Section A 6.2-03 **Annex Point IIA VI.6.2**		**Percutaneous absorption (*in vitro* test)**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Xxxxx, XX. (XXXX) The *in vitro* percutaneous absorption of radiolabelled Difethialone in two test preparations through rat and human skin. xxxxxxxxxxx, xxxxx, xxxxx. Laboratory report number xxxxx. Report date x Xxxxx XXXX (unpublished).	
1.2	**Data protection**	Yes	
1.2.1	Data owner	LiphaTech S.A.S.	
1.2.2	Companies with letter of access	None	
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**	Yes. OECD draft guideline 428: Skin absorption: *in vitro* method December 2000.	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2 for unlabelled material and ^{14}C-Difethialone	
3.1.1	Lot/Batch number	Radiolabelled batch no XXX XXXXX Non labelled batch XXXX XXXXXX	
3.1.2	Specification	As given in section 2. Deviating from specification given in section 2 as follows: The test material was radiolabelled.	

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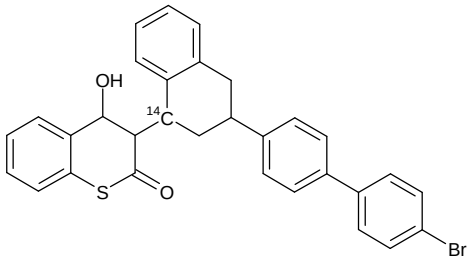
3.1.2.1 Description	Two test preparations were used: 0.5% w/w ^{14}C-Difethialone in wheatflour, considered equivalent to the dry concentrate and diluted 1:1 w/w in water for application. 1.25 g/L ^{14}C-Difethialone in glycol solvent. Descriptions of physical appearance of the two preparations are not presented in the report.	
3.1.2.2 Purity	Radiochemical purity of radiolabelled difethialone was 100%. There were two isomers, containing 66.2% and 33.8% of radioactivity. No information provided for purity of non-labelled difethialone.	
3.1.2.3 Stability	Not stated.	
3.1.2.4 Radiolabelling	^{14}C. Specific activity 52 mCi/mmol. The labelled product was [tetrahydronaphthyl-1-^{14}C]-Difethialone. ^{14}C label structural location of radio labelling,  	
3.2 Skin samples	Non-entry field	
3.2.1 Human	Full thickness skin samples (7 breast samples and 1 abdominal section) were obtained from patients (between 23 and 44 years old, typically cases of cosmetic surgery) after obtaining patient's consent for skin to be used for scientific research. The samples were transhipped on ice to the laboratory where they were cleaned of subcutaneous fat and connective tissue, washed in cold running water, dried, cut into smaller sections and wrapped in aluminium foil and then stored in plastic bags and frozen until required.	
3.2.2 Rats	Seven male rats of the Sprague Dawley Crl:CD(SD) IGS BR strain aged approximately 7 weeks and weighing 194-218g were obtained from Charles River UK. The animals were housed at the laboratory for three days and then terminated (carcass weight range at termination was (214±11g). Dorsal fur was removed and the dorsal skin excised and separated along the dorsal midline. Both pieces were wrapped in aluminium foil and then stored in plastic bags and frozen until required.	X
3.2.3 Split thickness samples	Frozen skin samples were removed from storage and allowed to thaw. The thickness of the uncut membranes was measured with a micrometer and then full thickness skin was pinned to a corkboard with the stratum corneum uppermost and a section cut at 200 to 400 µm with a Zimmer electric dermatome. After sectioning the thickness of the split membrane was rechecked with a micrometer.	
3.3 Administration/ Exposure	*In vitro* flow through diffusion cell	

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3.3.1	Preparation of test membranes	Membranes, ca 15 x 15 mm, prepared as detailed in section 3.2.3 were positioned on the diffusion cell receptor chamber and the donor chamber was then tightened onto the membrane and entire cell placed in heated manifold to maintain the skin at approximately 32.1ºC.	
3.3.2	Diffusion cell apparatus	An automated flow through cell was used. Peristaltic pumps were attached to the afferent ports and receptor fluid effluent was collected into scintillation vials on a fraction collector. The receptor cell volume was 0.25 mL and the exposed skin surface area was 0.64 cm^2. The flow rate through the cell was maintained at circa 1.5 mL/h.	
3.3.3	Receptor fluid	Physiological saline was used in the water permeability assessment for barrier integrity. Ethanol and water (1:1 v/v) was used in the test material assessment	
3.3.4	Barrier integrity evaluation	Tritiated water (250 µl, equivalent to approximately 100,000 d.p.m.) was applied to the surface of the rat or human skin sample and penetration of tritiated water assessed by collecting hourly samples from the receptor fluid for 2 hours. The fractions were then analysed by liquid scintillation counting. Permeability coefficients were calculated and any skin sample with a value greater than 2.5 x 10^{-3} cm/h was excluded from the subsequent test material evaluation.	
3.3.5	Dose formulation preparations	Difethialone was prepared as a stock solution of 1 mCi of radiolabelled difethialone in 2 mL of acetone. For preparation of the [^{14}C]-Difethialone in glycol formulation, an aliquot of stock was taken and acetone removed in a nitrogen stream. The [^{14}C]-Difethialone was then mixed with PEG 400 and propylene glycol to a final concentration of 1.27 mg/mL. For preparation of the [^{14}C]-Difethialone wheatflour in water formulation, an aliquot of the stock solution was added to further unlabelled Difethialone and additional acetone. The acetone was then removed in a nitrogen stream and water was added prior to vortex mixing. Aliquots were taken to determine concentration by LSC. Initial results indicated the achieved concentration was only 78% of the target 2.5 g/kg and more unlabelled Difethialone was added to bring the final concentration up to approximately 105% of the target concentration.	
3.3.6	Application and exposure period	0.64 µL was applied to the exposed stratum corneum. The donor chamber was left open to the atmosphere rather than occluded. The skin was exposed for 6 hours and then washed with a soap solution and skin washes were retained for LSC analysis.	X

