.5)	14 (2.6)
50	76 (14)	24 (3.2)	324 (18)	15 (2.3)	9 (2.6)
150	67 (10)	14 (2.5)	331 (10.5)	18 (2.5)	4 (1)
500	56 (10.6)	16 (2.5)	319 (7.5)	15 (4.9)	4 (2)
1500	59 (15.7)	19 (5.1)	325 (15.5)	18 (6.2)	8 (4)
5000	52 (4.4)	12 (4)	255 (49.4)	10 (1.5)	4 (1)
Positive control	461 (19.5)	277 (35.8)	1005 (34.7)	171 (3)	3813 (66.2)
Experiment 2					
0	82 (2.0)	18 (4.4)	287 (14.6)	13 (6.2)	10 (3.2)
50	66 (3.5)	13 (4.5)	272 (5.9)	16 (1.7)	6 (1.7)
150	53 (1.5)	16 (0.6)	258 (13.6)	11 (3.5)	3 (1.5)
500	49 (9.5)	9 (4)	256 (5.1)	10 (5.7)	3 (1)
1500	44 (10.3)	11 (2.1)	269 (22.5)	17 (1.5)	4 (1.5)
5000	44 (5)	11 (5.2)	244 (11.1)	12 (2)	3 (0.6)
Positive control	350 (19.1)	261 (106.7)	890 (35.1)	100 (9.5)	2691 (390.8)

Table A6_6_1-1. Mean revertants/plate (±SD) in the presence of S9

Treatment (µg/plate)	Strain				
	TA100	TA1535	TA102	TA98	TA1537
Experiment 1					
0	79 (1.7)	15 (1.2)	378 (26.7)	31 (5.0)	13 (7.0)
50	86 (5.2)	15 (3.5)	402 (10.8)	29 (5.2)	13 (4.5)
150	69 (8.1)	12 (1.2)	363 (17)	29 (2.5)	6 (0.6)
500	66 (7.5)	11 (5.5)	355 (10.8)	21 (2.5)	11(2)
1500	61 (8.1)	7 (0.6)	343 (5.1)	18 (4.6)	4 (1.5)
5000	50 (4.9)	5 (1.2)	257 (35.9)	12 (2.9)	4 (0.6)
Positive control	2120 (54.5)	260 (50.1)	1048 (34.5)	218 (18.3)	495 (28.6)
Experiment 2					
0	89 (15.2)	12 (3.5)	313 (12.9)	32 (5.8)	18 (1.5)
50	82 (14.4)	14 (2.6)	324 (6.7)	27 (10.2)	10 (7.6)
150	57 (10.8)	11 (2)	344 (14.1)	22 (0.6)	4 (1.2)
500	62 (8.5)	10 (2.3)	322 (21.4)	21 (2.3)	5 (4)
1500	51 (9)	7 (3.5)	301 (24.4)	16 (4.4)	5 (3.2)
5000	57 (6.0)	6 (3)	256 (29.5)	15 (4.6)	4 (2.1)
Positive control	2641 (75.2)	157 (27.8)	715 (45.1)	218 (32.4)	493 (10.5)

Section A6.6.2 Genotoxicity in vitro

Annex Point IIA VI.6.2

In vitro mammalian cytogenetic test

Official use only

1. REFERENCE

1.1	**Reference**	XXXXX (2002) DIFENACOUM: Chromosome aberration test in human lymphocytes *in vitro.* XXXXX report 1558/001
1.2	**Data protection**	Yes
5.3.3	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force
5.3.4	Companies with Access to data	PelGar International Ltd. Activa srl
1.3	**Criteria for data protection**	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I

2. GUIDELINES AND QUALITY ASSURANCE

2.1 Guideline study	OECD 473
2.2 GLP	Yes
2.3 Deviations	No

3. MATERIALS AND METHODS

3.1	**Test material**	As given in section 2
3.1.1	Lot/Batch number	03652
3.1.2	Specification	As given in section 2
3.1.2.1	Description	White powder
3.1.2.2	Purity	99.7% (difenacoum)
3.1.2.3	Stability	Stable under test conditions
3.2	**Study Type**	*In vitro* mammalian chromosome aberration test
3.2.1	Organism/cell type	Primary human lymphocytes
3.2.2	Deficiencies / Proficiencies	Not applicable
3.2.3	Metabolic activation system	S9 mix Lot Number PB/BNF S9 13/10/01 was prepared in-house at SafePharm Laboratories from the livers of male Sprague-Dawley rats weighing approximately 250g. These had received three daily oral doses of a mixture of phenobarbitone (80 mg/kg) and β-naphthoflavone (100mg/kg), prior to S9 preparation on the fourth day. The S9 was stored at -196°C in a liquid freezer.
3.2.4	Positive control	- S9 – mitomycin C (Sigma, Batch No. 116K2508) at 0.4 µg/ml dissolved in Minimal Essential Medium + S9 – cyclophosphamide (Sigma, Batch No. 91K1176) at 10 µg/ml dissolved in dimethyl sulphoxide
3.3	**Administration / Exposure; Application of test substance**	

Section A6.6.2 Genotoxicity in vitro

Annex Point IIA VI.6.2

In vitro mammalian cytogenetic test

3.3.1	Concentrations	Expt. 1: -S9: 0*, 18.75, 37.5*, 75*, 150*, 225, 300, Mitomycin C 0.4* µg/ml +S9: 0*, 18.75, 37.5*, 75*, 150*, 225, 300, Cyclophosphamide 10* µg/ml *Dose levels selected for metaphase analysis
3.3.2	Way of application	Dissolved in dimethyl sulphoxide
3.3.3	Pre-incubation time	Not applicable
3.3.4	Other modifications	None
3.3.5	Examinations	*See tables in appendix for examinations and results*
3.3.6	Number of cells evaluated	200 cells per dose level for aberrations 2000 cells per dose level for mitotic index

4. RESULTS AND DISCUSSION

4.1 Genotoxicity

4.1.1	without metabolic activation	Statistically significant ($p<0.01$) increase in the frequency of aberrant cells (without S9) at 150 µg/ml.
4.1.2	with metabolic activation	Statistically significant ($p<0.001$) increase in the frequency of aberrant cells (with S9) at 150 µg/ml.
4.1.3	Cytotoxicity	Yes -S9: 225 µg/ml +S9: 225 µg/ml

5. APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The study was conducted in accordance to OECD 473.

Primary human lymphocyte cultures were used to detect structural chromosome aberrations (chromosome-type and chromatid-type) induced by the test substance.

Whole blood cultures were established in culture medium 48 hours before exposure to the test material. The cells were incubated at 37°C, 5% CO_2 in humidified air and treated with the test material diluted both with and without metabolic activation for a 4-hour period.

Cytotoxic concentrations were identified using a preliminary cytotoxicity test with and without metabolic activation followed by a 20-hour recovery period and a continuous exposure of 24 hours without metabolic activation. The concentration used were in the range of 18.75 to 300 µg/ml for the 4 (20) –hour treatment both with and without S9.

Solvent treated cultures were used as negative controls, Mitomycin C in the absence of S9 and Cyclophosphamide in the presence of S9.

Experiment 1:

4-hour exposure to the test material without S9-mix followed by 20-hour culture in treatment free media prior to cell harvest.

Section A6.6.2 Annex Point IIA VI.6.2	Genotoxicity in vitro *In vitro* mammalian cytogenetic test
	4-hour exposure to the test material with S9 mix followed by 20 hour culture in treatment free media prior to cell harvest. Two hours prior to cell harvest cells were treated with demecolcine (Colcemid 0.1µg/ml). After incubation with demecolcine, the cells were centrifuged, the culture medium was drawn off and discarded, and the cells resuspended hypotonic 0.075M hypotonic KCl and fixed in methanol/acetic acid (3:1 v/v). The fixative was changed at least three times and the cells stored at 4°C for at least four hours to ensure complete fixation. Slides were dried at and stained in 5% Gurrs Giemsa solution for 5 minutes, rinsed, dried and cover slipped using mounting medium. For the evaluation of the mitotic index 2000 cells were evaluated on the slides of each test concentration. Chromosomal aberrations were determined by analysing 200 metaphases of each test concentration. Polyploid metaphases were recorded separately if noted but were not considered for analysis of chromosomal aberrations. The frequency of cells with aberrations excluding gaps and the frequency of polyploidy cells was compared, where necessary, with the concurrent vehicle control value using Fisher's Exact test.
5.2 Results and discussion	Preliminary toxicity test – Based on mitotic indices, the test material showed some evidence of toxicity at all doses tested without S9, but the index was at the level of the solvent control value at the lower dose levels without S9. However, appropriate levels of cytotoxicity was reached at the highest dose level analysed for chromosomal aberrations.. Chromosome aberration test – The toxicity was similar to that observed in the Preliminary toxicity test. There is a dose-related inhibition of mitotic index, of 50% or greater at 150ug/ml in both the absence and presence of S9. The maximum dose level selected for metaphase analysis was based on toxicity and was 150µg/ml for both exposure groups. The positive control treatments gave statistically significant increases in the frequency of cells with aberration to show the function of the metabolic S9 system. Difenacoum induced statistically significant increases in the frequency of cells with aberrations in the absence and presence of S9. Difenacoum did not induce a statistically significant increase in the numbers of polyploidy cells at any level in either of the exposure groups.
5.3 Conclusion	Difenacoum was shown to be clastogenic to human lymphocytes *in vitro.*
5.3.1 Reliability	1
5.3.2 Deficiencies	No
	Evaluation by Competent Authorities Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE

Section A6.6.2 Genotoxicity in vitro

Annex Point IIA VI.6.2

In vitro mammalian cytogenetic test

Date	12 May 2006, revised 7 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Difenacoum induces chromosomal aberrations in cultured human lymphocytes.
Reliability	1
Acceptability	Acceptable
Remarks	Key study

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_6_2-1. Table for Cytogenetic *in vitro*-Test: Chromosomal Analysis (-S9)

Treatment Group	Replicate	Mitotic index (%)	Number of Cells Scored	Number of Aberrations						Total number of aberrations		Frequency of aberrant cells (%)	
				Gaps	Chromatid		Chromosome		Others X	+ Gaps	-Gaps	+ Gaps	-Gaps
					Breaks	Exchanges	Breaks	Exchanges					
Vehicle control	A	2.95	100	0	0	0	0	0	0	0	0	0	0
	B	3.35	100	1	1	0	1	0	0	3	2	2	1
	Total	(100)	200	1	1	0	1	0	0	3	2	2 (1.0)	1 (0.5)
37.5µg/ml	A	2.60	100	1	0	0	0	0	0	1	0	1	0
	B	2.35	100	0	1	0	0	0	0	1	1	1	1
	Total	(79)	200	1	1	0	0	0	0	2	1	2 (1.0)	1 (0.5)
75 µg/ml	A	2.25	100	2	0	0	0	0	0	2	0	2	0
	B	2.35	100	1	0	0	0	0	0	1	0	1	0
	Total	(73)	200	3	0	0	0	0	0	3	0	3 (1.5)	0 (0)
150 µg/ml	A	0.85	100	6	7	3	0	0	0	16	10	10	7
	B	1.30	100	1	6	0	0	0	0	7	6	5	5
	Total	(34)	200	7	13	3	0	0	0	23	16	15 (7.5)	12** (6)
225 µg/ml	A	0	TOXIC										
	B	0	TOXIC										
	Total	(0)											
Positive control 0.4 MMC µg/ml	A	1.00	50[a]	6	27	14	2	0	0	49	43	28	27
	B	1.35	50[a]	0	29	11	5	0	1	46	46	26	26
	Total	(37)	100	6	56	25	7	0	1	95	89	54 (54)	53** (53)

X = >10 aberrations per cell (not included in total aberrations)
(figures in brackets) = aberrations per 100 cell
MMC = Mitomycin C
a = slide evaluation terminated at 50 cells because approximately 50% cells with aberrations had been observed
** = $p<0.01$
*** = $p<0.001$

Table A6_6_2-2. Table for Cytogenetic *in vitro*-Test: Chromosomal Analysis (+S9)

Treatment Group	Replicate	Mitotic index (%)	Number of Cells Scored	Number of Aberrations						Total number of aberrations		Frequency of aberrant cells (%)	
				Gaps	Chromatid		Chromosome		Others X	+ Gaps	-Gaps	+ Gaps	-Gaps
					Breaks	Exchanges	Breaks	Exchanges					
Vehicle control	A	2.65	100	0	0	0	0	0	0	0	0	0	0
	B	3.15	100	0	1	0	0	0	0	1	1	1	1
	Total	(100)	200	0	1	0	0	0	0	1	1	1(0.5)	1 (0.5)
37.5µg/ml	A	3.45	100	1	1	0	0	0	0	2	1	2	1
	B	2.90	100	0	2	0	0	0	0	2	2	2	2
	Total	(110)	200	1	3	0	0	0	0	4	3	4 (2.0)	3 (1.5)
75 µg/ml	A	3.90	100	0	1	0	1	0	0	2	2	2	2
	B	3.15	100	2	1	0	0	0	0	3	1	3	1
	Total	(122)	200	2	2	0	1	0	0	5	3	5 (2.5)	3 (1.5)
150 µg/ml	A	2.10	100	7	10	1	0	0	0	18	11	13	7
	B	1.10	100	1	7	2	0	0	0	10	9	10	9
	Total	(55)	200	8	17	3	0	0	0	28	20	23 (11.5)	16***(8)
225 µg/ml	A	0	TOXIC										
	B	0	TOXIC										
	Total	(0)											
Positive control 10CP µg/ml	A	1.55	100	3	2	0	0	0	0	5	2	4	2
	B	1.95	100	1	13	2	0	0	0	16	15	13	13
	Total	(60)	200	4	15	2	0	0	0	21	17	17 (8.5)	15*** (7.5)

X = >10 aberrations per cell (not included in total aberrations)
(figures in brackets) = aberrations per 100 cell
MMC = Mitomycin C
*** = $p<0.001$
CP = Cyclophosphamide

Table A6_6_2-3. Mean Frequency of Polyploid Cells (%)

Dose Level 1 µg/ml	Harvest Time 24 Hours	
	4 hours without S9	4 hours with S9
0	0.0	0.5
37.5	0.0	1.0
75	1.0	0.0
150	0.5	1.0
MMC 0.4	0.0	NA
CP 10	NA	0.0

MMC = Mitomycin C
CP = Cyclophosphamide
NA = Not applicabke

Section A6.6.3 Genotoxicity in vitro

Annex Point IIA VI.6.6.3 *In vitro* gene mutation assay in mammalian cells

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2004) DIFENACOUM: L5178Y TK+/- Mouse Lymphoma Assay. XXXXX report 1558/002	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with Access to data	PelGar International Ltd. Activa srl	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 476	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	white powder	
3.1.2.2	Purity	99.7% (difenacoum)	
3.1.2.3	Stability	Stable under test conditions	
3.2	**Study Type**	Mouse lymphoma assay	
3.2.1	Organism/cell type	Mouse lymphoma L5178Y cells L5178Y TK+/- 3.7.2c mouse lymphoma cells	
3.2.2	Deficiencies / Proficiencies	Not applicable	
3.2.3	Metabolic activation system	S9 mix PB/βNF S9 was prepared in-house at Safepharm Laboratories Limited from the livers of male Sprague-Dawley rats weighing ~250g. These had received orally, three consecutive daily doses of phenobarbitone/β-naphthoflavone (80/100 mg per kg per day) before to S9 preparation on the fourth day. The S9 was stored at -196°C in a liquid nitrogen freezer. 10% S9-mix was prepared by mixing S9, NADP (5mM), G6P (5mM), KCl (33mM) and MgCl2 (8mM) in R0. Final concentration of S9 was 1% throughout the study.	
3.2.4	Positive control	- S9 – ethylmethanesulphonate at 800 µg/ml for 3 hour exposure and 150 µg/ml for 24 hour exposure dissolved in dimethyl sulphoxide + S9 – cyclophosphamide at 4 µg/ml dissolved in dimethyl sulphoxide Solvent Dimethyl sulphoxide (DMSO) was used as the vehicle controls	

Section A6.6.3 Genotoxicity in vitro

Annex Point IIA VI.6.6.3 *In vitro* gene mutation assay in mammalian cells

3.3	**Administration / Exposure; Application of test substance**	
3.3.1	Concentrations	Preliminary toxicity test: - 5, 15, 50, 150, 500, 1500 and 5000 µg/ml in 3 hour exposure with and without S9 - 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/ml in 24 hour exposure without S9 Expt. 1: +/- S9: 2.34, 4.69, 9.38, 18.75, 37.5, 50 µg/ml Expt. 2: +/-S9: 2.34, 4.69, 9.38, 18.75, 37.5, 50 µg/ml
3.3.2	Way of application	Dissolved in dimethyl sulphoxide
3.3.3	Pre-incubation time	Not applicable
3.3.4	Other modifications	None
3.4	**Examinations**	*See tables in appendix for examinations and results*
3.4.1	Number of cells evaluated	Not applicable

4 RESULTS AND DISCUSSION

4.1	**Genotoxicity**	
4.1.1	without metabolic activation	Statistically and toxicologically significant dose-related increase (linear trend) in the mutant frequency
4.1.2	with metabolic activation	Statistically and toxicologically significant dose-related increase (linear trend) in the mutant frequency
4.2	**Cytotoxicity**	Yes Range-finder: +/- S9 >= 50 µg/ml, 24 hours –S9 = 12.5 µg/ml Experiment 1: +/- S9 >= 18.75 µg/ml Experiment 2: + S9 >= 4.69 µg/ml -S9 > = 9.39 µg/ml

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	OECD 476 Point mutations and chromosome deletions at the Thymidine Kinase (TK) locus in chromosome 11b are measured. The mutant frequency is determined by culturing cells in a selective agent (trifluorothymidine) during the selection period. Range finder: Cells from the L5178Y mouse lymphoma cell line were cultured at a density of 1.5 or 4.00 E-05 cells/ml in the preliminary toxicity test..

Section A6.6.3 Genotoxicity in vitro

Annex Point IIA VI.6.6.3 *In vitro* gene mutation assay in mammalian cells

.Cells were incubated for 3 hours in the presence of the test material (5, 15, 50, 150, 500, 1500 and 5000 µg/ml) with and without S9 mix and for 24 hours with 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/ml of test material without S9 mix. The test article was diluted in DMSO. As a negative control cells were treated with DMSO. The relative suspension growth value was determined.

Mutation- test

Expression period: Test concentrations used for Experiment 1 were 2.34, 4.69, 9.38, 18.75, 37.5 and 50 µg/ml. Cells were treated for 3 hours with the test material. Positive controls were treated with EMS (- S9) or CP (+ S9). After treatment cells were maintained for a 48-hour expression period by daily cell counts and dilution to 2 E05 cells per ml.

Selection period: Following the expression period, the cells were plated to determine toxicity and mutation frequency:

1) The cells cultured in test flasks were harvested and seeded at a density of 2000 cells/well in each of two 96-well microtitre plates in medium containing 4 µg/ml TFT as the selective agent.

2) 2 cells/well in each of two 96-well microtitre plates were seeded in medium without TFT in order to determine any cytotoxic effects and the cloning efficiency during the selection period.

After 10 to 14 days the numbers of survival and mutation colonies were counted.

The entire experiment was repeated independently except that a 24 hour exposure in the absence of S9 was used instead of a 4 hour exposure.

Cloning efficiency was calculated at the end of the selection period and was expressed as relative suspension growth (%RSG).

The mutant frequency was calculated expressed as mutant frequency per survivor. All results of the mutant frequency values were tested for significance using the statistical methods recommended by the UKEMS.

5.2 Results and discussion

Range-finder:

No survival of cells was observed in the range finder test after 4 hours treatment at dose levels greater than 50 µg/.

Experiment 1:

-S9: Dose-related reductions in %RSG values were noted at and above 18.75µg/ml. The mutation frequency values were all within the range of historical control data. A statistically significant ($p<0.05$) linear trend value was considered to be not biologically significant.

+S9: Dose-related reductions in %RSG values were noted at and above (treatment excluded due to excessive heterogeneity) 18.75µg/ml. The mutation frequency was significantly increased at the upper three test concentrations compared to negative controls but did not exceed the 3-fold increase criteria for a positive response. The dose response was bell-shaped but the linear trend was highly significant ($p<0.01$).

Section A6.6.3 Genotoxicity in vitro

Annex Point IIA VI.6.6.3 *In vitro* gene mutation assay in mammalian cells

	Experiment 2: -S9: Dose-related reductions in %RSG values were noted at and above 9.38 µg/ml. The mutation frequency value was significantly increased at 37.5 µg/ml but the value itself was modest. A highly statistically significant ($p<0.001$) linear trend value was noted. +S9: Dose-related reductions in %RSG values were noted at and above 4.69µg/ml. The mutation frequency was not significantly increased at any test concentration compared to negative controls. This result was contrary to that seen in Experiment 1. The positive controls induced a significant response under the chosen conditions in both experiments
5.3 Conclusion	The results of both experiments were taken to indicate that the test article is weakly mutagenic under the conditions of the study
5.3.1 Reliability	1
5.3.2 Deficiencies	No

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	29 May 2006, revised 7 February 2007
Materials and Methods	Agree with applicant's version
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Difenacoum induced mutations in mouse lymphoma cells. The proportion of small colonies suggests clastogenic activity.
Reliability	1
Acceptability	Acceptable
Remarks	Key study
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_6_3-1. Table for cell gene mutation test In-Vitro-Test (Expt.1)

Treatment (µg/ml)	3- Hour - S9			Treatment (µg/ml)	3- hour +S9		
	% RSG	RTG	MF		% RSG	RTG	MF
0	100	1.00	49.41	0	100	1.00	86.38
2.34	103	1.04	50.15	2.34	102	1.04	114.96
4.69	112	1.11	44.98	4.69	95	0.92	99.53
9.38	112	1.12	65.78	9.38 $$	86	(0.77)	(113.19)
18.75	88	1.03	57.68	18.75	84	0.68	208.51*
37.5	42	0.54	73.45	37.5	43	0.43	172.98*
50	15	0.16	71.43	50	18	0.16	143.57*
Linear trend			*	Linear trend			**
EMS 800	81	0.44	1273.60	CP 4	73	0.39	1407.17

Table A6_6_3-2. Table for cell gene mutation test In-Vitro-Test (Expt. 2)

Treatment (µg/ml)	24 Hour - S9			Treatment (µg/ml)	3hour +S9		
	% RSG	RTG	MF		% RSG	RTG	MF
0	100	1.00	60.07	0	100	1.00	77.46
2.34	96	1.20	60.53	2.34	94	0.91	73.91
4.69	103	1.12	94.09	4.69	88	0.74	74.38
9.38	83	0.87	73.64	9.38	64	0.62	63.29
18.75	63	0.76	88.68	18.75	72	0.79	88.25
37.5	19	0.11	167.60*	37.5 $$	52	0.48	(68.60)
50 X	0	0.01	289.47	50	29	0.23	92.77
Linear trend			***	Linear trend			NS
EMS 150	80	0.51	2020.25	CP 4	66	0.30	971.33

$$ = Treatment excluded due to excessive heterogeneity

% RSG = Relative suspension Growth
RTG = Relative Total Growth corrected for post treatment toxicity

EMS = Ethylmethanesulphonate
CP = Cyclophosphamide
MF = 5-TFT resistant mutants/10^6 viable cells 2 days after treatment

X = Treatment excluded from test statistics due to toxicity
* = $p<0.05$
** = $p<0.01$
*** = $p<0.001$

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 Micronucleus Test in the Mouse

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (1995) Difenacoum: An evaluation in the mouse micronucleus test. XXXXX – Report number MLS/10029	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.3.1	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 474	
2.2	**GLP**	Yes	
2.3	**Deviations**	According to the OECD 474 guideline valid at the time of performance of this study, the ratio of immature/mature erythrocytes should have been determined by counting 1000 erythrocytes per animal. However, in the revised guideline, the number is 200 at the minimum.	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	TCP 0027/95	
3.1.2	Specification	Deviating from specification given in section 2 as follows: Purity in study is 98.2%. Purity specification in section 2 is 99.2%	
3.1.2.1	Description	Off-white powder	
3.1.2.2	Purity	98.2%	
3.1.2.3	Stability	Stable	
3.1.2.4	Maximum tolerable dose	5000 mg/kg bw	
3.2	**Test Animals**		
3.2.1	Species	Mice	
3.2.2	Strain	CDI	
3.2.3	Source	Charles River Laboratories, Wilmington, Mass, USA	
3.2.4	Sex	Male and Female	
3.2.5	Age/weight at study initiation	8-12 weeks Weights 17.2g – 27.6g	
3.2.6	Number of animals per group	5 /sex/group/time to kill	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Oral	

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 Micronucleus Test in the Mouse

3.3.1	Number of applications	Single dose	
3.3.2	Interval between applications	Not applicable	
3.3.3	Postexposure period	72 hr after treatment	
		Oral	
3.3.4	Type	Gavage	
3.3.5	Concentration	3125 and 5000 mg/kg bw	
3.3.6	Vehicle	Corn oil	
3.3.7	Concentration in vehicle	10ml/kg bw	
3.3.8	Total volume applied	3125 or 5000 mg/kg bw	X
3.3.9	Controls	Positive control – cyclophosphamide Vehicle - Corn oil	
3.4	**Examinations**		
3.4.1	Clinical signs	No	
3.4.2	Tissue	bone marrow	
3.4.3	Number of animals:	20	
3.4.4	Number of cells:	1000	
3.4.5	Time points:	24, 48, 72 h after treatment	
3.4.6	Type of cells	erythrocytes in bone marrow	
3.4.7	Parameters:	Polychromatic/normochromatic erythrocytes ratio and the number of micronucleated polychromatic erythrocytes was determined.	
3.5	**Further remarks**		
		4 RESULTS AND DISCUSSION	
4.1	**Clinical signs**	No mortalities were observed in any dose group by day 4 but all animals had died or were killed in extremis by day 9. These observations were made during the pre-test for median lethal dose.	
4.2	**Haematology / Tissue examination**	There was no examination of haematology or tissue in this study	
4.3	**Genotoxicity**	There was no evidence of genotoxic effect in this study	
4.4	**Other**	There was no evidence of other effect in this study	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The study was conducted according to OECD 474. The median lethal dose (MLD) over a four day period was determined but since no deaths were observed over this period, 5000 mg/kg was the highest dose level used. Five animals per sex per group were given a single oral dose of either 5000 mg/kg or 3125 mg/kg bw of difenacoum. Animals were killed by cervical dislocation at either 24, 48, or 72 hours after the dose. The	

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 Micronucleus Test in the Mouse

		femurs were removed and 1000 polychromatic erythrocytes were examined and the number containing micronuclei scored. Slides were examined for evidence of cytotoxicity which may have effected the ratio of differenct cell types in the bone marrow. This was assessed by determining the percentage of polychromatic to normochromatic erythrocytes in a total pollution of 500 erythrocytes. Micronuclei were identified according to Schmid (1976) In: A Hollander (Ed.). Chemicals Mutagens: Principals and Methods for their Detection. Vol 4. Plenum, New York 31-43. Results were analysed using a one-sided Students 't' test for difference from the negative control group.
5.2	**Results and discussion**	No significant increases in the polychromatic erythrocytes PCE's containing micronuclei were seen after treatment with difenacoum at either dose rate or at any sampling times. The date data indicated that difenacoum is not clastogenic in the mouse bone marrow micronucleus assay. Cyclophosphamide, the positive control, produced significant increases in PCE's at all three sampling times.
5.3	**Conclusion**	Difenacoum is not clastogenic in the mouse bone marrow assay.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	29 May 2006, revised 7 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's conclusion. Difenacoum did not induce micronuclei under the test conditions.
Reliability	1
Acceptability	Acceptable
Remarks	Key study Point 3.3.8: Very high doses of difenacoum were given to mice. However, due to the characteristic delay in effects, mortality was not observed during the four day observation period in the pre-test for median lethal dose. Animals died or were killed by day 9. However, statistically significant ($p<0.05$) reduction in the mean percentage of polychromatic erythrocytes at the lower dose indicates appropriate cytotoxicity.

COMMENTS FROM ...

Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*

Section A6.6.4 Genotoxicity in vivo

Annex Point IIA6.6.4 Micronucleus Test in the Mouse

Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_6_4-1. Table for Micronucleus Test In Vivo, mean incidence of micronuclei

Group	**Compound**	**Dose**	**Incidence of Micronuclei/1000 cells**		
			24 hrs	48hrs	72 hrs
1	Control (corn oil)	10 ml/kg	2.0	1.4	1.5
2	Difenacoum	3125 mg/kg	1.5	1.0	2.0
3	Difenacoum	5000 mg/kg	1.5	1.2	1.6
4	Cyclophosphamide	65 mg/kg	16.4**	18.0**	2.7*

* statistically significant increase in polychromatic erythrocytes containing micronuclei at p<0.05 students 't' test (one-sided)

** statistically significant increase in polychromatic erythrocytes containing micronuclei at p <0.01 students 't' test (one-sided)

Table A6_6_4-2. Table for Micronucleus Test In Vivo, mean polychromatic erythrocytes

Group	**Compound**	**Dose**	**% Polychromatic erythrocytes**		
			24 hrs	48hrs	72 hrs
1	Control (corn oil)	10 ml/kg	29.9	31.5	28.4
2	Difenacoum	3125 mg/kg	27.6	24.3*	21.2*
3	Difenacoum	5000 mg/kg	30.3	25.5	31.5
4	Cyclophosphamide	65 mg/kg	27.2	25.9	24.3

* statistically significant reduction in polychromatic erythrocytes at p<0.05 students 't' test (one-sided)

Section A6.6.5 Genotoxicity in vivo

Annex Point IIA VI6.6.5 DNA Synthesis in Rat Hepatocytes in vivo

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (1995) Difenacoum: Assessment for the induction of unscheduled DNA synthesis in rat hepatocytes, XXXXX – Report number MLS/10021	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.3.2	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No. Method as described by Mirsalis and Butterworth (1980) and modified by Ashby et al (1985-1987). Technique was in broad agreement with those described by Butterworth et al (1987) as an ASTM Guideline.	X
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	TCP 0004/95	
3.1.2	Specification	Deviating from specification given in section 2 as follows: Purity in study is 97.4%. Purity specification in section 2 is 99.2%	
3.1.2.1	Description	Off-white powder	
3.1.2.2	Purity	97.4%	
3.1.2.3	Stability	Stable	
3.1.2.4	Maximum tolerable dose	800 mg/kg bw	
3.2	**Test Animals**		
3.2.1	Species	Rat	
3.2.2	Strain	Wistar	
3.2.3	Source	Charles River Laboratories, Wilmington, Mass, USA	
3.2.4	Sex	Young adult male	
3.2.5	Age/weight at study initiation	Not stated	
3.2.6	Number of animals per group	5/dose group/post exposure time/experiment	

Section A6.6.5 Genotoxicity in vivo

Annex Point IIA VI6.6.5 DNA Synthesis in Rat Hepatocytes in vivo

		3/Control group/experiment
3.2.7	Control animals	Yes
3.3	**Administration/ Exposure**	Oral
3.3.1	Number of applications	Single dose
3.3.2	Interval between applications	Not applicable
3.3.3	Postexposure period	4 & 12 hr after treatment
		Oral
3.3.4	Type	Gavage
3.3.5	Concentration	200, 400 and 800 mg/kg bw
3.3.6	Vehicle	Corn oil
3.3.7	Concentration in vehicle	20, 40 and 80 mg/ml
3.3.8	Total volume applied	10 ml/kg bw
3.3.9	Controls	Positive control: 4hr – N-nitrosoddimethylamine (NDMA) 12hr – 6-p-dimethylaminophenylazobenzthiazole (6BT) NegativeVehicle - Corn oil
3.4	**Examinations**	
3.4.1	Clinical signs	No
3.4.2	Tissue	Liver
		Number of animals: 84 Number of cells: 100 per animal Time points: 4 and 12 h after treatment Type of cells Hepatocytes Parameters: Nuclear grain count Cytoplasmic grain count
3.5	**Further remarks**	There are no further remarks
		4 RESULTS AND DISCUSSION
4.1	**Clinical signs**	There was no examination of clinical signs in this study
4.2	**Haematology / Tissue examination**	There was no examination of haematology or tissue in this study
4.3	**Genotoxicity**	There was no evidence of genotoxic effect in this study
4.4	**Other**	There was no evidence of other effect in this study
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	Method as described by Mirsalis and Butterworth (1980) and modified by Ashby et al (1985-1987). Technique was in broad agreement with those described by Butterworth et al (1987) as an ASTM Guideline.