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3.3.7 Sampling time	The receptor fluid was collected at hourly intervals for the first 6 hours following dosing and then over two hour intervals from 6 to 24 hours after dosing. Skin washes were retained for analysis. A tissue paper swab used to dry the washed skin was retained in a Combustocone for analysis. At the end of the 18 hour post-exposure observation period the diffusion cell was dismantled, the underside of the treated skin was rinsed with receptor fluid and washings retained for analysis. The cell (donor and receptor chambers) was cleaned and washings retained for analysis. 15 successive tape strips were used to remove the stratum corneum. The tapes were pooled in a Combustocone for each skin section and analysed by combustion/LSC.	
3.3.8 Analysis of Samples	Samples were combusted in a Model 307 Tri-Carb Automatic Sample Oxidiser and resulting $^{14}CO_2$ collected in Carbosorb and mixed with scintillation fluid. Liquid samples were all analysed directly in scintillation fluid. Samples were generally counted for 5 minutes with representative blanks and automatic external quench control. The limit of quantification was 30 dpm above background.	
	4 RESULTS AND DISCUSSION	
4.1 Calculations	The Permeability Coefficient (K_p) of water was calculated from cumulative absorption of tritiated water. The absorption rate ($dpm/cm^2/h$) was calculated from the slope of the absorption versus time curve = $\frac{\text{Slope (dpm/h)}}{\text{Exposed area (cm}^2\text{)}}$ The Permeability Coefficient was then calculated by dividing the absorption rate by the application rate (dpm/cm^3). The absorbed dose ($\mu g\ equiv.cm^{-2}$) of Difethialone was calculated as = $\frac{\text{Sample radioactivity (dpm)}}{\text{Specific activity (dpm.}\ \mu g^{-1}\text{) x exposure area (cm}^2\text{)}}$ The absorbed dose (%) = $\frac{\text{Sample radioactivity (dpm)}}{\text{Applied dose (dpm)}} \times 100$ Dermal delivery was defined as the sum of the absorbed dose and the amount in the skin.	

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4.2 Recovery of labelled compound	The mass balance, dislodgeabledose, unabsorbed and absorbed dose for ^{14}C-Difethialone in glycol solvent applied to human skin were 13.65, 10.46, 13.21 and 0.08 µg equiv.cm^{-2}. At 24 hours the mean total unabsorbed dose was 99.80% and absorbed dose was only 0.06%. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 3.29%. The mass balance, dislodgeabledose, unabsorbed and absorbed dose for ^{14}C-Difethialone in glycol solvent applied to rat skin were 13.25, 8.67, 11.97 and 0.47 µg equiv.cm^{-2}. At 24 hours the mean total unabsorbed dose was 89.95% and absorbed dose was 3.53%. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 9.71%. The mass balance, dislodgeabledose, unabsorbed and absorbed dose for ^{14}C-Difethialone in wheatflour applied to human skin were 27.65, 26.91, 27.61 and <0.01 µg equiv.cm^{-2}. At 24 hours the mean total unabsorbed dose was 99.70% and absorbed dose was only 0.02%. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 0.13%. The mass balance, dislodgeabledose, unabsorbed and absorbed dose for ^{14}C-Difethialone in wheatflour applied to rat skin were 27.41, 23.40, 26.88 and 0.26 µg equiv.cm^{-2}. At 24 hours the mean total unabsorbed dose was 96.68% and absorbed dose was 0.94%. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 1.94%.	
	5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1 Materials and methods	The study was conducted in accordance with OECD Guideline for testing of chemicals, draft new guideline 428: skin absorption: in vitro method (December 2002). Two test preparations of ^{14}C-Difethialone were prepared in wheatflour (equivalent to the dry concentrate) and in glycol solvent (equivalent to the liquid concentrate) to achieve target doses of 12.5 µg/cm^2 or 25.0 µg/cm^2 respectively. Dose volumes of 10 µL/cm^2 were applied to rat and human split thickness skin samples for an exposure period of six hours. The samples were mounted *in vitro* on flow through diffusion cells and receptor fluid samples were collected over hourly intervals for the first six hours and then over two hourly intervals up to 24 hours after completion of exposure. Samples were analysed by liquid scintillation counting and skin permeability and absorption values calculated.	
5.2 Results and discussion	^{14}C- Difethialone was applied in two test formulations to rat and human split thickness skin membranes mounted *in vitro* in flow through diffusion chambers. The two preparations were applied at 10 µL/cm^2 to achieve application rates of difethialone of circa 12.5 µg/cm^2 for the dry concentrate and circa 25 µg/cm^2 for the liquid concentrate. <u>^{14}C- Difethialone in glycol solvent applied to human skin</u> At 6 hours post dosing 71.54% of the applied dose was washed off and at 24 hours this dislodgeable dose to 78.83%. The mean total unabsorbed dose was 99.80% when stratum corneum levels were added to the cell wash, skin wash and tissue swab values. The absorbed dose	