Section A6.6.5 Genotoxicity in vivo

Annex Point IIA VI6.6.5 DNA Synthesis in Rat Hepatocytes in vivo

		Difenacoum was evaluated for its ability to induce unscheduled DNa synthesis (UDS) in a *in vitro* rat hepatocytes assay incorporating an autoradiographic technique. Male Wistar rats were given a single oral dose of difenacoum by gavage at 200, 400 and 800 mg/kg bw. The highest test treatment was the four day maximum tolerated dose (MTD) for difenacoum. Two sampling times were used, four hours and twelve hours following administration of the substance. Two independent experiments were carried out at each time point, validated by concurrent control treatments of rats with the solvent used to formulate the difenacoum test solutions and the carcinogen 6-p-dimethylaminophenylazobenzthiazole (6BT) or N-nitrosodimethylamine (NDMA). Difenacoum was assessed from UDS at the two dose levels 400 and 800 mg/kg.
5.2	**Results and discussion**	Difenacoum was evaluated in two independent experiments at 4 and 12 hours following a single oral dose of either 400 or 800 mg/kg bw. No signs of excessive cytotoxicity were observed, cells were of normal morphology and with similar number of pyknotic cells present compared to the solvent control cultures. Difenacoum caused no induction of UDS on the basis of both mean net nuclear grain count and percentage of cells in repair. The sensitivity of the assay was confirmed by the detection of the known genotoxins 6BT and NDMA.
5.3	**Conclusion**	When test up to the maximum tolerated dose, difenacoum did not induce DNA repair in rat hepatocytes exposed *in vivo.*
5.3.1	Reliability	1
5.3.2	Deficiencies	No

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	30 May 2006, revised 7 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Under the test conditions, difenacoum did not induce DNA damage that is detectable by this test.
Reliability	1
Acceptability	Acceptable
Remarks	Key study Point 2.1: The performance of the test is in line with the OECD guideline 486.
	COMMENTS FROM ...
Date	*Give date of comments submitted*

Section A6.6.5 Genotoxicity in vivo

Annex Point IIA VI6.6.5 DNA Synthesis in Rat Hepatocytes in vivo

Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_6_5-1. Table for Unscheduled DNA Synthesis in Rat hepatocytes In Vivo

State mean ± standard deviation state individual numbers for critical findings	control group	low dose	high dose	Positive control
Sampling time (h)	12	12	12	12
Mean nuclear grain count	5.13 ± 0.55	5.93 ± 0.49	6.43 ± 0.70	27.54 ± 7.3
Mean cytoplasmic grain count	8.09 ± 0.60	8.85 ± 0.57	9.11 ± 1.02	8.84 ± 1.58
Mean net nuclear grain count	-2.95 ± 0.21	-2.92 ± 0.47	-2.68 ± 0.59	+18.70 ± 8.55
Mean % cells in repair	1.33	0.5	1.0	88.5
Sampling time (h)	4	4	4	4
Mean nuclear grain count	8.59 ± 1.70	9.16 ± 1.45	9.29 ± 2.12	37.38 ± 7.94
Mean cytoplasmic grain count	11.82 ± 2.83	12.76 ± 2.50	13.45 ± 3.21	12.40 ± 2.84
Mean net nuclear grain count	-3.21 ± 1.19	-3.60 ± 1.24	-4.16 ± 1.20	+24.98 ± 5.24
Mean % cells in repair	0.9	1.6	1.3	89.7

Section A6.6.6 Annex Point IIA VI.6.6.6	Genotoxicity in vivo (germ cell effects)	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	Results of study in Section A6.6.4 Genotoxicity in vivo - Micronucleus Test in the Mouse and an in vivo rat study were both negative for Genotoxicity, therefore based on this result and animal welfare concerns, it is deemed unnecessary to conduct this study.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	30 May 2006	
Evaluation of applicant's justification	The applicant has followed the testing strategy to reveal any genotoxic effects of difenacoum according to the requirements laid down in the TNsG on Data Requirements, and as no genotoxicity was observed in the *in vivo* micronucleus test or UDS test, no more testing is required.	
Conclusion	Applicant's justification is acceptable.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.6.7 Annex Point - *III-0§*	Genotoxicity in vivo (further test if metabolites of concern are formed in mammals)	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	No positive findings in *in vivo* genotox studies. No metabolites of concern are noted for Difenacoum in the literature or for any other analogue. See Section A6.2	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	30 May 2006	
Evaluation of applicant's justification	The applicant has performed adequate *in vitro* and *in vivo* testing of difenacoum and no metablolites of concern are known to be formed in mammals.	
Conclusion	Applicant’s justification is acceptable.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.7 Annex Point IIA VI.6.7	Carcinogenicity	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [X]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	The compound belongs to a well-known and closely analogous group of anticoagulants with very similar properties. The mode of action of the Difenacoum is fully known. All studies on vertebrates show the same effects, primarily loss of blood coagulation, and these are shown clearly in acute studies. To avoid acute effects, doses in repeat dose studies must be kept very low and it is considered infeasible to keep alive animals receiving any appreciable dose for more than a few months. The potential for exposure to rodenticides is limited by the nature of their use, and there is no exposure as a result of residues of the substance, or as a result of long-term exposure to vapour. A waiver for carcinogenicity study on anti-coagulant rodenticides is scientifically justified, based on the absence of any other effects that may lead to non-genotoxic carcinogenesis, and the absence of any carcinogenic effects following long-term exposure of Difenacoum to humans. A QSAR program called 'DEREK' for word was performed to see if Difenacoum contains any structural alerts that are of concern for genotoxicity and mutagenicity. The result was negative and no structural alerts for this endpoint were highlighted. Difenacoum was not mutagenic in a bacterial mutagenicity study but did show some genotoxic activity in two *in vitro* mammalian cell assays. However, the level of activity that was observed was very weak, showed little evidence of a dose response relationship and was had a low level of reproducibility. Furthermore, the weak responses were only observed at dose levels close to the toxic limit in each case and a strong possibility exists that they were artefactual in nature. Two acute in vivo studies showed no evidence of genotoxic activity in either the rat or mouse. The chemical structure of Difenacoum does not contain any significant structural alert for genotoxicity or carcinogenicity (as shown by DEREK for windows) and has a strong similarity to the structure of Brodifacoum and Bromadiolone, neither of which showed any evidence of genotoxic activity in vitro. Rodents are used in toxicity testing because they are small, readily available, and have a relatively short life span. In the case of testing the carcinogenic potential of difenacoum, which is designed to kill the rats in low dose, long-term testing of the target species will be very difficult to perform. By looking at these factors a carcinogenicity studies is considered unethical and unjustified.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	7 February 2007	

Section A6.7 Annex Point IIA VI.6.7	Carcinogenicity
Evaluation of applicant's justification	Although not agreeing in every detail of the applicant's justification RMS can agree on the conclusion that waiving of carcinogenicity studies is scientifically justified. MSs have accepted a refined waiving concept for rodenticides due to the unique nature of these substances as being used to kill the animals in nature, which should also be used to test the human toxicity of the compound. This fact together with the known mode of action (anticoagulation due to vitamin K_1 deficiency) of difenacoum, leads inevitably to major technical difficulties in performing valid long-term (or even studies with much shorter duration) toxicity tests in mammals. Based on available toxicity data there is no indication of carcinogenic potential to humans. Although the frequency and duration of exposure of professional users is above the level of lower concern and secondary exposure of small children does not meet the criteria of "negligible", RMS considers that scientific and ethical reasons dominate to support waiving of chronic toxicity studies with difenacoum.
Conclusion	Waiving of carcinogenicity studies in rodents is accepted due to ethical reasons and known mode of action. Based on current knowledge of absence of carcinogenic effects difenacoum or analogous compounds difenacoum should not be classified as carcinogenic to humans.
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.8.1 (1) Teratogenicity Study

Annex Point IIA6.8.1 Oral developmental toxicity to the rat

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (1995) DIFENACOUM: Development toxicity to the rat. XXXXX, Report MLS/10013	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.3.3	Companies with access to data	PelGar International Ltd. Activa srl	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	OECD 414	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	TCP 0004	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Off-white powder	
3.1.2.2	Purity	Difenacoum 98.7%	X
3.1.2.3	Stability	Stable	
3.2	**Test Animals**		
3.2.1	Species	Rats	
3.2.2	Strain	Wistar	
3.2.3	Source	Charles River Laboratories, Wilmington, Mass., U.S.A.	
3.2.4	Sex	Female (virgin)	
3.2.5	Age/weight at study initiation	12 weeks Female 237 – 293g	X
3.2.6	Number of animals per group	20	X
3.2.7	Control animals	Yes	
3.2.8	Mating period	Overnight	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of exposure	rat: day 7-16 post mating	

Section A6.8.1 (1) Teratogenicity Study

Annex Point IIA6.8.1 Oral developmental toxicity to the rat

3.3.2	Postexposure period	6 days
3.3.3		**Oral**
3.3.4	Type	Gavage
3.3.5	Concentration	0.01, 0.03 and 0.09 mg/kg
3.3.6	Vehicle	Polyethylene glycol 300
3.3.7	Concentration in vehicle	0.001, 0.003 and 0.009 mg/ml
3.3.8	Total volume applied	1ml/100g bw
3.3.9	Controls	Vehicle
3.4	**Examinations**	
3.4.1	Body weight	Yes, arrival, day 3, 7, 9, 11, 13, 15, 18 and 22 of gestation
3.4.2	Food consumption	No
3.4.3	Clinical signs	Yes, twice daily
3.4.4	Examination of uterine content	Gravid uterine weight Number of corpora lutea Number of implantations Individual foetal weights
3.4.5	Examination of foetuses	
3.4.5.1	**General**	Litter Size, Nr. Of dead Foetuses, Foetal Weight, Sex, external, skeletal and soft tissue abnormalities.
3.4.5.2	**Skeletal**	Yes, all foetuses .
3.4.5.3	**Soft tissue**	Yes, all foetuses .
3.5	**Further remarks**	Blood samples were collected from a tail vein of dams in each group on days 1,6,11, 17 and 22 for the measurement of prothrombin (PT) and kaolin-cephalin (KCT) times.
		4 RESULTS AND DISCUSSION
4.1	**Maternal toxic Effects**	Three females fromn the 0.09 mg/kg dose group were killed for human reasons on days 16 and 17. All were pregnant. Piloerection and signs of diarrhoea was seen in all treatment groups including the control and was therefore no related to treatment with Difenacoum. At the highest dose rate signs of staining around the nose and coloured faeces were evident and related to the test material. In addition, decreased activity, dehydration and paleness and increased incidence of piloerection was observed at the highest dose of 0.09 mg/kg bw/day. There was no significant differences in weight gain across the treatment groups and control. There was no effect in coagulation times in animals dosed with the test material compared to the control
4.2	**Teratogenic / embryotoxic effects**	There was no compound-related effects in litter data. The major defects seen in one foetus dosed at .0.09mg/kg was microphthalmia of both

Section A6.8.1 (1) Teratogenicity Study

Annex Point IIA6.8.1 Oral developmental toxicity to the rat

		eyes. There was an increased incidence of incomplete ossification of 5th Sternebrae at 0.09 mg/kg. There was a reduced incidence of full ossification for cervical vertebrae – 7th at 0.03 mg/kg of the test material. There was no evidence of a compound-related effect on the incidence of variants.	
4.3	**Other effects**	No evidence of teratogenicity or other development effects seen.	X
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The study was conducted according to OECD 414. Groups of time-mated female rats were dosed by oral gavage with 0, 0.01, 0.03, or 0.09 mg/kg of Difenacoum using polyethylene glycol 300 (PEG 300) as the vehicle. The rats were dosed on days 7 to 16 of the gestation period. On day 22 of gestation, the rats were killed and there uteri examined for live foetuses and intra-uterine deaths. The foetuses were weighed, examined for both external and visceral abnormalities, sexed, eviscerated and stained for skeletal examination.	
5.2	**Results and discussion**	Three of the 20 females at the top dose were killed on days 16 or 17 of gestation due to the clinical symptoms. There had been no effects on bodyweights or food consumption indicative of toxicity prior to this time. There were no effects of compound seen in animals dosed with 0.01 or 0.03 mg/kg. Macroscopic findings in the females from the 0.09 mg/kg dose group killed and examined before day 22, were consistent with anticoagulant poisoning but there were no other compound-related effects. There was no evidence of teratogenicity and no other effects on foetal development at any dose with no evidence of haemorrhage in the foetuses and no increase in post-implantation loss. No maternal toxicity toxicity was seen at either the 0.01 or 0.03 mg/kg.	X
5.3	**Conclusion**	No overt signs of toxicity were seen in animals that survived to the end of the study. No evidence of teratogenicity or other indications of developmental toxicity was seen. No evidence of teratogenicity or other development effects seen.	X
5.3.1	LO(A)EL maternal toxic effects	0.09 mg/kg/day	
5.3.2	NO(A)EL maternal toxic effects	0.03 mg/kg/day of difenacoum	X
5.3.3	LO(A)EL embryotoxic / teratogenic effects	No effects seen	
5.3.4	NO(A)EL embryotoxic / teratogenic effects	0.09 mg/kg/day of difenacoum	
5.3.5	Reliability	1	X
5.3.6	Deficiencies	No	

Evaluation by Competent Authorities	
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
EVALUATION BY RAPPORTEUR MEMBER STATE	

Section A6.8.1 (1) Teratogenicity Study

Annex Point IIA6.8.1

Oral developmental toxicity to the rat

Date	June 19th, 2006, revised 7 February 2007
Materials and Methods	Point 3.1.2.2.: Reference to Section 2 of the dossier is made for specificarion, but the test is performed with a substance of little lower purity. Point 3.2.5 and 3.2.6: Indicated values are on arrival day, = day 1 of gestation.
Results and discussion	Agree with applicant's version, but see RMS Conclusion and Remarks.
Conclusion	Other conclusions: NOEL/NOAEL for maternal toxicity was 0.03 mg/kg bw/day (clinical signs indicating bleeding, but no effect was seen in coagulation times). Maternal coagulation times were not increased even at the highest dose level where three dams were killed in extremis due to humane reasons. This suggests that coagulation times were not good indications of maternal toxicity. At 0.09 mg/kg bw/d dose level, there was one foetus with microphthalmia, one foetus with discoloured adrenals and some minor skeletal effects in foetuses. There findings were not considered treatment-related. In this study, developmental toxicity was not observed. However, based on information on analogical compounds it should be classified as Repr. Cat 1; R61 (see Remarks below).
Reliability	1 (see Remarks below)
Acceptability	Acceptable (but see Remarks below)

Section A6.8.1 (1) Teratogenicity Study

Annex Point IIA6.8.1

Oral developmental toxicity to the rat

Remarks	Based on the similar mode of action and structural similarity (coumarin moiety) to warfarin, it is possible that difenacoum, like warfarin, is teratogenic in humans. Coumarin derivates, particularly warfarin, are associated with the induction of malformations in human. The results of developmental toxicity studies with warfarin in rats, mice and rabbits have been confusing (Schardein 2000), but the teratogenicity of warfarin was confirmed in rats using a special study design where net extrahepatic vitamin K deficiency was achieved (Howe and Webster, 1990, 1992; Howe et al., 1992). Maternal bleeding was prevented by giving warfarin along with high vitamin K doses and dosing of warfarin was continued after birth. Very high warfarin doses were needed to induce similar maxillonasal hypoplasia and other skeletal disturbances to those observed in humans at therapeutic dose levels. Similar approach would have been needed for difenacoum to reveal the possible teratogenic effect in rats. Based on warfarin data, human foetuses seem to be much more vulnerable to vitamin K deficiency than rodent foetuses (Howe and Webster, 1994, 1997). It is obvious that possible teratogenic effects of coumarin related compounds (coumarin embryopathy) can not be detected using conventional OECD 414 approach. Thus, without vitamin K dosing or other methods to prevent maternal bleeding, rodents are not good models for studying developmental effects of coumarin-derivate compounds. In addition, the different time schedule on bone development in rats and humans should be taken into account. Howe AM and Webster WS (1990): Exposure of the pregnant rat to warfarin and vitamin K1: an animal model of intraventricular hemorrhage in fetus. Teratology 42:413-420. Howe AM and Webster WS (1992): The warfarin embryopathy: a rat model showing maxillonasal hypoplasia and other skeletal disturbances. Teratology 46:379-390. Howe AM, Webster WS, Lipson AH, Halliday JL, Sheffield LJ (1992): Binder's symdrome due to prenatal vitamin K deficiency: a theory of pathogenensis. Aust Dent J 37:453-460. Howe AM and Webster WS (1994): Vitamin K – its essential role in craniofacial development. A review of the literature regarding vitamin K and craniofacial development. Aust Dent 39:88-92. Howe AM, Lipson AH, de Silva M, Ouvrier R, Webster WS (1997). Severe cervical dysplasia and nasal cartilage calcification following prenatal warfarin exposure. Am J Med Genet 71:391-396. Schardein JL (2000). Chemically induced birth defects. Marcel Dekker, Inc., New York, pp 124 Key study
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*

Section A6.8.1 (1) Teratogenicity Study

Annex Point IIA6.8.1

Oral developmental toxicity to the rat

Remarks

Table A6_8-1. Table for Teratogenic effects (separate data for all dosage groups)

Maternal effects

Parameter	control data		low dose	medium dose	high dose	dose-response + / -
	historical	study				
Number of dams examined		20	20	20	20	
Mortality of dams *state %*		0	0	0	3	
Abortions		0	0	0	0	
Resorption		0	0	0	0	
Pregnancy *pregnancy rate or %*		100	100	100	100	

Table A6_8-2. Table for Teratogenic effects (separate data for all dosage groups)

Litter response (Caesarean section data)

Parameter	control data		low dose	medium dose	High dose	dose-response + / -
	historical	study				
Corpora lutea *mean*		16.1	16.3	16.6	15.4	
Implantations *mean*		14.8	14.8	14.5	13.5	
pre-implantation loss *state %*		8.1	9.2	13	12.1	
post-implantation loss *state %*		6.8	4.1	6.5	5.8	
live fetuses *mean*		12.8	13.6	12.6	12.8	
fetus weight (mean) *[g]*		4.93	4.89	4.83	4.80	
Mean gravid uterus weight *[g]*		83.8	92.5	87.1	88.5	
Fetal sex ratio *[state ratio m/f]*		50	47.4	47.2	57.8	

Table A6_8-3. Table for Teratogenic effects (separate data for all dosage groups)

Examination of the fetuses

Parameter	**control data**		**low dose**	**medium dose**	**High dose**	**dose-response + / -**
	historical	study				
External malformations* *[%]*		0	0	0	0.5	
External anomalies* *[%]*		1.7	1.9	1.2	0.4	
Skeletal malformations* *[%]*		0	0	0	0	
Skeletal anomalies* *[%]*		18.8	19.9	16.8	22.9	
Skeletal variants* *[%]*		88.9	86.3	85.9	85.8	
Variants visceral* *[%]*		8.2	8.1	9.1	4.4	

Section A6.8.1 (2) Teratogenicity Study

Annex Point IIA6.8.1

Teratology study of Difenacoum technical in rabbits

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX, (2004) Teratology study of the test item Difenaocum Technical in Rabbits, XXXXX, Study code: 03/738-105N	
1.2	**Data protection**	Activa / PelGar brodifacoum and difenacoum Task Force	
1.2.1	Data owner	PelGar International Ltd. Activa srl	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes, OECD 414	
2.2	**GLP**	Yes	
2.3	**Deviations**	Yes In some animals the individual body weight was not measured on the gestation day 22. In some cases the placental weights were measured with accuracy 0.1g. These deviations do not affect the integrity of the study.	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.2	**Description**	Greyish-white powder	
3.3	**Purity**	99.7%	
3.4	**Stability**	Stable	
3.5	**Test Animals**		
3.5.1	Species	Rabbit	
3.5.2	Strain	New Zealand	
3.5.3	Source	Ferenc Sàndor breeder, H-2173 Kartal, Vörös Hadsereg ūt 131 Hungary	
3.5.4	Sex	females	
3.5.5	Age/weight at study initiation	Age: at least 4 months Body weight: 3506-4438g	
3.5.6	Number of animals per group	22	
3.5.7	Control animals	Yes	
3.5.8	Mating period	Inseminated	
3.6	**Administration/ Exposure**	Oral	
3.6.1	Duration of exposure		

Section A6.8.1 (2) Teratogenicity Study

Annex Point IIA6.8.1

Teratology study of Difenacoum technical in rabbits

		rabbit: day 7-28 post insemination
3.6.2	Postexposure period	1 day
		Oral
3.6.3	Type	Gavage
3.6.4	Concentration	0, 1, 3, 10 µg/kg bw/day
3.6.5	Vehicle	1.25% aqueous methyl cellulose containing 2% of ethanol
3.6.6	Concentration in vehicle	0, 2, 6 and 20 µg/ml
3.6.7	Total volume applied	0.5 ml/kg bw/day.
3.6.8	Controls	Vehicle
3.7	**Examinations**	
3.7.1	Body weight	Yes, gestation days: 1, 4, 7, 10, 13, 14, 16, 19, 21, 22, 25, 28 and 29
3.7.2	Food consumption	Yes, gestation days: 4, 7, 10, 13, 14, 16, 19, 21, 22, 25, 28 and 29
3.7.3	Clinical signs	Yes, daily
3.7.4	Examination of uterine content	Gravid uterine weight Number of corpora lutea Number of implantations
3.7.5	Examination of foetuses	
3.7.5.1	General	Litter Size, Number of dead Foetuses, Foetal Weight, Sex Ratio, Crown-rump length of foetuses, and,external malformations
3.7.5.2	Skeletal	Yes
3.7.5.3	Soft tissue	Yes
3.8	**Further remarks**	Blood samples were collected on autopsy in control and 10 µg/kg dose groups for the measurement of partial thromboplastin time (PTT) and prothrombin time (PT).