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	(receptor fluid and receptor rinse values) was only 0.6% of administered dose after 24 hours. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 3.29%. ^{14}C- Difethialone in glycol solvent applied to rat skin At 6 hours post dosing 62.37% of the applied dose was washed off and at 24 hours this dislodgeable rose to 64.97%. The mean total unabsorbed dose was 89.85% when stratum corneum levels were added to the cell wash, skin wash and tissue swab values. The absorbed dose (receptor fluid and receptor rinse values) was 3.53% of administered dose after 24 hours. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 9.71%. ^{14}C- Difethialone in wheatflour in water applied to human skin At 6 hours post dosing 96.61% of the applied dose was washed off and at 24 hours this dislodgeable rose to 97.19%. The mean total unabsorbed dose was 99.70% when stratum corneum levels were added to the cell wash, skin wash and tissue swab values. The absorbed dose (receptor fluid and receptor rinse values) was only 0.02% of administered dose after 24 hours. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 0.13%. ^{14}C- Difethialone in wheatflour in water applied to rat skin At 6 hours post dosing 83.37% of the applied dose was washed off and at 24 hours this dislodgeable rose to 84.20%. The mean total unabsorbed dose was 96.68% when stratum corneum levels were added to the cell wash, skin wash and tissue swab values. The absorbed dose (receptor fluid and receptor rinse values) was only 0.94% of administered dose after 24 hours. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 1.94%. ^{14}C- Difethialone in wheatflour was more effectively washed off the skin of both species than the glycol formulation (dislodgeable dose 97.19% human and 84.20% rat for wheatflour and 78.83% human and 64.79% rat for glycol). However, the different levels retained in the stratum corneum accounted for much of the variation so that the total unabsorbed material was similar for each formulation in both species. Absorption and dermal delivery were higher in the rat than human for each formulation and higher for the glycol formulation than the water based wheatflour formulation. Washing the skin (rat and human) apparently enhanced absorption of both formulations for the rat but only affected the absorption of the glycol formulation in human skin. The dermal delivery to human skin of ^{14}C- Difethialone in glycol solvent was 11 fold the delivery of the wheatflour preparation (0.43 µg equiv./cm^2 compared to 0.04 µg equiv./cm^2). The dermal delivery to rat skin of ^{14}C- Difethialone in glycol solvent was 2.3 fold the delivery of the wheatflour preparation (1.28 µg equiv./cm^2 compared to 0.54 µg equiv./cm^2).	
5.3 Conclusion	Topical application of ^{14}C-Difethialone in wheatflour (0.5% w/w) diluted 1:1 in water by volume or a formulation in glycol solvent (1.25 g/L) to human or rat split thickness skin samples maintained *in vitro* resulted in dermal delivery and total absorption that was higher in the rat than human skin for both formulations. Absorption of the glycol solvent formulation was higher in both species than the wheatflour formulation and more of the glycol solvent formulation was contained in	

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	the stratum corneum for both species. Removal of the wheatflour formulation by skin washing was easier than the glycol based formulation. The total absorption of ^{14}C-Difethialone in glycol solvent was 3.53% in rat skin and 0.6% in human skin. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 3.29% for human skin and 9.71% for rat skin. Total absorption of ^{14}C-Difethialone in wheatflour was 0.94% in rat skin and 0.02% in human skin. The dermal delivery, made up of absorbed dose, exposed skin and unexposed skin values was 0.13% for human skin and 1.94% for rat skin.	
5.3.1 Reliability	1	
5.3.2 Deficiencies	No	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	25 January 2005, revised 6 December 2006	
Materials and Methods	Agree with applicant´s summary and conclusion. **Comments (3.2.2):** In the study report the weight of the animals (3 days following delivery) is stated to be 241 $\pm$ 11. However, in the study summary by the applicant the weight is 214 $\pm$ 11, which appears more likely. **Comments (3.3.6):** 6.4 µL (and not 0.64 µL as stated) was applied to the exposed stratum corneum.	
Results and discussion	Agree with applicant´s version	
Conclusion	Agree with applicant´s version	
Reliability	1	
Acceptability	Acceptable	
Remarks	-	