4 RESULTS AND DISCUSSION

4.1	**Maternal toxic Effects**	Mortality, clinical signs: Control group: Two animals died (on gestation day 13 and 22), no prior signs of clinical symptoms. Dose group 1 µg/kg: One animal showed reduced activity on gestation day 22 and died the next day. Dose groups 3 µg/kg: Three animals died between gestation days 19 and 21. In two animals haemorrhage was observed around the vagina and rectum between gestation days 16 and 19, and the third animal appeared to have pale mucous membranes on gestation day 18. Dose group 10 µg/kg: One animal was killed in extremis on gestation day 9 due to previous injury. Haemorrhage around the vagina and rectum was seen in another animal on gestation days 15 and 16. Bodyweight: No significant difference in average body weight between

		the control and treated groups.	
		Food consumption: No significant difference in food consumption between the control and treated groups	
		The partial thromboplastin time and prothrombin time was significantly higher in the dose group of 10µg/kg as compared to the control.	
4.2	**Teratogenic / embryotoxic effects**	No significant difference was found in the average number of corpora lutea, average number of implantations, viable foetuses and their sex distribution. The incidence of preimplantation loss, postimplantation loss and total intrauterine mortality was at similar for the control level in the dose groups 1 and 3 µg/kg. In the dose group 10ug/kg, the preimplantation loss was lower than in the control.	X
		In all groups, thymus misshapen, absent or rudimentary intermedial lung lobe and double or rudimentary gall-bladder were observed on the foetuses.	
		In dose group 1 and 10µg/kg, the number of skeletal variations was higher as compared to the control. The occurrence of abnormalities was without dose dependency, and therefore not considered as related to the test material.	
4.3	**Other effects**		
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The study was conducted according to OECD guidelines 414. The test material, Difenacoum technical, was tested on New Zealand white rabbits. The females were artificially inseminated and the day of insemination was considered as the first day of pregnancy. The animals were treated daily with 1, 3, and 10µg/kg, by oral gavage from the 7th to 28th day of gestation. Each group had 22 females. The test item was dissolved in ethanol absolute and diluted by methyl cellulose. The control received ethanol absolute diluted by methyl cellulose.	
		Caesarean sections were made on animals on gestation day 29 and the foetuses were examined for any abnormalities. On the days of the autopsies in control and 10µg/kg dose group, partial thromboplastin time (PTT) and prothrombin time (PT) was measured from plasma samples derived by heart puncture with anticoagulant dosium citrate.	
		Statistical evaluation was performed using the program package SPSS PC+ 4.0. The homogeneity of variance between groups was checked by Bartlett's homogeneity of variance test. Where no significant heterogeneity was detected one-way analysis of variance (ANOVA) was carried out.	
5.2	**Results and discussion**	1 µg/kg dose group	
		Toxic symptoms were observed in one animal. The body weight, the body weight gain and the food consumption were normal. Treatment related pathological alterations were found in seven animals. The incidence of foetuses retarded in crown-rump length was lower than in the control. Treatment related external, visceral or skeletal abnormalities were not recorded. The intrauterine development of conceptuses was not affected.	
		3 µg/kg dose group	
		Toxic symptoms were observed in three animals. The body weight, the body weight gain and the food consumption were normal. Treatment	

		related pathological alterations were found in five animals. The incidence of foetuses retarded in crown-rump length was similar to the control. The intrauterine development of conceptuses was not affected. 10 µg/kg dose group Toxic symptoms were observed in one animal. The body weight, the body weight gain and the food consumption were normal. Significant treatment related pathological alterations were found in seventeen animals. The incidence of foetuses retarded in crown-rump length was similar to the control. The partial thromboplastin time and prothrombin time was significantly higher as compared to the control. The 7th number of lumbar and sacral vertebrae of foetuses was higher than in the control. Treatment related external, visceral or skeletal abnormalities were not recorded. The intrauterine development of conceptuses was not affected. Pathological alterations observed in seven, five and 17 animals at 0.001, 0.003 and 0.010 mg/kg bw/day were related to internal haemorrhages. At the highest dose, haemorrhages in the uterus were found in five animals. PPT and PT were increased by 35 and 28%, respectively. Postimplantation loss did not correlate with maternal PPT or PT values. PTT and PT values did not correlate with pathological alterations observed.	
5.3	**Conclusion**		X
5.3.1	LO(A)EL maternal toxic effects	1µg/kg	
5.3.2	NO(A)EL maternal toxic effects	<1 µg/kg	
5.3.3	LO(A)EL embryotoxic / teratogenic effects	Not found	
5.3.4	NO(A)EL embryotoxic / teratogenic effects	Not found	
5.3.5	Reliability	1	X
5.3.6	Deficiencies	No	

Section A6.8.1 (2) Teratogenicity Study

Annex Point IIA6.8.1

Teratology study of Difenacoum technical in rabbits

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 19th, 2006, revised 7 February 2007; table A6_8-1 revised on June 22, 2009.
Materials and Methods	Agree with applicant's version.
Results and discussion	Point 4.2: Agree with applicant's version, but would like to add that postimplantation loss and dead foetuses was slightly increased both at 0.003 and 0.010 mg/kg bw/day.
Conclusion	Other conclusions: Agree with applicant's version. In this study, clear developmental toxicity was not observed. However, based on information on analogical compounds it should be classified as Repr. Cat 1; R61 (see Remarks below).
Reliability	1 (but see Remarks below)
Acceptability	Acceptable, but see remarks below

Section A6.8.1 (2) Teratogenicity Study

Annex Point IIA6.8.1

Teratology study of Difenacoum technical in rabbits

Remarks	Based on the similar mode of action and structural similarity (coumarin moiety) to warfarin, it is possible that difenacoum, like warfarin, is teratogenic in humans. The teratogenicity of warfarin is not easily detected in animals because it causes foetal effects only at highly maternotoxic doses. Warfarin has caused malformations in humans that are related to skeletal ossification (coumarin embryopathy, warfarin embryopathy) after exposure during early pregnancy and other malformations after exposure during later pregnancy. The mechanism for skeletal and other abnormalities is through vitamin K dependent carboxylation of proteins in bone matrix and other organs. Some of the malformations may be related to foetal bleeding or other mechanisms, too. Human foetus seem to be very sensitive to slight changes in vitamin K balance because coumarin embryopathy has been observed after exposure to therapeutic dose levels of warfarin. In rodents, maternal bleeding has been overwhelming at teratogenic doses. However, teratogenicity of warfarin was confirmed in rats using a special study design (Howe and Webster 1992). In this study, vitamin K was dosed to dams and neonatal animals to prevent bleeding, but achieve a net extrahepatic vitamin K deficiency. In addition, measurement dimensions of skull and bones and a different dosing schedule was necessary to study skeletal changes similar to those observed in humans. Developmental toxicity of warfarin has not been studied in rabbits, but there is at least one study with coumadin (Hirsh et al., 1970). In this study, stillborn foetuses with widespread subcutaneous haemorrhages were observed after coumadin administration (1-3 mg/kg bw i.m. every second or third day from approximately 1 week after conception until term). The mean maternal protrombin time (activity?) at delivery was 20% of normal level. When coumadin administration was given up to caesarean sections or stopped 4-5 days before delivery, all foetuses were alive and without haemorrhages. Other foetal damages were not evaluated and reported, but apparently gross malformations were not observed. However, based on the results of this study, it can be evaluated that doses causing 10-40 % of normal maternal prothrombin time (activity?), did not reveal any gross malformations in rabbits. There is no data for evaluating whether the conventional or modified OECD 414 study design would have revealed the teratogenicity of warfarin, but based on the Hirsh et al. study (1970), it seems unlikely at doses that causes significant changes in maternal prothrombin time. In line with this, the difenacoum doses used may be too low to reveal its possible teratogenicity through vitamin K deficiency. It is obvious that possible teratogenic effects of coumarin related compounds (coumarin embryopathy) are not easily detected using conventional OECD 414 approach without a modified study design. Hirsh J, Cade JF, Gallus AS (1970): Fetal effects of coumadin administered during pregnancy. Blood 36:623-627. Howe AM and Webster WS (1992): The warfarin embryopathy: a rat model showing maxillonasal hypoplasia and other skeletal disturbances. Teratology 46: 379-390. Key study
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*

Section A6.8.1 (2) Teratogenicity Study

Annex Point IIA6.8.1

Teratology study of Difenacoum technical in rabbits

Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6_8-1. Table for Teratogenic effects (separate data for all dosage groups)

Maternal effects

Parameter	**control data**		**low dose**	**medium dose**	**high dose**	**dose-response + / -**
	historical	study				
Number of dams examined		22	22	22	22	
Clinical findings and autopsy findings(%)						
Reduced activity		0	5	14	0	
Pale mucous membranes		0	0	5	0	
Haemorrhage around the rectum and vagina		0	0	9	5	
Pathological alterations related to internal haemorrhages a)		0	7	5	17	
Mortality of dams *state %*		0	5	5	0	
Abortions		0	0	0	0	
Body weight gain						
1-7		291.4	162.1	200.9	185.9	
7-14		129.9	190.6	136.7	158.8	
14-21		141.6	125.5	169.4	148.8	
21-29		159.3	134.1	135.6	143.6	
Food consumption (g/animal/day)						
1-7		178.3	170.8	185.1	178.2	
7-14		182.8	171.2	172.0	177.3	
14-21		174.8	172.2	172.6	176.3	
21-29		137.9	128.4	132.3	135.6	
Water consumption *if test substance is applied with drinking water*		NA	NA	NA	NA	
Pregnancies *pregnancy rate or %*		91	68	95	77	

a) added by RMS

Section A6.8.1 (2) Teratogenicity Study

Annex Point IIA6.8.1

Teratology study of Difenacoum technical in rabbits

Table A6_8-2. Table for Teratogenic effects (separate data for all dosage groups)

Litter response (Caesarean section data)

Parameter	**control data**		**low dose**	**medium dose**	**High dose**	**dose-response + / -**
	historical	study				
Corpora lutea *state total/number of dams*		196/18	157/14	197/18	166/16	
Implantations *state total/number of dams*		173/18	142/14	182/18	158/16	
Resorptions *state total/number of dams*		23/18	15/14	15/18	9/16	-
total number of fetuses		165	134	170	143	
pre-implantation loss *state %*		12	10	8	5	
post-implantation loss *state %*		5	6	7	9	
total number of litters		18	14	18	16	
fetuses / litter		166/18	134/14	174/18	146/16	
live fetuses / litter *state ratio*		165/18	134/14	170/18	143/16	
dead fetuses / litter *state ratio*		1/18	0/14	4/18	3/16	
fetus weight (mean) *[g]*		38.2	37.4	39.2	39.2	
placenta weight (mean) *[g]*		6.9	6.7	7.1	6.8	
crown-rump length (mean) *[mm]*		9.6	9.5	9.7	9.7	
Fetal sex ratio *[state ratio m/f]*		77/88	70/64	75/95	72/71	

Section A6.8.1 (2) Teratogenicity Study

Annex Point IIA6.8.1

Teratology study of Difenacoum technical in rabbits

Table A6_8-3. Table for Teratogenic effects (separate data for all dosage groups)

Examination of the fetuses

Parameter	control data		low dose	medium dose	High dose	dose-response + / -
	historical	study				
External malformations* *[%]*		1	0	0	0	
External anomalies* *[%]*		5	4	6	6	
Skeletal malformations* *[%]*		1	0	0	0	
Skeletal anomalies* *[%]*		6	11	7	11	
Skeletal variants* *[%]*		5	11	7	11	
Visceral malformations* *[%]*		0	0	0	0	
Visceral anomalies* *[%]*		5	4	4	6	
Variants visceral* *[%]*		5	4	4	6	

Table added by RMS:

Table A6_8-4. Summary of blood clotting

Parameter	Control	0.010 mg/kg bw/day
PTT (sec)	16.58	22.43**
PT (sec)	7.87	10.10**

** $p<0.01$, Mann-Whitney U test

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX, (2004) Two generation reproduction toxicity study of test item Difenacoum, XXXXX, Study code: 03/738-202P	
1.2	**Data protection**	Activa / PelGar brodifacoum and difenacoum Task Force	
1.2.1	Data owner	PelGar International Ltd. Activa srl	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes, OECD 416	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.2	**Description**	Greyish white powder	
3.3	**Purity**	99.7%	
3.4	**Stability**	Stable	
3.5	**Test Animals**		
3.5.1	Species	Rat	
3.5.2	Strain	CRL: (WI)BR	
3.5.3	Source	Charles River (Europe) Laboratories Inc	
3.5.4	Sex	Males & Females	
3.5.5	Age/weight at study initiation	Age: Males 6 weeks Females 8 weeks Weight: Males (mean) 223-227 Females (mean) 229-231	
3.5.6	Number of animals per group	25/sex/group	
3.5.7	Mating	*See table below*	
3.5.8	Duration of mating	12 days	
3.5.9	Deviations from standard protocol	Body weight and food consumption of dams was weighed on gestational days 1, 8, 15, 21 and 29 (F1) and 1, 8, 15 and 21 (F2). This deviation does not affect the integrity of the study.	
3.5.10	Control animals	Yes	
3.6	**Administration/ Exposure**	Oral	

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

3.6.1	Animal assignment to dosage groups	*See table below*
3.6.2	Duration of exposure before mating	70 days
3.6.3	Duration of exposure in general P, F1, F2 males, females	**P generation** Males: 82 days Females:134 days **F_1 generation** Males: 98 days Females: 143 days
		Oral
3.6.4	Type	Gavage
3.6.5	Concentration	Gavage Start of the study: 0, 20, 40 and 80µg/kg/day reduced to 0, 10, 20 µg/kg at the completion of the study
3.6.6	Vehicle	Distilled water with ethanol
3.6.7	Concentration in vehicle	Difenacoum technical was administered in concentrations of 4µg/ml, 8 µg/ml and 16µg/ml prepared in distilled water with alcohol for 17 days. After dose reduction 4, 8 and 12µg/ml concentrations was used. After second dose reduction (day 40), 2 µg/ml and 4µg/ml concentrations were applied.
3.6.8	Total volume applied	5ml/1000g
3.6.9	Controls	Vehicle
3.7	**Examinations**	
3.7.1	Clinical signs	Yes, daily
3.7.2	Body weight	Yes, Males: P: once a week prior to and during the mating F1: postnatal days 1, 5, 8, 15, 22, 29 and weekly thereafter Females: P: once a week prior to and during the mating, gestation days 1, 8, 15, and 21, postnatal days 1, 8, 15, 22 and 29 F1: postnatal days 1, 5, 8, 15, 22 29 and once a week prior to and during the mating, gestation days 1, 8, 15, 21 and postpartal days 1, 5, 8, 15 and 22
3.7.3	Food consumption	Weighed once a week before mating period. Gestation period; 1-8, 8-15, 15-21 Postpartal period: 1-8, 8-15, 15-22 (P and F1 gen) and 22-29 (P gen)
3.7.4	Oestrus cycle	Daily during the premating period (2 weeks) and mating period