Table A6.2-9: Table for percutaneous absorption (*in vitro* test)

	Test preparation			
	Glycol solvent		Wheatflour	
	Human	Rat	Human	Rat
Target application (µg/cm^2) Actual application (µg equiv/cm^2)	12.5 13.7	12.5 13.3	25.0 27.7	25.0 27.4
Target concentration (g/L or g/kg) Actual concentration (g/L or g/kg)	1.25 1.33	1.25 1.33	2.50 2.52	2.50 2.52
Dislodgeable dose (µg equiv/cm^2) Dislodgeable dose (%applied dose)	10.46 78.83	8.67 64.97	26.91 97.16	23.40 84.20
Unabsorbed dose (µg equiv/cm^2) Unabsorbed dose (%applied dose)	13.21 99.80	11.97 89.95	27.61 99.70	26.88 96.68
Dermal delivery (µg equiv/cm^2) Dermal delivery (%applied dose)	0.43 3.29	1.28 9.71	0.04 0.13	0.54 1.94
Absorbed dose (µg equiv/cm^2) Absorbed dose (%applied dose)	0.08 0.60	0.47 3.53	<0.01 0.02	0.26 0.94
Mass balance (µg equiv/cm^2) Mass balance (%applied dose)	13.65 103.09	13.25 99.56	27.65 99.84	27.41 98.61

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	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [x]** **Scientifically unjustified [x]**	
Limited exposure [ ]	**Other justification [ ]**	

Detailed justification:

Waiver for carcinogenicity/toxicity studies in rodents on Difethialone.

The following is a series of rationales to waive the requirement to perform carcinogenicity/chronic toxicity studies on the anticoagulant rodenticide active substance Difethialone under the Biocidal Products Directive 98/8/EEC.

6 INTRODUCTION.

The Biocidal Products Directive (98/8/EEC 'the Directive') requires long-term testing in rodents as part of the suite of toxicology tests in order to assess the possible adverse consequences of chronic exposure (i.e., chronic toxicity and carcinogenicity) to the biocidal active substance Difethialone.

It is a unique feature of the rodenticides that the test species used in long-term toxicity and carcinogenicity studies is also the target species, and that the active substances are lethal in the target species at very low levels. This gives rise to several questions: Is it relevant to consider the possible use of long term rodent studies to predict possible effects of rodenticides in humans. Is it scientifically feasible? Can the data be derived using other species? Given that at one rodenticide molecule has been used for over forty years in human medicine, are there data in the human that are more relevant than animal data would be? Are there other data that demonstrate the potential, or lack of potential, carcinogenic properties of active substances used as rodenticides?

The Directive states in Article 8 (5) that "*information which is not necessary owing to the nature of the biocidal product or of its proposed uses need not be supplied. The same applies where it is not scientifically necessary or technically possible to supply the information. In such cases, a justification, acceptable to the competent authority must be submitted…*". A more detailed waiver concept is given in the TNsG on data requirements.

The TNsG gives the strong recommendation "*to minimise testing on vertebrate animals or to avoid unnecessary suffering of experimental animals the data should not be generated*".

The TNsG recommendations were further refined in an Addendum to the TNsG entitled Refined waiving concept for rodenticides (TMII03-item9a-CA-Jun03-Doc9-TNsG.doc). These include:

The study is technically not possible to perform,

Use of other data,

Data evaluated with regard to agricultural use

Read-across from data on related substances

Evaluation of acceptable human data,

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The study is not scientifically necessary

The choice of species is not appropriate

The study is not necessary owing to limited exposure and toxicity profile.

The Notifier has prepared a scientific justification based on this guidance to waive the requirement for these studies. Before the waiving arguments are given, it will be useful to review the way the coagulation system works in mammals and the mechanism by which the anticoagulant rodenticides function.

7 FUNCTION

Anticoagulant rodenticides such as Difethialone function by inhibiting the ability of the blood to clot at the site of a haemorrhage, by blocking the regeneration of vitamin K in the liver.

Blood clots form when the soluble protein fibrinogen, normally present in the blood, is converted by the enzyme thrombin to the insoluble fibrous protein fibrin, which binds platelets and blood cells to form a solid mass referred to as a blood clot, sealing the site of the haemorrhage and preventing further blood loss. Fibrinogen is present in the blood, but thrombin is not. Thrombin factor IIa in the scheme below) is formed at the site of injury from prothrombin (factor II), which is present in the blood. Conversion of prothrombin to thrombin occurs via the coagulation cascade, in which the blood clotting factors are employed. Without these blood factors clotting cannot take place, and the haemorrhage will not be controlled by clot formation. If the blood vessel is large and/or serves a vital organ, the haemorrhage will be fatal. The synthesis of a number of blood coagulation factors (factors II [prothrombin], VII [proconvertin] IX [Christmas factor], X [Stuart-Prower factor] and the coagulation inhibiting proteins C and S) is dependent upon vitamin K, which acts as a co-enzyme.