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

3.7.5	Sperm parameters	testis weight
		epididymis weight
		sperm count
		sperm motility
		sperm morphology
3.7.6	Offspring	number and sex of pups
		stillbirths
		live births
		presence of gross anomalies
		weight gain
		physical or behavioural abnormalities
3.7.7	Organ weights P and F1	Uterus
		ovaries
		testes
		epididymides
		prostate
		seminal vesicles with coagulation glands
		brain
		liver
		kidneys
		spleen
		pituitary
		thyroid
		adrenal glands
		One random selected pup/sex/litter both in F1 and F2 generation
		Barain, spleen thymus
3.7.8	Histopathology P and F1	Vagina
		Uterus with cervix
		ovaries
		testis
		epididymis
		seminal vesicle
		prostate
		coagulating gland
		liver
		kidney
		organs showing abnormalities
		all organ of animals which died or were in moribund condition

3.7.9	Histopathology F1 not selected for mating, F2	One pup/sex/litter Thymus Spleen Kidneys Liver Orgnas with undiagnosed alterations
3.8	**Further remarks**	
		4 RESULTS AND DISCUSSION
4.1	**Effects**	
4.1.1	Parent males	Mortality 20 (40) µg/kg/day – eight male animals and 60 (80) µg/kg/day - 25 male Clinical symptoms: bleeding from nose, mouth, eye, ear, head, swollen cheek and ear, sanguineous urine, sanguine eye, exophthalamos, haemotma, cyanotic scrotum, abnormal body position, paralytic hindlimbs, decreased activity, decreased reflexes, decreased body tone, piloerection paleness, hunched back, dyspnoea.
4.1.2	Parent females	60 (80) µg/kg/day – 3 females animals The number of animals with irregular cycles increased dose dependently as compared to the control. There was no test item related alteration in the delivery data of dams as compared to the control value.
4.1.3	F1 males	Clinical symptoms related to the test item effect were not found in the pre-weaning period. Three males from dose group 20µg/kg/day died during the post-weaning period.
4.1.4	F1 females	No females died. No difference in the body weight gain in the testing period. Food consumption was not affected.
4.1.5	F2 males	There was no significant effect on the mortalilty rate linked to the test item.
4.1.6	F2 females	Slightly increased brain weight was observed in female pups of 20µg/kg/day compared to the control.
4.2	**Other**	
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	The study was conducted according to OECD guidelines 416. Twenty five CRL: (WI)BR rats/sex/group were involved in the study. The dose levels initially were (20, 40 and 80µg/kg) but were reduced because of the death of animals in 80µg/kg and 40µ/kg. 80 was reduced to 60µg/kg on treatment day 20. The 20 and 40µg/kg were halved on treatment day 40, because of the death of all male animals dosed with 60µg/kg of Difenacoum Technical. The study was completed at dose levels of 0, 10µg/kg, and 20µg/kg. Treatment was carried out orally in a 5ml/1000g body weight volume once a day. All animals of the P generation and animals of the F1 generation selected for mating were dosed at least for 10 weeks prior to mating, thoughout 2 weeks mating, gestation and lactation periods. Treatment of F1 animals started after weaning, on postnatal day 29. Observations included mortality, clinical symptoms, body weight, food consumption, gross necropsy, organ weight, sperm analysis, and

Section A6.8.2	Multigeneration Reproduction Toxicity Study	
Annex Point IIA6.8.2	*Two generation reproduction toxicity study of Difenacoum technical in Rats*	
	histopathological examinations. The dams were allowed to litter, and rear their youngs up to weaning on day 29 postpartum. Development tests were evaluated on litters (surface righting reflex, suckling, pinna detachment, eye opening, testicular descent, vaginal opening, water maze performance). On postnatal day 29, 1-3 males and 1-3 females per litter were selected for subsequent evaluation of reproductive performance following pairing in F1 generation and the observation of the F2 generation up to postnatal day 22. One randomly selected pup/sex/litter from both the F1 and F2 generations were subjected to gross pathology, histopathology, and organ weights of brain, thymus and spleen.	
5.2 Results and discussion	Difenacoum technical caused death in CRL: (WI) BR rats in doses of 20 (F1 generation), 20(40)µg/kg and 60(80)µg/kg (P generation). The clinical symptoms observed before the death were consistent with the known anticoagulant activity of the test material. Bleeding from nose, mouth, eye, head, swollen cheek and ear, sanguineous urine, sanguine eye, exophthalmos, haematoma, cyanotic scrotum, abnormal body position, paralytic hindlimbs, decreased activity, decreased reflexes, decreased body tone, piloerection, paleness, hunched back, dyspnoea were observed. The number of animals with irregular cycles increased dose dependently when compared to the control and the number of cycles was slightly less in the 20µg/kg group than in the control in the P generation. Sperm analysis showed a decrease in the total sperm count in the 20µg/kg of P generation, which could be related to the test item.	X
5.3 Conclusion	Difenacoum technical induced adverse parental effects at 20µg/kg dose levels in the rats. This included clinical symptoms, death caused by general haemorrhagic diathesis. Male animals showed to be more sensitive than female animals in the P generation. Reproductive performance of males and females was unaffected by treatment with Difenacoum technical. There was no effect on postnatal development of pups either in F1 or F2 generations. At 10µg/kg dose level no adverse effect was observed in the P, F1 and F2 generations.	X
5.3.1 LO(A)EL		
5.3.1.1 Parent males	20µg/kg/day	
5.3.1.2 Parent females	20 µg/kg/day	
5.3.2 NO(A)EL		
5.3.2.1 Parent males	10µg/kg/day	
5.3.2.2 Parent females	10µg/kg/day	
5.3.2.3 Reproductive performance of males	20µg/kg/day	
5.3.2.4 Reproductive performance of females	20µg/kg/day	
5.3.2.5 Developmental toxicity	20µg/kg/day	
5.3.3 Reliability	1	
5.3.4 Deficiencies	No	

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 19th, 2006, revised 7 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version, but would like to add that in histopathology ovarian cysts were found at 0.06 mg/kg bw/day further indicating some (hormonal?) disturbances in females.
Conclusion	Agree with applicant's version, but would like to add that the changes in oestrous cycles and sperm counts that may be treatment-related. **There was no NOEL-value** because of changes of oestrous cycles at 0.010 and 0.020 mg/kg bw/day in both generations. Liver weight was decreased also at both dose levels in F1-females. In female pups thymus weight slightly increased. Postimplantation loss was very high in all groups in F0-generation and slightly increased in dosed groups in F1-generation. The was no NOEL
Reliability	1
Acceptability	Acceptable (see Remarks)

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

Remarks	Additional Tables are included by RMS (Tables A6_8_2-3, A6_8_2-4, A6_8_2-5). NOAEL for parent females was 0.010 mg/kg bw/day due to death of one F0-dam and two F1-dams. Otherwise there was no clear maternal toxicity at 0.020 mg/kg bw/day. The results of this study do not indicate clear effects on fertility although there were signs of disturbed oestrous cycles in both generations, slightly decreased sperm count in F0 males, slightly prolonged precoital period in F0 generation and slightly increased postimplantation loss in F1 generation. Based on the warfarin data (and other coumarin compounds), anticoagulants may increase the risk of abortions and early pregnancy loss in humans and animals. These effects may be due to embryonic/foetal death or effects on placenta and may be related to bleeding, vitamin K deficiency or other (unknown) mechanisms. The possible effects of warfarin on oestrous cycles or fertility have not been studied or reported in animals. The rat model is not considered suitable to study long-term effect of anticoagulants, because of the lethality due to bleeding at low dose levels that may hide other effects that may be relevant for humans. This seems to be the case in developmental toxicity. Therapeutic doses of warfarin have caused foetal malformations in humans but in animals, malformations were observed at lethal doses only. Difenacoum did not decrease fertility in rats in this study but it may have endocrinological effects as indicated, e.g., by disturbed oestrous cycles and ovarian cysts. A single sublethal dose of flocumafen enhanced follicular atresia, affected lipid metabolism in ovary and caused transient infertility (Sangha et al., 1992). Thus, it may be possible that also other coumarin compounds have similar effects than flocumafen although there may be differences in potency. Because difenacoum did not have clear effect on fertility and there is no warfarin data indicating decreased fertility, classification for fertility was not considered necessary for difenacoum. However, **R48/23/24/25 is proposed**, for one thing, because of disturbed ovarian function as indicated by disturbed oestrous cycle at doses 0.010 and 0.020 mg/kg bw/day and ovarian cysts at 0.060 mg/kg bw/day. Sangha GK, Bilaspuri GS and Guraya SS (1992): Effects of oral administration of rodenticide flocoumafen on the rat ovary. Pesticide Biochemistry and physiology 44: 15-20. Key study
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

Table A6_8_2-1. Table for animal assignment for mating

		Number of animals			
		Controls	**Low Dose, 10 (20)µg/kg**	**Medium Dose, 20 (40)µg/kg**	**High Dose, 60 (80)µg/kg**
Parents	**m**	25	25	25	25
	f	25	25	25	25
F_1	**m**	30	30	30	-
	f	30	30	30	-

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

Table A6_8_2-2. Table for reproductive toxicity study

Parameter			control		low dose		medium dose		High dose			
		Generation	m	f	m	f	m	f	m	f	m	f
Mortality	incidence	**P**	0/25	1/25	0/25	0/25	8/25	1/25	25/25	3/25		
		F_1	0/30	0/30	0/30	0/30	0/30	2/30				
Food consumption, % of control	Premating period	**P**	-	-	96	90	97	110				
		F_1	-	-	98	98	101	96				
	Gestation period	**P**	-	-	-	91	-	102				
		F_1	-	-	-	98	-	103				
	Lactaction period	**P**	-	-	-	104	-	100				
		F_1	-	-	-	105	-	103				
Body weight gain, % of control	Premating period	**P**	-	-	94	90	94	110				
		F_1	-	-	96	104	101	104				
	Gestation period	**P**	-	-	-	98	-	99				
		F_1	-	-	-	95	-	102				
	Lactation period	**P**	-	-	-	85	-	99				
		F_1	-	-	-	103	-	12				
Clinical Observations	Incidence (%)											
Decreased activity		**P**	-	4	-	-	12	4	64	12		
Decreased reflexes			-	-	-	-	-	-	24	-		
Decreased body tone			-	-	-	-	-	-	12	-		
Piloerection			-	4	-	-	8	4	48	12		
Paleness			-	4	-	-	12	4	32	4		
Hunched back			-	-	-	4	16	-	48	12		
Dyspnoea			-	-	-	-	12	-	36	4		
Bleeding			-	-	-	-	20	4	68	4		
Swollen cheek/ear			-	-	8	-	8	-	16	-		
Organ weights	% of control	**P**										
Liver			-	-	92	94	96	105				
Spleen			-	-	93	89	89	93				
		F1										
Testes			-	-	106	-	111	-				
Liver			-	-	-	93	-	92				
Uterus			-	-	-	104	-	119				

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two generation reproduction toxicity study of Difenacoum technical in Rats*

Histopathologic examination General haemorrhagic diathesis	Incidence	**P**	-	-	-	-	32	-	100	-		
Ovaries, cysts			-	8	-	-	-	4	-	44		
Reproductive Performance												
Mating index		**P**	92	-	96	-	90	-				
		F1	83	-	87	-	86	-				
Fertility index		**P**	-	78	-	71	-	94				
		F1	-	88	-	88	-	96				
Number of implantation	Mean	**P**	-	251	-	216	-	264				
		F1	-	359	-	353	-	358				
Duration of pregnancy	Mean	**P**	-	22.3	-	22.3	-	22.5				
		F1	-	22.4	-	22.5	-	22.4				
Live birth index		**P**	-	100	-	100	-	100				
		F1	-	100	-	100	-	100				
Gestation index		**P**	-	78	-	82	-	76				
		F1	-	100	-	100	-	100				
Litter size	Mean	**P**	-	10.6	-	11.8	-	11.3				
		F1	-	14.9	-	13.2	-	13.0				
Litter weight	Mean	**F1**	-	7.2	-	7.1	-	7.3				
Viability index		**F1**	-	99	-	100	-	96				
		F2	-	97	-	97	-	97				
Lactation index		**P**	-	98	-	100	-	96				
		F1	-	96	-	97	-	97				
Sperm characterization												
Number, 10^6/g	% of control	**P**	-	-	-	-	83	-				
		F1	-	-	-	-	101	-				
Deformations, immotile	% of control	**P**	-	-	-	-	102	-				
		F1	-	-	-	-	94	-				

Tables prepared by RMS:

Table A6_8_2-3. Two-generation reproductive toxicity study, F0-generation

Parameter	Control	10 µg/kg	20 µg/kg	60 µg/kg
F0-generation (M/F)	25/25	25/25	25/25	25/25
Mortality				
- males	0	0	8/25 (32%)	25/25 (100%)
- females	1/25 (4%)	0	1/25 (4%)	3/25 (12%)
Body weight gain				
- gestation (days 1-21)	112.5	110.8	111.7	-
- lactation (days 1-29)	-22.9	-19.6	-22.6	-
Clinical signs (incidence, M/F)				
- bleeding	0/0	0	20%/4%	68%/4%
- other signs	0/< 4%	<8%<4%	<16%/<4%	<64%/<12%
Organ weights				
-Spleen, males (g)	0.91	0.85	**0.81***	-
-Spleen, relative to bw, males	0.159	0.154	0.148	-
Histopathological changes				
-general haemorrhagic diathesis	0	0	32% of males	100% of males
-ovaries, cysts	2/25 (8%)	0	1/25 (4%)	11/25 (44%)
-lack of corpora lutea	0	0	0	2/25 (8%)
Estrous cycle				
-number of cycles	2.5	2.2	**1.9**	-
-days in proestrus	1.5	1.4	1.5	-
-days in estrus	2.5	**4.1***	**3.9***	-
-days in diestrus	6.3	**3.4***	**4.3***	-
-animals in prolonged estrus	2/25 (8%)	6/25 (24%)	4/25 (16%)	-
-animals in prolonged diestrus	5/25 (20%)	1/25 (4%)	3/25 (12%)	-
-animals with irregular cycles	6/25 (24%)	9/25 (36%)	14/25 (56%)	-
Sperm analysis				
- number of cells x 10^6/g	81.2	-	67.4	-
- deformations (%)	1.00	-	1.20	-
Precoital days	2.2	2.9	3.3	-
Mating index a)	92%	96%	96%	-
Copulatory index b)	92%	96%	90%	-
Fertility index c) (M/F)	78%/78%	71%/71%	94%/88%	-
Pregnant, not delivered	4/18 (22%)	3/17 (18%)	5/21 (24%)	-
Gestation index d)	78%	82%	76%	-
No. of implantation sites	13.9	12.7	12.6	-
Duration of pregnancy	22.3	22.3	22.5	-
Postimplantation loss	**41%**	**24%***	**32%**	-
Females with total resorptions	4	3	5	-
Resorptions	1 (6%)	2 (12%)	1 (5%)	-
Foetal deaths	0	1 (6%)	1 (5%)	-
Live birth index (PND 1)	100	100	100	-
Litter size	10.6	11.8	11.3	-
Pup weight (PND 1)	7.1	7.0	7.1	-
Sex ratio (male/female)	71/77	73/92	93/87	-
Survival index (lactation index)				
- PND 29	98	100	96	-

M/F = Males/females, PND = postnatal day, * = p<0.05

a) Number of females mated/number of females with males

b) Number of males copulated/number of males with females

c) For females: Number of females pregnant/number of females mated,
for males: Number of females pregnant/number of copulated males

d) Number of females with live litter/number of females pregnant

Table A6_8_2-4. Two-generation reproductive toxicity study, F1-generation

Parameter	Control	10 µg/kg	20 µg/kg	60 µg/kg
F1-generation (M/F)	30/30	30/30	30/30	-
Mortality				
- males	0	0	0	-
- females	0	0	2/30	-
Body weight gain				
- gestation (days 1-21)	132.6	125.7	153.0	-
- lactation (days 1-22)	6.0	6.2	-0.7	-
Organ weights				
-Liver, females (g)	16.35	**15.15***	**15.09***	-
-Liver, relative to bw, females	4.661	**4.287****	**4.193****	-
-Liver, relative to brain weight, F	788.98	**718.82****	**716.10****	-
-Uterus (g)	0.48	0.50	**0.57***	-
-Uterus, relative to bw	0.136	0.142	0.159	-
-Uterus, relative to brain weight	23.07	23.61	**27.14***	-
-Testes (g)	3.40	3.62	**3.78****	-
-Testes, relative to bw	0.597	0.657	0.656	-
-Testes, relative to brain weight	151.13	162.24	**168.17****	-
-Spleen, relative to bw, males	0.164	0.155	**0.146***	-
Estrous cycle				
-number of cycles	3.0	2.5	2.6	-
-days in proestrus	0.8	1.0	0.6	-
-days in estrus	3.0	2.4	2.6	-
-days in diestrus	7.1	7.7	8.0	-
-animals in prolonged estrus	0	1/30 (1%)	**1/28 (4%)***	-
-animals in prolonged diestrus	2/30 (7%)	**5/30 (17%)***	**5/28 (18%)***	-
-animals with irregular cycles	3/30 (10%)	**10/30 (33%)****	**6/28 (21%)***	-
Sperm analysis				
- number of cells x 10^6/g	64.2	-	65.1	-
- deformations (%)	1.40	-	1.10	-
Precoital days	5.2	3.8	**3.1***	
Mating index a)	83%	87%	86%	-
Copulatory index b)	83%	87%	86%	-
Fertility index c) (M/F)	88%/88%	88%/88%	**96%*/96%***	-
Pregnant, not delivered	0	0	0	-
Gestation index d)	100%	100%	100%	-
Dams with prolonged pregnancy	0	1	0	
No. of implantation sites	16.3	15.3	15.6	-
Duration of pregnancy	22.4	22.5	22.4	-
Postimplantation loss	9%	14%	16%	-
Live birth index (PND 1)	100	100	100	-
Litter size	14.9	13.2	13.0	-
Pup weight (PND 1)	7.2	7.1	7.3	-
Sex ratio (male/female)	151/176	144/160	137/160	-
Survival index (lactation index)				
- PND 29	96	97	97	-