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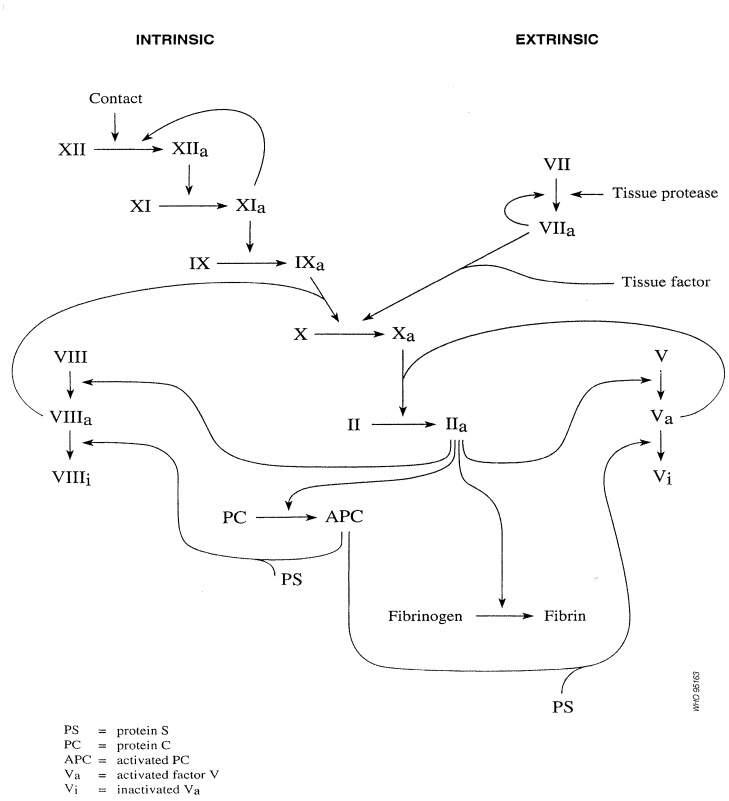
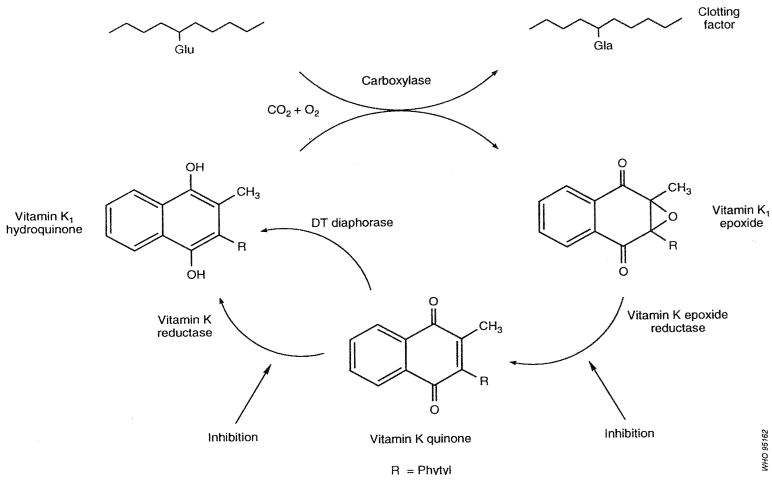


Vitamin K hydroquinone is the active co-enzyme, and its oxidation to vitamin K 2,3-epoxide provides the energy required for the carboxylation reaction where glutamate (Glu) in the precursor is converted to γ-carboxyglutamate (Gla) to make the activated clotting factor.



The anticoagulant rodenticide active substances such as Difethialone work by blocking the regeneration of vitamin K 2,3-epoxide to vitamin K hydroquinone. The Glu→Gla conversion does not take place.

The action is cumulative, increasing levels of the anticoagulant leading to increased clotting times, such that in the event of a significant haemorrhage, death occurs. The amount of vitamin K in the body is finite, and progressive blocking of the regeneration of vitamin K will lead to an increasing probability of a fatal haemorrhage. In general terms, progressive intake of anticoagulants results in death. The active

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substances are highly toxic and bioaccumulative. The oral LD_{50} of Difethialone is 0.56 mg/kg. Rodenticide baits generally contain 25 ppm Difethialone and are fatal after one meal.

8 TECHNICAL FEASIBILITY

Carcinogenicity/toxicity studies seek to determine the consequences of long-term (near life-span) exposure to the active substance by the daily, dietary administration for two years of (typically) three increasing doses to groups of rats or mice, and observing their effects in comparison to a similar group of untreated animals (the control group).

8.1 Dose-setting and the Maximum Tolerated Dose

In order to demonstrate the validity of long-term carcinogenicity/toxicity study, the highest dose should induce some form of toxicity. This toxic effect is not necessarily carcinogenicity *per se* but should be a difference from the control group that can be demonstrated experimentally (e.g. reduced body-weight gain, altered enzyme levels, changes in function of an organ exhibited by either weight change or histopathology). This measurable indicator of toxicity should be present in the high dose level, ideally at a level that does not affect the animals sufficiently to affect survival adversely over the length of the study. This high dose level referred to as the Maximum Tolerated Dose (MTD) and, conventionally, should not cause more than 10% mortality above that observed in the control group.

Studies without an MTD are considered invalid by many regulatory authorities. The intention is to administer sufficient test material such that the animal has to respond to the chemical burden i.e. it is placed under toxic stress. The implication is that if the animal does not respond to the stress by showing increased incidence of tumours, then the chemical is considered unlikely to be carcinogenic in man. Secondly, if the animal is not stressed sufficiently to show MTD response, it has not been stressed sufficiently to demonstrate the potential to cause increased incidence of tumours.

A difficulty in the administration of an MTD in a two-year study is caused by the fact that the anticoagulants are not excreted rapidly. Terminal half-lives in the liver are relevant, as the liver is the site of vitamin K regeneration, and these half-lives are very long. See Table 6.5-1.

Warfarin has the lowest half-life at 42 hours in human plasma. Human liver data are not available (because liver biopsy is too hazardous for routine investigation in humans), but the liver half life is predicted to be several days, where ‘several’ is probably greater than ten but less than one hundred). Absorbed doses accumulate, and lethality occurs when a threshold dose is exceeded. This may occur after one or two large doses, or several smaller doses.

It is feasible to conduct short-term animal studies with these substances because it is possible to ensure that the accumulated dose does not exceed lethal levels. However, the LD_{50} of these molecules is very low and, since the level for low lethality (e.g. LD_{10}) will be lower still, the amount to be administered daily over a two year study, in order to deliver (but not to exceed) an LD_{10}, would technically impossible to achieve. For example, for bromadiolone, the LD_{50} in rats is >0.56 mg/kg but < 0.84 mg/kg. A reasonable estimate of the LD_{10} (a value that would theoretically induce 10% mortality allowed in a long-term rodent study) is 0.6 mg per animal during the study. Using excretion data for

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bromadiolone, and computer software it can be shown that over the 730 days of a typical rat carc/tox study, to reach the LD_{10} by termination would require daily doses (at food intake of 25 g/rat/day) of 0.2 ppm. This is not a feasible level of dietary inclusion.

8.2 Route of Administration of the Test Substance

Dietary admixture is the only practical long-term route for administration of the test substance. It is not feasible accurately to prepare homogenous rodent test diets (to the standards required by GLP and Guidelines) at the very low concentrations needed for the MTD (i.e. 0.2 ppm as shown above). Even lower concentrations would be required for the other dose levels and these would approach the analytical method limit of detection of 0.02 ppm. It may be argued that a regulator would not expect accurate formulations, but that a study should be performed anyway. However, if inhomogeneous diet were administered, some rats would be given a feed ration that contained too much active substance, which could simply be fatal to that entire cage of five rats. Even if the rats were housed singly, the risk of fatality over a two-year period would be too great to anticipate enough animals surviving to the end of the study to provide meaningful data.

An alternative to dietary administration is the use of oral gavage. However, handling for gavage can lead to minor haemorrhage in the nasal passages (shown as brown facial staining), and the act of introducing the plastic or rubber gavage tube or steel cannula may cause minor haemorrhage in the buccal cavity and oesophagus. The use of this procedure daily for two years is considered unfeasible for an anticoagulant. Injection is also not worth considering for similar reasons. The active substances are mostly only sparingly soluble in water, so that administration in drinking water is not feasible. See Table 6.5-2

Similarly, inhalation is not feasible. Whole body exposure would lead to oral intake from grooming, resulting in death, and nose-only administration is not feasible because the increased handling and restraint of the test animals would promote the likelihood of haemorrhage. Dermal administration is also not feasible: rats need to be shaved frequently to expose the skin. Shaving is inevitably associated with minor cuts and haemorrhage.

8.3 Choice of species

Rodents are used in safety testing because they are small (easy to handle and house), readily available (large numbers can be bred in captivity), and they have a relatively short life span (studies are of shorter duration than with longer-lived species). In the case of rodenticides, designed to kill the wild form of the test species at low doses, long-term testing of the target species is inherently difficult. It is logical to see if there are alternative species, suitable for long-term tests that are less sensitive to these active substances. A comparison of LD_{50} values in other mammals shows that for each active substance the range of tolerance between species is generally one order of magnitude, and all are very low in absolute terms. See Table 6.5-3.

It has been shown above that a dose intended to achieve LD_{10} in two years for Bromadialone would be equivalent to 0.2ppm in the diet. A slightly less sensitive species such as the dog would need a dose of 2 ppm (by simple pro-rata increase of the dose in proportion to the ratio of LD_{50}s) to reach LD_{10}. Dietary concentrations of 2 ppm are still very difficult to achieve accurately.

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There are also practical considerations in performing carcinogenicity studies in large animals such as dogs, pigs or cats. In theory, a carcinogenicity study should be performed over the life span of an animal. This is two years in the rat, but is seven to ten years in the dog and pig, and ten to fifteen years in the cat. Studies of one year duration are performed on pesticides in the dog, but these are considered extensions of the 90-day subchronic study, rather than chronic studies. Dogs are amenable to laboratory housing over lengthy periods; cats are not. They require frequent handling if they are not to revert to feral behaviour and they do not respond well to being caged.