M/F = Males/females, PND = postnatal day. * $p<0.05$, ** $p<0.01$

a) Number of females mated/number of females with males

b) Number of males copulated/number of males with females

c) For females: Number of females pregnant/number of females mated,
for males: Number of females pregnant/number of copulated males

d) Number of females with live litter/number of females pregnant

Table A6_8_2-5. Two-generation reproductive toxicity study, findings in pups

Parameter	Control	10 µg/kg	20 µg/kg	60 µg/kg
F1-pups	148	165	180	-
- males	71	73	93	-
- females	77	92	87	
Mortality				
- males	1	0	4	-
- females	2	0	4	
Clinical signs (selected)				
- cold pups	13 (9%)	1 (1%)	27 (15%)	-
- pups with haemorrhage	0	2 (2%)	6 (3%)	-
Organ weights				
- thymus, absolute, females (g)	0.35	0.39	**0.43** *↑(23%)	-
- thymus, relative, females	0.396	**0.474**	**0.461** (↑16%)	-
- thymus, relative to brain, F	21.14	24.21	25.35 (↑20%)	-
Eye opening, PND 15	141/148 (97%)	137/165 (83%)	140/180 (81%)	-
Vaginal opening PND 34	29/30 (97%)	28/30 (93%)	29/30 (97%)	-
Testicular descent PND 30	30/30 (100%)	30/30 (100%)	30/30 (100%)	-
Water maze test (latency sec)	57.0	31.0*	34.0*	-
F2-pups	327	304	299	
Clinical signs (selected)				
-pups with haemorrhage	2 (1%)	3 (1%)	0	
-not suckled	1 (0%)	5 (2%)	3 (1%)	
Organ weights				
- thymus, absolute, females (g)	0.22	0.23	0.25	
-thymus, relative, females	0.469	0.455	0.480	
-thymus, relative to brain, F	15.87	15.95	17.13	
Eye opening, PND 15	252/315 (80%)	230/295 (78%)	240/289 (83%)	-

M/F = Males/females, PND = postnatal day

Section A6.9 Annex Point *IIIA VI.1*	Neurotoxicity study	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	There has been no evidence of neurotoxic effects in any studies conducted. Consideration of the chemical structure does not suggest that it would induce neurotoxic effects, such as an organophosphate. There have been no neurotoxic effects shown by analogues in any species. Hence, conducting a neurotoxicity study would be scientifically unjustified and would not provide any new data. Based on this and animal welfare grounds it is deemed unnecessary to conduct a neurotoxicity study.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	14 June 2006	
Evaluation of applicant's justification	Agree with applicant's justification.	
Conclusion	Study not needed.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.10 Annex Point *IIIA VI.1*	Mechanistic study – any studies necessary to clarify effects seen in toxicity studies	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	The effects of administration of anticoagulants has been extensively investigated and summaries above and reported under metabolism data Please refer to section IIIA 6.2.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	14 June 2006	
Evaluation of applicant's justification	Agree with applicant's justification. The mode of action of difencoum is adequately elucidated and well known.	
Conclusion	Studies not needed.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.11 Annex Point *IIIA VI..7*	Studies on other routes of administration (parental routes)	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	Compound is highly toxic by the oral route to all mammalian species. It is a large, lipophilic molecule, which is poorly absorbed through the skin. It is of very low water solubility and low vapour pressure. The mode of action is common to all mammals and is well understood as a vitamin K antagonist, without secondary effects. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is thought to be the inhibition of K_1 epoxide reductase. The anticoagulants accumulate and are stored in the liver until broken down. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. It is only used as baits for the control of rodents. Manufacturing takes place in closed or controlled environments with full protective clothing and use as a rodenticide necessarily involves wearing gloves, overalls and other protective clothing because of the biological hazards involved and associated hygiene requirements. Data on other routes of administration are considered an unjustifiable waste of experimental animals since the compound is shown to be highly toxic by the oral route and other routes of administration are not relevant to the current and proposed uses of the compound.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	8 June 2006	
Evaluation of applicant's justification	Applicant’s justification is acceptable. Parenteral administration of difenacoum is unlikely to bring any new insight into the mode of action or effects of difencaoum.	
Conclusion	Studies using parenteral administration are not needed.	
Remarks	Skin is the main route of exposure and exposure to small amounts of difenacoum is possible depending on the actual formulation type of the product. However route-to-route extrapolation of effects is considered possible.	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	

Section A6.11 Annex Point *IIIA VI..7*	Studies on other routes of administration (parental routes)
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.1 Annex Point IIA VI.6.9.1	Medical surveillance data on manufacturing plant personnel if available	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	Variation: The following information is based on a letter from Dr Tezza, dated 11 Feb 2005 and supercedes the study summary information submitted in November 2004, which was based on informal communication. There are no medical surveillance records. However, there is a statement from the Italian plant, Tezza Rodenticide production facility, as follows: Only two workers are involved in the synthesis of hydroxycoumarin anticoagulant rodenticides in the facility. Production is on a campaign basis that means the workers only work in this part of the facility for a total of five days each two months. Appropriate protective equipment is used and hence workers do not come into direct contact with hazardous materials. Good manufacturing practice is followed throughout the facility. Production started in 1979 and no chronic or acute medical problems have occurred, that can be associated with this process, during the whole of that period.	X
	The average production of difenacoum is 75kg per year. Workers have a general health screen, which includes specific tests for anticoagulant poisons, every three months. This includes respiratory, blood and urine tests. A specialist occupational health practitioner carries out these tests. The results of the tests are not provided to the manufacturing facility due to doctor/patient confidentiality, the workers are simple declared 'fit for work' or 'not fit for work'.	X
	As is normal in any chemical production process waste products are purified, recycled and re-used. In the particular process used in the production of rodenticide actives, it is solvents that fall into this category. It should be noted that during the synthesis of rodenticide actives, it is only the final stage, when the intermediate is couple with 4-hydroxycourmarin, that the material becomes highly toxic. This final stage is conducted in a sealed vessel and the technical material is then diluted with glycol in the same vessel to avoid the possibility of contact with the technical material.	

Section A6.12.1 Annex Point IIA VI.6.9.1	Medical surveillance data on manufacturing plant personnel if available
	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	2 June 2006
Evaluation of applicant's justification	This general information on health screening of workers involved in manufacture of anticoagulant active substances, including difenacoum, can be considered sufficient. Based on the information it seems that protective equipment, good manufacturing practice and sealed processes guarantee adequate protection of workers form adverse health effects.
Conclusion	
Remarks	The applicant has provided general information on health screening of workers involved in rodenticide production in one facility. Based on the letter, regular testing of workers for anticoagulant effects by respiratory, blood and urine tests has not revealed any adverse effects. Since 1979, no acute or chronic medical problems related to anticoagulants have been reported. Monitoring data is not included. According to section A2.10, worker monitoring started in 1975, and no health problems related to anticoagulants has been observed. There is a discrepancy between information given in this summary compared to section A2.10, where it is stated that since 1995, haematochimical and urine examen began annual.
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.2 (1) Annex Point IIA VI.6.9.2

Direct observation, e.g. clinical cases, poisoning incidents <u>if available</u>

Human – brodifacoum, Bromadiolone, chlorphacinone, diphacinone, pivalyl)

			Official use only
		1 REFERENCE	
1.1	**Reference**	Smolinske SC et al (1987) Long-acting anticoagulant rodenticide ingestion in children, Veterinary and human toxicology, volume 29, number 6, pg 492	
1.2	**Data protection**	No, published paper.	
1.2.1	Data owner	Public domain	
1.2.2	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	The guideline study is not stated in the published paper.	
2.2	**GLP**	The GLP status of the study is not stated in the published paper	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Brodifacoum, Bromadiolone, chlorphacinone, diphacinone, pivalyl	
3.1.1	Lot/Batch number	Batch numbers not stated in the published paper.	
3.1.2	Specification	Not stated in the published paper	
3.1.3	Description	*Not described*	
3.1.4	Purity	Not stated in the published paper	
3.1.5	Stability	A specific statement on stability is not provided within the paper.	
3.1.6	Radio labelling	No	
3.2	**Test Animals**		
3.2.1	Species	Human	
3.2.2	Strain	Yes	
3.2.3	Source	Hospital patients	
3.2.4	Sex	Male and female	
3.2.5	Age/weight at study initiation	Prospective study was done on children, ages are not specified..	
3.2.6	Number of humans	110	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Preparation of test site	accidental	
3.3.2	Concentration of test substance	Not relevant	
3.3.3	Specific activity of test substance	Not relevant	
3.3.4	Volume applied	Not relevant or stated	

Section A6.12.2 (1) **Annex Point IIA VI.6.9.2**	**Direct observation, e.g. clinical cases, poisoning incidents <u>if available</u>** *Human – brodifacoum, Bromadiolone, chlorphacinone, diphacinone, pivalyl)*
3.3.5 Sampling time	Prothrombin times: 24 and 48h
3.3.6 Samples	Blood
	4 RESULTS AND DISCUSSION
4.1 Result of study	At least one Prothrombin time (PT) was obtained for all patients; 26 had ≥ 2 values.
	5 APPLICANT'S SUMMARY AND CONCLUSION
5.1 Materials and methods	A prospectives study was done from August 1986-April 1987 of 110 pediatric cases of accidental ingestion of long-acting anticoagulant rodenticides – namely brodifacoum, Bromadiolone, chlorphacinone, diphacinone and pivalyl. Prothrombin times (PT) were recorded in all patients at 24 and 48 h post ingestion. Atleast one PT value was obtained for all patients; 26 had ≥ 2 values. A PT ratio (patient/control) was calculated.
5.2 Results and discussion	8/110 patients had one or more abnormal PT ratios (1.2-1.44). In 4 cases, the PT raio was normal at 24hr and abnormal at 48h. In 2, the first value obtained was at 48h. In 1, the PT ratio was abnormal at 24h and normal at 48h. The development of an abnormal PT was significantly associated with brodifacoum (7/77 cases). One child vomited spontaneously. One 2 year old child with a normal PT ratio at 48 and 60h had transient abdominal pain, vomiting, and heme and stools. One child with a 24 h PT ratio of 1.08 was diagnosed with acute myelocytic leukaemia 2 months later, a cause-effect relationship was not proven.
5.3 Conclusion	Children who ingest anticoagulant rodenticides, particularly brodifacoum, can develop anticoagulation, which usually does not cause symptoms.
5.3.1 Reliability	2
5.3.2 Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	2 June 2006, revised 6 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version. Only a short abstract of this study is available. This gives general, not substance specific, information only.
Conclusion	Based on the abstract, it can only be concluded that in some cases accidental ingestion (amount unknown) of anticoagulant rodenticides by children causes changes in blood clotting parameters. Sometimes even clinical symptoms are evident. No further conclusions can be made based on this small sample.
Reliability	2

Section A6.12.2 (1) Annex Point IIA VI.6.9.2

Direct observation, e.g. clinical cases, poisoning incidents <u>if available</u>

Human – brodifacoum, Bromadiolone, chlorphacinone, diphacinone, pivalyl)

Acceptability	Acceptable
Remarks	Identification of substances causing poisoning is not described in the abstract. Difencoum is not mentioned in the list of substances under study. The WHO publication, EHC 75, 1995, provides a comprehensive review of poisoning cases around the world. The applicant could have made a reference to that publication also.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.2 (2) Annex Point IIA VI.6.9.2 — Direct observation, e.g. clinical cases, poisoning incidents if available

			Official use only
		1 REFERENCE	
1.1	**Reference**	Barlow AM, Gay AL, Park BK (1982) Difenacoum (Neosorexa) poisoning, British Medical Journal, Vol 285, pg 541	
1.2	**Data protection**	No, published paper.	
1.2.1	Data owner	Public domain	
1.2.2	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	The guideline study is not stated in the published paper.	
2.2	**GLP**	The GLP status of the study is not stated in the published paper	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Difenacoum, Warfarin	
3.1.1	Lot/Batch number	Batch numbers not stated in the published paper.	
3.1.2	Specification	Not stated in the published paper	
3.1.3	Description	*Not described*	
3.1.4	Purity	Not stated in the published paper	
3.1.5	Stability	A specific statement on stability is not provided within the paper.	
3.1.6	Radio labelling	No	
3.2	**Test Animals**		
3.2.1	Species	Case report: Human Animals study: Rabbits	
3.2.2	Strain	New Zealand White	
3.2.3	Source	Not stated	
3.2.4	Sex	Animal study: Male	
3.2.5	Age/weight at study initiation	Animal study: 2.5-3.0 kg	
3.2.6	Number of animals	Not stated	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**		
3.3.1	Preparation of test site	Not stated	
3.3.2	Concentration of test substance	Animal study: 0.85mg/kg	
3.3.3	Specific activity of test substance	Not relevant	
3.3.4	Volume applied	Not relevant or stated	
3.3.5	Sampling time	Prothrombin complex activity	

Section A6.12.2 (2) Annex Point IIA VI.6.9.2 — Direct observation, e.g. clinical cases, poisoning incidents if available

3.3.6	Samples	Blood
		4 RESULTS AND DISCUSSION
4.1	**Result of study**	The duration of anticoagulation produced by Difenacoum in the rabbit, is much longer than that produced by warfarin.
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	Case report: A 17 year old girl made several suicide attempts in 1978. She consumed 550g rat poison and swallowed broken razor blades and pins. Animal study: Single dose of Difenacoum (0.85 mg/kg) was given to male New Zealand white rabbits and the blood clotting activity was measured. For the first 21 dfays vitamin K1 (2mg/kg) was administered intraperitoneally every two days to prevent death from haemorrhage; during this period prothrombin complex activity was determined before the administration fo the vitamin. In the second experiment, the effect of a single intravenous injection of vitamin K1 (0.5 mg/kg) on clotting activity in rabbits anticoagulated with either warfarin (63 mg/kg) or Difenacoum (0.85 mg/kg) was determined.
5.2	**Results and discussion**	Human case report: Coagulation tests gave an initial british corrected ratio of 15. Phytomenadione (Vitamin K1) was given both by mouth and intravenously at internal and the ingested poison was cleared from the system. Animals study: Prothrombin complex activity below 50% was recorded 45 days after a single dose of Difenacoum. Difenacoum is a more effective antagonist of vitamin K1 than warfarin in the rabbit. 18 hours after administration of vitamin k1 prothrombin complex activity was significantly lower ($p<0.001$) in animals pretreated with 0.85 mg Difenacoum/kg than in animals pretreated with 63 mg warfarin/kg .
5.3	**Conclusion**	The clinical effect of ingestion of Difenacoum in man is similar to that observed in animal experiments. Difenacoum is a much more persistent and potent antagonist of vitamin k1 than warfarin. In poisoning cases with rodenticides, vitamin k1 should be administered at frequent intervals until british corrected ratio returns to normal.
5.3.1	Reliability	2
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	2 June 2006, revised 6 February 2007

Section A6.12.2 (2) Annex Point IIA VI.6.9.2 — Direct observation, e.g. clinical cases, poisoning incidents if available

Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version. Both the case report on suicide attempts with difenacoum and the animal experiment prove the role of vitamin K in the action of difenacoum and other anticoagulants. Vitamin K_1 is the antidote in human poisonings.
Reliability	2
Acceptability	Acceptable
Remarks	The data is described in a short report. The WHO publication, EHC 75, 1995, provides a comprehensive review of poisoning cases around the world. The applicant could have made a reference to that publication also.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.3 Human Case Report

Annex Point IIA VI.6.9.3

Health records from industry and other sources.

			Official use only
		1 REFERENCE	
1.1	**Reference**	Davanzo F et al. (2001) Difenacoum: Information about and toxicity of anticoagulant rat poisons: Case Histories from Milan Poisons Centre 1996-1998. l Servizo Di Anestrsia E Rianimazione Centro Antiveleni	
1.1.1	Data owner	Activa srl	
1.3.4	Companies with access to data	PelGar (only for use in Annex I listing of difenacoum)	
2.0	**Guidelines and Quality Assurance**	Not applicable	
		2 MATERIALS AND METHODS	
2.1	**Substance**	Difenacoum (amount cannot be verified from the report).	
2.2	**Persons exposed**		
2.2.1	Sex	Both sexes were exposed but the gender most exposed was male.	
2.2.2	Age/weight	60% of cases were children aged 0 to 4 years old.	
2.2.3	Known Diseases	Not stated	
2.2.4	Number of persons	63 calls made to Milan Poisons Centre regarding clinical cases involving humans and animals with Difenacoum	
2.2.5	Other information		
2.3	**Exposure**	Oral/Inhalation/Dermal The contact routes were (94%) by ingestion, (1%) by inhalation, 5% by other routes.	
5.3.3	Reason of exposure	The circumstances of the intoxication were in most cases accidental (65%) but there were reports of voluntary intoxication (14%). Analysing the details of these circumstances, the most frequent cause was the incapacity to rationalise, i.e. by children in the age range 0-4 years.	
2.3.1	Frequency of exposure	Not stated	
2.3.2	Overall time period of exposure	Not stated	
2.3.3	Duration of single exposure	Not stated	
2.3.4	Exposure concentration/dose	not available	
2.3.5	Other information		
2.4	**Examinations**	The risk assessment at the time of the telephone consultation is based on the active ingredient, on the case history data of the human or animal involved, on the contact mode, on the circumstances and on the symptoms found.	