There is also the statistical power of such a study. The EC Guidelines for carcinogenicity (B.32, B.33, Directive 87/302/EEC) recommend 100 rodents per group (50 male and 50 female), with at least three treated groups plus one control. One year dog studies are typically performed with four males and four females per group.

The following statistical proof (from Quantics Consulting, 2004, based on 'The design and analysis of long term animal experiments', Gart JJ, Krewski D, Lee PN, Tarone RE, Wahrendorf J.1986. IARC Scientific Publications no 79. IARC, Lyon) shows that unless there are approximately 50 animals per group, it would not be possible to detect excess tumour incidences of less than 20%.

If there are N animals in each of four treatment groups: control and 3 doses.

Per organ at post mortem examination, the number of animals with at least one tumour in that organ is counted. Incidence in that group is percentage of animals with at least one tumour.

Each treated group is compared with the control group in turn. See Table 6.5-4.

It can be seen that with a background incidence of 5%, at least 46 animals would be needed per group to detect an excess of 25% (i.e. total incidence of 30%) in the treated group. Such studies are not feasible in larger (non-rodent) mammals.

In addition, there would be virtually no background control tumour incidence data on the species chosen, as such studies are rarely if ever performed in the larger mammals.

European legislation militates against the use of animals in unnecessary experimentation; the use of large mammals in such studies, particularly cats and dogs, would be considered unethical in most jurisdictions.

8.4 Antidotal treatment

Studies are presented in the dossier which administer vitamin K as an 'antidote'. These studies variously show that it is possible to use vitamin K in the treatment of low single doses of anticoagulants.

For Difethialone, rats were given diet containing the active substance at 25 ppm for either 24, 48 or 72 hours, followed by daily administration of vitamin K. The animals treated for 48 and 72 hours all died, despite the antidote, but the animals treated for 24 hours survived (reference A 6.10-03). In the dog, animals were given a single oral dose of 40 mg/kg, and vitamin K was administered as soon as prothrombin time was elevated to 2 to 4 times above background. Vitamin K administration was continued for 30 days. All dogs survived (reference A 6.10-04).

The anticoagulant active substances are highly lipophilic. They have been shown to accumulate in the liver. The inhibition of the

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regeneration of vitamin K occurs by blocking, i.e. competitive binding of the active substance and the vitamin K reductase enxyme (see above) to form a lipophilic complex, which will accumulate in the liver in the same manner as the active substance. Long term co-administration of vitamin K as an antidote, would result in the accumulation in the liver of the lipophilic complex; not the active substance. As there would be no free active substance present the test would not be valid.

8.5 Absence of carcinogenic risk

The anticoagulant action is the sole pharmacological action of the materials. The mode of action has been described in detail. It is difficult to demonstrate that this is the sole mode of action, as administration is acutely lethal, but it is supported by the available short-term toxicology data and an investigation into possible pharmacological or neurotoxicological effects (see IIIA 6.9-01). The absence of any other toxic effect indicates that the probability of a physiological effect (such as chronic irritation of gut walls leading to hyperplasia, or adaptive proliferation of liver or kidney cells in response to increased workload) leading to non-genotoxic carcinogenesis is low. Indeed the very long half-lives and accumulation within the liver indicate that the liver is unable actively to excrete the active substances, further indicating that a proliferative or adaptive response is unlikely in that organ. The 90-day rat study showed no indications of any adverse hyperplasia or hypertrophy in the target organ, the liver, at near-lethal levels of administration.

The absence of carcinogenic potential is further supported by the fact that mutagenicity studies on the active substances are negative. Given that the materials are not mutagenic/genotoxic, the likely mechanisms of carcinogenicity are limited to those resulting from effects such as hepatic hypertrophy, or irritance, and short-term studies show that there are no responses of that nature. It is reasonable to conclude that the active substances have no carcinogenic potential. This is supported by human data (see below).

9 USE OF OTHER DATA

9.1 Data evaluated with regard to agricultural use

Difethialone is not registered for agricultural uses. All of the available data are presented in the BDP dossier: no other data have been derived specifically to defend agricultural uses.

9.2 Long-term human data

There is long term experience in humans with warfarin, widely used in anti-clotting therapy in humans for over forty years, with no association with increased incidence of cancer.

Warfarin was the first of the anti-vitamin K rodenticides. The anticoagulant rodenticides fall into two categories: inandones, such as chlorophacinone, and hydroxycoumarins such as warfarin, bromadialone and difethialone.