Section A6.12.3 Human Case Report

Annex Point IIA VI.6.9.3 Health records from industry and other sources.

2.5	**Treatment**	In attempted suicides which showed altered coagulation, vitamin K therapy over several weeks was found beneficial. Therapy is based on the prevention of absorption. If a few grains of bait have been ingested it is sufficient to adminsister powdered activated charcoal. If more significant quantities have been ingested, it is useful to administer ipecac syrup as an emetic. Gastric Lavage Gastric lavage consists of the removal of gastric contents using a probe which is either swallowed by the patient or delieved to the stomach passing through the nasal channels. It is useful in the ingestion of significant quantities of product, especially with attempted suicide in adults.
2.6	**Remarks**	
		3 RESULTS
3.1	**Clinical Signs**	Coagulopathy develops after intoxication by difenacoum. Haemorrhaging is the most common symptom of intoxication and may appear some time after exposure. Neurological effects Intracranial haemorrhage with cephalea Reduced state of awareness Convulsions Coma followed by death (Ornstein & al 1999) Gastroneteric symptomatlogy abdominal pains spontaneous vomitting gastroenteric bleeding with melanotic or haemorrhagic stools Genitoutinary symptomatology haematuria vaginal bleeding
3.2	**Results of examinations**	Not stated
3.3	**Effectivity of medical treatment**	
3.4	**Outcome**	
3.5	**Other**	
		4 APPLICANT'S SUMMARY AND CONCLUSION
4.1	**Materials and methods**	The paper presents the experience of the anticoagulant intoxication requiring consulting from the Milan Poisons Centre from the Milan Poison Centre from 1996 to 1998.
4.2	**Results and discussion**	80 calls regarding difenacoum were received between 1996 to 1998. The gender most affected was male.

Section A6.12.3 Human Case Report

Annex Point IIA VI.6.9.3

Health records from industry and other sources.

4.3 Conclusion	Clinical effects appear when there is a massive overdose with rapid and persistent diminution of the prothrombin activity associated or otherwise with the presence of haemorrhagic diathesis. Children in the age range of 0-4 years were the most likely to be intoxicated. Monitoring of INR or the prothrombin time must be effected at 24-48 hours after ingestion, in asymptomatic children. Treatment will involve the prevention of absorption but the method of prevention will depend on the amount ingested and how long after ingestion treatment is being received. Emesis is more efficient the earlier it is effected. Vitamin K1 phytonadione, is the antidote of choice which must be administered on to patients with extended PT or INR and must be administered via intramuscular injection if ingestion is of medium entity.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	2 June 2006, revised 6 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version. The summary reflects the information provided in the reference document.
Conclusion	This is an interesting and useful piece of information which shows that both suicide attempts and accidental poisonings to rodenticides do happen. Among those cases recorded by the poison control centre, symptoms from accidental ingestion were mostly mild ones.
Remarks	This document provides information on difenacoum intoxications (symptoms, incidence, age and sex distribution of affected persons etc) recorded in an Italian poison control centre between 1996 and 1998. It also describes the medical advice practices provided by the centre.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.4 Annex Point IIA VI.6.9.4	Epidemiological studies on the general population, if available	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification:	No data available	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	2 June 2006	
Evaluation of applicant's justification	Applicant's justification is acceptable.	
Conclusion	No data available.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.12.5 Annex Point IIA VI.6.9.5 — Diagnosis of poisoning including specific signs of poisoning and clinical tests, if available.

			Official use only
		1 REFERENCE	
1.1	**Reference**	Park BK et al (1986) Abnormal vitamin k metabolism in the presence of normal clotting factor activity in factory workers exposed to 4-hydroxymcoumarins, British Journal of Clinical pharmacology, vol 21, pg 289-294	
1.2	**Data protection**	No, published paper.	
1.2.1	Data owner	Public domain	
1.2.2	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	The guideline study is not stated in the published paper.	
2.2	**GLP**	The GLP status of the study is not stated in the published paper	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Difenacoum and brodifacoum	
3.1.1	Lot/Batch number	Batch numbers not stated in the published paper.	
3.1.2	Specification	Not stated in the published paper	
3.1.3	Description	*Not described*	
3.1.4	Purity	Not stated in the published paper	
3.1.5	Stability	A specific statement on stability is not provided within the paper.	
3.1.6	Radio labelling	No	
3.2	**Test Animals**		
3.2.1	Species	Human	
3.2.2	Strain	Yes	
3.2.3	Source	Factory workers	
3.2.4	Sex	Male	
3.2.5	Age/weight at study initiation	Aged 26 & 28	
3.2.6	Number of animals	2	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**		
3.3.1	Preparation of test site	Not relevant	
3.3.2	Concentration of test substance	Not relevant	
3.3.3	Specific activity of test substance	Not relevant	
3.3.4	Volume applied	Not relevant or stated	
3.3.5	Sampling time	0.5, 1, 2 , 4, 8 hr	

3.3.6 Samples

Activity of clotting factors, Blood

4 RESULTS AND DISCUSSION

4.1 Result of study

The activities of the individual clotting factors are expressed as a percentage of the normal value obtained using pooled serum. The normal range for the factors are: II (60-167%), VII (58-162%), IX (56-175%), X (58-124%)

	II	VII	IX	X
Case 1				
Pre	100	115	76	98
0.5h	100	98	74	100
1.0	100	110	69	102
2.0	92	145	76	105
4.0	100	150	72	102
8.0	96	135	79	102
Case 2				
Pre	90	135	69	115
0.5h	106	145	67	120
1.0	130	135	84	125
2.0	130	160	83	120
4.0	105	160	117	120
8.0	106	145	90	115

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Case 1

The prothrombin time was routinely examined in a 26 year old Caucasian male who work in the manufacture of coumarin anticoagulants. After intermittent abnormal results, oral vitamin K1 was given. Even after daily vitamin k1 intake, his prothrombin time was abnormal, and hence subsequently stopped.

Case 2

28 year old Caucasian male working with coumarin anticoagulants was admitted to hospital with spontaneous bruising and haematuria, prothrombin time of 100 sec and partial thromboplastin time of 80 secs. Platelet count, thrombin time and fibrinogen levels were normal. Fresh frozen plasma and vitamin k1 was given and was discharged from hospital.Vitamin K_1 metabolism was studied twice after the prothrombin time had been normal for three years

5.2 Results and discussion

Case 1

Even after daily vitamin k1 intake, his prothrombin time was abnormal, and hence subsequently stopped. He developed haematuria, bleeding gums and easy bruising was apparent.

He was admitted into a hospital, and it was revealed that Difenacoum at 60ng/ml was present in the plasma, a prothrombin time of 53 sec (control 12.5sec), a partial thromboplastin time of 88 sec (control 25 sec), normal platelet count, thrombin time and fibrinogen level.

He was given fresh frozen plasma, oral vitamin K1 and consequently discharged from the hospital. Vitamin K1 was still given for the prothrombin time to remain normal. The prothrombin time became elevated (30-50 ng/ml of difenaoucm and brodifacoum in plasma) and

Section A6.12.5 Annex Point IIA VI.6.9.5 — Diagnosis of poisoning including specific signs of poisoning and clinical tests, if available.

	decided to leave work and had no further industrial contact with 4-hydroxycoumarims. The determine the biochemical effect of coumarin exposure, vitamin K1 and vitamin K1 2,3-epoxide in plasma after intravenous administration of 10 mg of the vitamin on several occasions. Concentrations of vitamin K1 2,3-epoxide (330-500 ng/ml) were measured from 2-24 hr after vitamin K1 administration. The intravenous vitamin K1 administration reduced prothrombin time from 66 to 30 sec within 12 hours. Case 2 Vitamin K_1 metabolism was studied twice in this individual after his prothrombin time had been normal for 3 years.Vitamin K1 2,3-epoxide was detected in plasma (100-200 ng/ml) after intravenous administration of vitamin k1 (10mg). Vitamin k1-dependent clotting factor activities were in the normal range.
5.3 Conclusion	Investigation of vitamin k metabolism may be a more sensitive assessment of industrial coumarin exposure than monitoring prothrombin time.
5.3.1 Reliability	2
5.3.2 Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	8 June 2006, revised 6 February 2007
Materials and Methods	This study summary template is not best suited to describe this kind of data. The methods of the study (parameters determined) shall be described more precisely under materials and methods. Please, use the study summary template for Human Case Reports.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's conclusion. Effects on vitamin K metabolism may be more sensitive to coumarin effects than prothrombin time. However, routine diagnostics and monitoring are based on the latter so far.
Reliability	2
Acceptability	Acceptable
Remarks	This paper describes two poisoning cases within occupational settings which have been investigated more thoroughly for vitamin K pharmacokinetics and the activity of individual clotting factor activities etc. The study provides interesting information on the effects of anticoagulants. In order to get a more comprehensive picture of clinical signs of poisoning and details of clinical tests useful for diagnostic purposes, appropriate information in the International Programme on Chemical Safety, Environmental Health Criteria, Anticoagulant Rodenticides, WHO report 1995, shall be described under this section point also.

Section A6.12.5 Annex Point IIA VI.6.9.5 — Diagnosis of poisoning including specific signs of poisoning and clinical tests, if available.

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.6 Annex Point IIA VI.6.9.6	Sensitisation/allergenicity observations, if available	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X] **Limited exposure [X]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [ ]**	
Detailed justification:	No evidence of allergenic effect seen in man or animals in commercial and experimental use by difenacoum or analogues. High toxicity and hygiene means that gloves must be worn at all times during rodent control operations.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	2 June 2006	
Evaluation of applicant's justification	Applicant's justification is acceptable.	
Conclusion	No data available.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.12.7

Annex Point IIA6.12.7

Specific treatment in case of an accident or poisoning: first aid measures, antidotes and medical treatment, if known.

			Official use only
		1 REFERENCE	
1.1	**Reference**	International Programme on Chemical Safety, Environmental Health Criteria, Anticoagulant Rodenticides, WHO report 1995.	
		2 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)	
		3 MATERIALS AND METHODS	
3.1	**Substance**	Difenacoum	
3.2	**Persons exposed**	N/A	
3.2.1	Sex	Not stated	
3.2.2	Age/weight	Not stated	
3.2.3	Known Diseases	Not stated	
3.2.4	Number of persons	Generic treatment	
3.2.5	Other information		
3.3	**Exposure**	Not known	
5.3.4	Reason of exposure	N/A	
3.3.1	Frequency of exposure	N/A	
3.3.2	Overall time period of exposure	N/A	
3.3.3	Duration of single exposure	N/A	
3.3.4	Exposure concentration/dose	N/A	
3.3.5	Other information		
3.4	**Examinations**	Not stated	

Section A6.12.7

Annex Point IIA6.12.7

Specific treatment in case of an accident or poisoning: first aid measures, antidotes and medical treatment, if known.

3.5 Treatment	All poisoned patients should immediately receive medical attention. Prothrombing time and evidence for bleeding should be determined and monitored for several weeks. Gastric lavage or emesis induction should be performed if it a recent incident or is possibly lethal. Repeated administration of activated charcoal may be useful. Cathartics can also be administered. The specific antidote isVitamin K1. If the patient is severely bleeding, 25mg of vitamin K1 (phytomenadione) should be given by slow intraveneous injection. Prothrombin time should be checked at 3 hours intervals in severe cases or 8-10hrs in less severe cases. Vitamin K1 should be given repeatedly if there are no improvement in the condition. Dose of up to 125-200 mg/kg have been given without any adverse effects. After initial parenteral vitamin k1 administration, oral treatment can be continued for a prolonged period of time. Additionally, it is also prudent to monitor prothrombin time after cessation of treatment to ensure that there is no regression. Whole blood, fresh frozen plasma and fresh blood can be given in acute severe bleeding to restore the blood factors, or factor concentrate may considered if the amount of plasma is too great
3.6 Remarks	
	4 RESULTS
4.1 Clinical Signs	*Not stated*
4.2 Results of examinations	*Not stated*
4.3 Effectivity of medical treatment	*Not stated.*
4.4 Outcome	*Not stated*
4.5 Other	*Not stated*
	5 APPLICANT'S SUMMARY AND CONCLUSION
5.1 Materials and methods	*See above*
5.2 Results and discussion	*See above.*
5.3 Conclusion	The information given is a clear indication of the kind of treatment needed following anticoagulant poisoning.

Section A6.12.7

Annex Point IIA6.12.7

Specific treatment in case of an accident or poisoning: first aid measures, antidotes and medical treatment, if known.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	8 June 2006, revised 6 February 2007
Materials and Methods	
Results and discussion	
Conclusion	
Remarks	Recommended treatment of poisoning cases is described as stated in the reference.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.8 Prognosis following poisoning

Annex Point IIA6.12.8

		Official use only
	1 REFERENCE	
1.1 Reference	International Programme on Chemical Safety, Environmental Health Criteria, Anticoagulant Rodenticides, WHO report 1995.	
	2 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)	
	3 MATERIALS AND METHODS	
3.1 Substance	This sunnary is a review of 3 cases of Difenacoum poisoning.	
3.2 Persons exposed		
3.2.1 Sex	Case 1: Not stated Case 2: Male Case 3: Not stated	
3.2.2 Age/weight	Case 1: Not stated Case 2: 59 years Case 3: Not stated	
3.2.3 Known Diseases	Not stated for any cases	
3.2.4 Number of persons	Case 1: 1 person Case 2: 1 person Case 3: 2 persons	
3.2.5 Other information	n/a	
3.3 Exposure	Not known	
3.3.1 Reason of exposure	Case 1: Attempted suicide Case 2: Accidental exposure Case 3: Occupational exposure	
3.3.2 Frequency of exposure	Case 1: Twice Case 2; Once Case 3: Chronic nature 2 and 4 years respectively	
3.3.3 Overall time period of exposure	Case 1: Several months Case 2: Not specified Case 3: As stated in 3.3.2	
3.3.4 Duration of single exposure	Not stated.	
3.3.5 Exposure concentration/dose	Case 1: 25mg of difenacoum (500g rat bait) followed several months later by 1800g-rat bait. Case 2: Not specified Case 3: In the first patient plasma analysis revealed 30-50µg/litre of both difenacoum and brodifacoum.	
3.3.6 Other information		

Section A6.12.8 Prognosis following poisoning

Annex Point IIA6.12.8

3.4 Examinations	Not stated
3.5 Treatment	Case 1: Treated with vitamin K_1 (phytomendione) for 48 days and 42 days respectively. Case 2: Not specified Case 3: Not specified
3.6 Remarks	
	4 RESULTS
4.1 Clinical Signs	Case 1: Not specified Case 2: Unusual coagulopathy. Subacute tetraparesis following severe sudden neck pain Case 3: Not specified
4.2 Results of examinations	Case 1: Not specified Case 2: Clinical examination showed a subdural cervical haematoma. Prothrombin complex activity was low and difenacoum was present in the plasma. Case 3: Unexpectedly high concentrations of vitamin K_1 2,3-epoxide were found in the presence of normal clotting factor activities and antigen levels.
4.3 Effectivity of medical treatment	Not specified
4.4 Outcome	Case 1: Pharmacological effect ceased. Case 2: Complete recovery Case 3: Not specified
4.5 Other	Not stated
	5 APPLICANT'S SUMMARY AND CONCLUSION
5.1 Materials and methods	This report describes in brief several cases of difencoum poisoning: Case 1: Attenpted suicide with 25mg of difencoum (500g rat bait) followe several months later by 1800g of rat bait. Case 2: Accidental exposure to a difenacoum rodenticide Case 3: Two cases of occupational exposure to brodifacoum and difenacoum of a chornic nature (2 and 4 years respectively)

Section A6.12.8 Prognosis following poisoning

Annex Point IIA6.12.8

5.2 Results and discussion	Case 1: Treatment with K1 (phytomenadione) for 48 and 42 days respectively resulted in the pharmacological effects of difenacoum to cease. Case 2: The patient had a subdural hematoma, low prothrombin complex activity and prescence of difenacoum in the plasma. Specific medical management led to a complete recovery. Case 3: In both patients unexpectedly high concentrations of vitamin K_1 2,3-epoxide were found in the presence of normal clotting factor activities and antigen levels which suggested the presence of coumarin anticoagulants in the liver.
5.3 Conclusion	It is possible for a humans to survive poisoning at the levels specified in the acute poisoning case following prolonged treatment with vitamin K. Chronic poisoning at the levels detected did not result in death.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	8 June 2006, revised 6 february 2007
Materials and Methods	
Results and discussion	
Conclusion	A few human poisoning cases are presented as described in the reference.
Remarks	
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.13 Annex Point IIIA VI.2	Toxic effects on livestock and pets	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	The mode of action is common to all mammals and is well understood as a vitamin K antagonist, without secondary effects. It is only used as baits for the control of rodents. Manufacturing takes place in closed or controlled environments with full protective clothing, without pets or livestock having access to the premises. Use as a rodenticide necessarily involves the possibility of pets and livestock gaining access to baits in situ and there are many reported cases of pets and livestock being poisoned by bromadiolone baits It is widely recognised that animals should be denied access to baits due to their toxicity and the reduction in bait available for target organisms. Other routes of administration are likely to be of minimal significance, as they are in humans. Data on the toxic effects in sheep, horses and dogs have been summarised above under Section A6.2 metabolism data. Data are also proved in report Difenacoum / T13, Please refer to report Difenacoum / T13, "Information about and toxicity of anticoagulant rat poisons: Case Histories from the Milan Poisons Centre 1996-1999" Studies on toxic effects to pets and livestock are considered an unjustifiable waste of experimental animals since the compound is shown to be highly toxic by the oral route to all mammals.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	8 June 2006	
Evaluation of applicant's justification	Agree with applicant's version. The mode of action in non-target mammals is shortly described and the potential to exposure is noticed. The applicant describes where elsewhere in the dossier further relevant data is provided.	
Conclusion	No new studies with pets and livestock needed.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.13 (1) Toxic effects on livestock and pets
Annex Point IIIA VI.2

Cattle - Diphenadione

			Official use only
		1 REFERENCE	
1.1	**Reference**	Roger W. Bullard, R. Daniel Thompson and Gilbert Holguin (1976) Diphenadione residues in Tissues of Cattle. Agric Food Chem., 1976 Vol. 24, No 2 : 261-263.	
1.2	**Data protection**	No, published paper.	
1.2.1	Data owner	Public Domain	
1.2.2	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	The guideline study is not stated in the published paper.	
2.2	**GLP**	The GLP status of the study is not stated in the published paper	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Diphenadione	
3.1.1	Lot/Batch number	Batch numbers not stated in the published paper.	
3.1.2	Specification	Not stated in the published paper	
3.1.3	Description		
3.1.4	Purity	Not stated in the published paper	
3.1.5	Stability	A specific statement on stability is not provided within the paper.	
3.1.6	Radio labelling	No	
3.2	**Test Animals**		
3.2.1	Species	Cattle	
3.2.2	Strain	Hereford heifers	
3.2.3	Source	Wild	
3.2.4	Sex	Female	
3.2.5	Age/weight at study initiation	Age not stated Weight approx 230kg	
3.2.6	Number of animals per group	6	
3.2.7	Control animals	Pre-treatment blood samples and samples of other tissues from a local butcher shop served as untreated controls	
3.3	**Administration/ Exposure**	Intraruminal injection to the cows. Dietary administration of livers from exposed cows to rats	
3.3.1	Preparation of test site	Not applicable	
3.3.2	Concentration of test substance	1 mg/kg	
3.3.3	Specific activity of		

	test substance	
3.3.4	Volume applied	
3.3.5	Sampling time	Two animals were killed and samples taken at 30, 60 and 90 days post treatment.
3.3.6	Samples	Blood plasma, liver, heart, kidney, brain, muscle form hindquarter and fat (1:1 mixture visceral and subcutaneous).

4 RESULTS AND DISCUSSION

4.1 Result of study

Detectable Diphenadione Residues (>0.01 ppm) in Tissues of Cattle Given a Single 1mg/kg Injection:

Days post-treatment	ppm found (mean ± SD)	
	Liver	Kidney
30	0.15 ± 0.01	0.08 ± 0.01
60	0.14 ± 0.01	0.10 ± 0.02
90	0.15 ± 0.00	0.08 ± 0.00

Secondary hazard determination in rats:
None of the test animals died or exhibited signs of chronic toxicity during the test or the following 14 day observation period. The prothrombin clotting times were as follows:

Days post treatment of cattle	Mean ± SD for 10 rats
30	17.7 ± 0.4
60	18.6 ± 0.6
90	17.4 ± 0.5
Untreated	18.7 ± 0.5

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

Treatment of animals:

Six Hereford heifers were dosed with 1mg/kg of diphenadione by injecting a Carbopol 941 suspension into the rumen. At 30, 60 and 90 days posttreatment two animals were randomly selected and killed. Samples of blood plasma, liver, heart, kidney, brain muscle from the hindquarter and fat were collected from each animal. GLC procedure was used for analysis.

Secondary hazard Determination.

Secondary hazards were evaluated by feeding the livers of each treated heifer to two groups of 5 adult Sprague-Dawley rats weighing approx 200g each. Two control groups of 5 rats were fed beef liver from a butcher shop. Each rat was fed a 70 kg human equivalent of 1.4 kg/day for 14 days. After 14 days of feeding one of the two test groups was returned to a diet of lab chow and observed an addition al 14 days for mortality and signs of toxicity. The reamining test group was killed and the prothrombin clotting time determined.

5.2 Results and discussion

Small quantities of diphenadione were present in liver and kidney samples from treated cattle but detectable levels (>0.01 ppm) could not be found in any of the other five tissues analysed or in any of the contro, samples. The almost constant residues found in kidney and liver from 30

Section A6.13 (1) Toxic effects on livestock and pets
Annex Point IIIA VI.2

Cattle - Diphenadione

		to 90 days post treatment may be due to diphenadione being susceptible to protein binding. Minute diphenadine residues in the tissue of treated cattle caused no observable effects in secondary consumers as was concluded from the second hazard determination in rats.	
5.3	**Conclusion**	Calculations based on residue levels in this study indicate that humans may safely eat the meat , including liver and kidney of treated cattle.	X
5.3.1	Reliability	2	
5.3.2	Deficiencies	No	

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	8 June 2006, revised 6 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version otherwise, but conclusions concerning safety to humans should not be made on the basis of this study.
Conclusion	Data provided by this article has no relevance for risk assessment of difenacoum
Reliability	3
Acceptability	Not acceptable
Remarks	
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.13 (2) Toxic effects on livestock and pets
Annex Point IIIA VI.2

Dogs - Flocoumafen

			Official use only
		1 REFERENCE	
1.1	**Reference**	G.E. Veenstra, D.E. Owen and K.R. Huckle (1991) Metabolic and Toxicological Studies on the Anticoagulant Rodenticide, Flocoumafen, Arch. Toxicol., Suppl. 14, 160-165.	
1.2	**Data protection**	No, published paper.	
1.2.1	Data owner	Shell Internationale Petroleum	
1.2.2	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	The guideline study is not stated in the published paper.	
2.2	**GLP**	The GLP status of the study is not stated in the published paper	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Flocoumafen	
3.1.1	Lot/Batch number	Batch numbers not stated in the published paper.	
3.1.2	Specification	Not stated in the published paper	
3.1.3	Description		
3.1.4	Purity	94.9%	
3.1.5	Stability	A specific statement on stability is not provided within the paper.	
3.1.6	Radio labelling	No	
3.2	**Test Animals**		
3.2.1	Species	Dogs	
3.2.2	Strain	Beagle	
3.2.3	Source	Not stated in the published paper	
3.2.4	Sex	Male and female	
3.2.5	Age/weight at study initiation	Not stated in article	
3.2.6	Number of animals per group	The total number of animals was eight (four males and four females)	
3.2.7	Control animals	No	
3.3	**Administration/ Exposure**	Single oral dose;	
3.3.1	Preparation of test site	Not applicable	
3.3.2	Concentration of test substance	Dosing 0.5 mg/kg Therapy: 2 or 5 mg/kg Vitamin K (Konakion 10, 1.1 ml ampoules) for 7 days followed by	

		2 or 5 mg/kg Vitamin K (Konakion 10 tablets) for 14 days followed by 1 or 2.5 mg.kg Vitamin K (Konakion 10 tablets) for 7 days followed by 0.5 or 1.25 mg/kg Vitamin K (Konakion 10 tablets) for 7 days
3.3.3	Specific activity of test substance	
3.3.4	Volume applied	Dosing 100mg/l
3.3.5	Sampling time	Blood – twice weekly.
3.3.6	Samples	Blood samples analysed for prothrombin time (PT) and activated partial thromboplastin time (APTT). Whole blood transfusion applied to dogs that showed advanced signs of anticoagulation. (200 ml/dog) Livers taken from all dogs at termination of study (6½ months) to test for flocoumafen.
		4 RESULTS AND DISCUSSION
4.1	**Result of study**	Treatment Phase Anticoagulation in 3 male and 2 female within 6 – 10 days. Remaining 1 male and 2 female given a second dose of 0.5mg/kg flocoumafen 7 weeks after the first dose. 2 of the 3 showed anticoagulation in 7 – 8 days. Body weight was not affected during any stage of the treatment even though food consumption was reduced during an episode of anticoagulation. Elevated PT values were seen 5 days after dosing. For dogs not showing signs of anticoagulation the maximum clotting times were recorded at day 8. By day 12 the PT values had returned to the base line and no further changes observed until after the second dosing. APTT values followed the same trend as PT values. Therapeutic Phase Vitamin K treatment was initiated immediately after observation of adverse clinical signs. 3 out of 7 animals did show signs of improvement after one or two doses of vitamin K and were given a blood transfusion. That resulted in immediate improvement and full recovery in 24 hours. Animals that did respond made a complete recovery in 48 hours. During the remainder of the treatment and observation period no further signs of anticoagulation occurred. Vitamin K treatment resulted in a rapid decrease in the PT values and a return to base line values within 48 hours. Liver residues Analysis of the liver at termination of the study showed that 8% of the administered dose was present in those animals given a single dose of 0.5 mg/kg whereas those given two doses the figure was 5%.
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	Metabolism study in dogs; antidotal therapy based on administration of Vitamin K and monitoring of blood chemistry; Analysis of livers at

Section A6.13 (2) Annex Point IIIA VI.2	Toxic effects on livestock and pets *Dogs - Flocoumafen*
	termination for residues of parent compound.
5.2 Results and discussion	Severe intoxication as observed in gross haemorrhaging was successfully treated by a whole blood transfusion. There was significant individual animal variation in the length of time to develop clinical signs of intoxication and in the degree of elevation of clotting times. No changes were, however, detected within 6 days of exposure in any of the animals.
5.3 Conclusion	For dogs there is no difference in antidotal response using either 2 or 5 mg/kg Vitamin K per day The therapeutic regime used in this study is effective and suitable for dogs intoxicated with flocoumafen. Flocoumafen is retained in the liver at concentrations similar to those in other species. The findings support the concept of the existence of a capacity limited hepatic binding site for flocoumafen
5.3.1 Reliability	2
5.3.2 Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	9 June 2006, revised 6 February 2007
Materials and Methods	Agree with applicant's version.
Results and discussion	Agree with applicant's version.
Conclusion	Agree with applicant's version.
Reliability	2
Acceptability	Acceptable (See remarks)
Remarks	As flocoumafen is a representative of the class of second generation anticoagulants, this study offers qualitative information on the effects of other second generation anticoagulants in dogs.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.14 Annex Point IIIA *III-XI.2*	Other test(s) related to the exposure of humans	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [X]	**Other justification [X]**	
Detailed justification:	Information regarding exposure to humans from anticoagulants are already well researched and fully elucidated, for this reason it is deemed to be scientifically unjustified to conduct a study for which the end points have been reasonable determined by previous studies conducted on analogous substances (bromadiolone and brodifacoum). These analogous substances have similar physico-chemical and toxicological properties to Difenacoum, hence other human exposure data can be read across to Difenacoum from the analogous substances. Hence, all endpoints have been reasonably assessed by the analogous substances. Difenacoum is a well-known compound which has been used extensively for many years. The properties of Difenacoum are understood as is its mode of action. Anticoagulant rodenticides such as Difenacoum are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent post translation processing before they are converted into the respective procoagulant zymogens. The specific point of action is thought to be the inhibition of K_1 epoxide reductase. The anticoagulants accumulate and are stored in the liver until broken down. The plasma prothrombin (procoagulant factor II) concentration provides a suitable guide to the severity of acute intoxication and to the effectiveness and required duration of the antidoting therapy (vitamin K_1), this process is seen in all other mammalian species tested, including humans in therapeutic use (warfarin) and in poisoning incidents in humans and animals. The technical active ingredient has a high level of purity and there are no other substances that are of concern included as impurities or additives. There are also no other known significant toxic effects. In addition, based on animal welfare grounds other test related to the exposure of humans is considered to be of no value as this additional animal testing would not provide any additional relevant data than is not already available from the analogous substances.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	9 June 2006	
Evaluation of applicant's justification	RMS agrees that prerequisites for waiving do exist, because in normal use of rodenticide products human exposure to possible substances, other than mammalian metabolites, generated from the active substance is not expected to be significant.	
Conclusion	Waiving of data for point A6.14 is acceptable.	

Section A6.14 **Annex Point IIIA *III-XI.2***	**Other test(s) related to the exposure of humans**
Remarks	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.15.1 **Annex Point *IIIA X.1.1, 1.3, 1.6***	**Food and feedingstuffs - Identification of the residues (identity and concentrations), degradation and reaction products and of metabolites of the active substance in contaminated foods or feedingstuffs**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	Difenacoum will be used around food and feedingstuff. However, it is not yet fully elucidated as it has not been decided as to what foodstuff should be tested on. Hence, at this present time it is not possible to conduct a study to identify the residues, degradation and reaction products and on metabolites of the active substance in contaminated foods or feedingstuffs.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	9 June 2006, reviewed 6 February 2007	
Evaluation of applicant's justification	RMS agrees with the applicant that difenacoum will be used around food and feeding stuff. Thus, some potential for human exposure through food and feeding stuff exists. However, proper baiting practices should minimise contamination of food with residues of rodenticides either directly or via rodent faeces. It is agreed between member states that maximum residue levels (MRLs) do not have to be determined for rodenticides.	
Conclusion	Since the potential secondary exposure from food and feeding stuffs is assumingly very limited, further studies or data at this point is considered unnecessary. Besides literature data, estimates by modelling would clarify the case. The applicant is asked to rewrite a combined justification for sections 6.15.1-6.15.5. It should contain a qualitative assessment of potential exposure through food and feeding stuff in general, based on available data.	
Remarks	This comment is valid for subsections 6.15.1-6.15.5.	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.15.2 **Annex Point *IIIA XI.1.2, 1.3, 1.5, 1.6***	**Food and feedingstuffs - Behaviour of the residues of the active substance, its degradation and reaction products and where relevant, its metabolites on the treated or contaminated food or feedingstuffs including the kinetics of disappearance**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	Difenacoum will be used around food and feedingstuff. However, it is not yet fully elucidated as it has not been decided as to what foodstuff should be tested on. Hence, at this present time it is not possible to conduct a study to identify the residues, degradation and reaction products and on metabolites of the active substance in contaminated foods or feedingstuffs.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	6 February 2007	
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*	
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*	
Remarks	See 6.15.1	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.15.3 **Annex Point *IIIA XI..1.4***	**Food and feedingstuffs - Estimation of potential or actual exposure of the active substance to humans through diet and other means**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ] Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	Difenacoum will be used around food and feedingstuff. However, it is not yet fully elucidated as it has not been decided as to what foodstuff should be tested on. Hence, at this present time it is not possible to conduct a study to identify the residues, degradation and reaction products and on metabolites of the active substance in contaminated foods or feedingstuffs.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	6 February 2007	
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*	
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*	
Remarks	See 6.15.1	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date		
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.15.4 Annex Point *IIIA XI.1.7*	Food and feedingstuffs - Proposed acceptable residues and the justification of their acceptability	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [X]**	**Technically not feasible [ ]** **Scientifically unjustified [X]** **Other justification [ ]**	
Detailed justification:	Difenacoum will be used around food and feedingstuff. However, it is not yet fully elucidated as it has not been decided as to what foodstuff should be tested on. Hence, at this present time it is not possible to conduct a study to identify the residues, degradation and reaction products and on metabolites of the active substance in contaminated foods or feedingstuffs.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	6 February 2007	
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*	
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*	
Remarks	See 6.15.1	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date		
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.15.5 Annex Point *IIIA XI.1.8*	Food and feedingstuffs - Any other available information that is relevant	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [ ]**	
Detailed justification:	Difenacoum will be used around food and feedingstuff. However, it is not yet fully elucidated as it has not been decided as to what foodstuff should be tested on. Hence, at this present time it is not possible to conduct a study to identify the residues, degradation and reaction products and on metabolites of the active substance in contaminated foods or feedingstuffs.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	6 February 2007	
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*	
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*	
Remarks	See 6.15.1	
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.15.6 Annex Point IIIA 6.15.6	Food and feeding stuffs – summary	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [X]**	
Detailed justification:	As it is considered not necessary to determine any of the endpoints under Sections 6.15.1 – 6.15.5, it is considered that a summary and evaluation of data submitted under point 6.15 is not justified.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	9 June 2006	
Evaluation of applicant's justification	Agree with applicant's justification.	
Conclusion	Summary not needed.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.16 **Annex Point IIIA VI.3.5 & XI.2**	**Any other tests related to the exposure of the active substance to humans, in its proposed biocidal products, that are considered necessary.**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [X]	**Other justification [X]**	
Detailed justification:	The risk assessment has not highlighted any requirements for further studies related to the exposure of the active substance to humans.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	June 9 2006	
Evaluation of applicant's justification	Agree with applicant's justification.	
Conclusion	No further studies needed under this point.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.17 **Annex Point IIIA VI.6**	**If the product is to be used in products for action against plants then tests to assess the toxic effects of metabolites from treated plants, if any, where different from those identified in animals shall be required.**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [ ]	**Other justification [X]**	
Detailed justification:	The product is not used for action against plants, so these studies are considered to be unnecessary.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	9 June 2006	
Evaluation of applicant's justification	Agree with applicant's justification.	
Conclusion	Studies not needed.	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		