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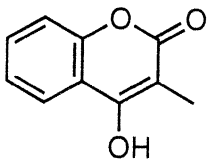
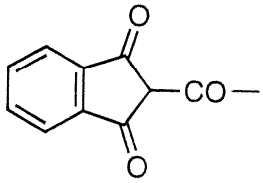
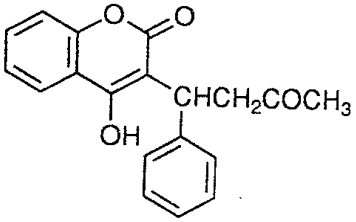
- hydroxycoumarins:



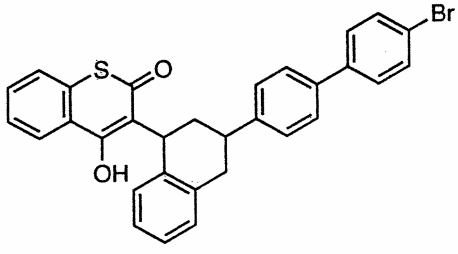
- indandiones:



The molecules all have significant structural similarity to the forms of vitamin K shown in Section 2 above. It can be seen that this structural similarity is responsible for the ability to interfere with i.e. block the enzymes used to regenerate vitamin K. The major differences in the active substances lie in the 'tail', which has varying degrees of lipophilicity. In general, the longer, and more lipophilic the 'tail' the longer the half-life, and more potent the active substance.



Warfarin



Difethialone

It has been established that the molecules are structurally similar, and all have the same mode of action. It is therefore appropriate to use information in humans in one molecule, warfarin, to support the risk

Section A6.5-01 **Long term toxicity in rats**
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assessment of Difethialone. This 'bridging' is an acceptable strategy under the TNG Risk assessment for human health (Section 3.2.2.5 '(Quantitative) structure-activity relationships ((Q)SARs)').

Warfarin is the most frequently prescribed oral anticoagulant human drug. It is the eleventh most frequently prescribed drug in the USA (EU figures not available), with annual sales of $500 million. It is used in stroke prevention, in treatment of vascular heart disease and deep vein thrombosis. For stroke and heart disease, including patients with prosthetic heart valves, duration is 'lifelong' i.e. the patient takes the drug for the rest of their life. (Horton, J., Bushwick, B.M., Warfarin therapy: Evolving strategies in anticoagulation. American Family Physician, February 1, 1999). Doses employed in humans are typically 3 – 9 mg/person/day (dose equivalent to 0.05 – 0.15 mg/kg/day for a 60 kg human [British National Formulary, March 2002]), with most doses being in the 4 – 6 mg/person/day range (Horton op cit). Treatment is associated with increased risk of bleeding episodes, but long-term use in predominantly elderly humans over forty years has not been associated with any increased risk of tumours. The sole long-term effect is bone protein depletion in female humans after 10-12 years of continuous use (WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995).

The absence of adverse effects in millions of humans following four decades of long term warfarin therapy is considered sufficient evidence that warfarin is not carcinogenic. The structural similarity of Difethialone to warfarin, together with the negative results in the guideline mutagenicity tests, indicates that Difethialone is not carcinogenic.

9.3 Exposure

The predominant use of anticoagulant rodenticides is at bait points, (varying in design for given situations to provide on a case-by-case basis for protection from enviromental factors such as sunlight or moisture, to prevent access to or interference by non-target animals/children/humans or to incorporate more formal physical obstruction e.g, enclosed boxes designed to be 'tamper-proof'), protected such that members of the general public cannot easily gain access to the baits within. This minimises the chances of secondary exposure, and reduces risk.

Where sale to the general public is permitted, block baits (and some pelleted and grain baits) are sold in plastic (LDPE) sachets, such that the user is not directly exposed to the bait. In theory, exposure could occur when partly used baits are cleared up. In this case, exposure should again be minimal, because the user should wear protective equipment (rubber gloves) to guard against rodent-born disease, such as leptospirosis and hepatitis. Amateur use is intermittent, typically occurring at a maximum of three times a year. This does not constitute long term exposure.

In terms of long-term risk, manufacturers regularly monitor the health of personnel, including regular assessment of clotting times. This immediately provides a warning if exposure is occurring, and allow for both vitamin K administration (if necessary to remedy the individual condition) and implementation of measures to prevent further exposure. Pest control operators are advised to wear protective clothing, not only because of the inherent acute toxicity of the active substances, but principally because the wild rodents themselves are significant disease vectors.

Section A6.5-01 Annex Point 6.5	Long term toxicity in rats
	10 CONCLUSION In conclusion, a waiver for long-term rodent studies on anticoagulant rodenticides is scientifically justified, based on lack of mutagenic/genotoxic effects, absence of any other effects that may lead to non-genotoxic carcinogenesis, and the absence of any carcinogenic effects following long-term administration of a closely-related molecule in humans. A waiver of the studies is further supported by the practical difficulties of performing a study, and the low risk of exposure in manufacturing and use. The practical difficulties of long-term administration of anticoagulants are such that an attempt at a study would be certain to fail; knowing this in advance is unethical and contrary to Directive 86/609/EEC. For the Biocidal Products Directive 98/8/EEC, a waiver for the requirement to submit rodent carcinogenicity/toxicity studies under Annex IIA, Section 6.7 is requested.
References	
Undertaking of intended data submission [ ]	*Give date on which the data will be handed in later (Only acceptable if test or study is already being conducted and the responsible CA has agreed on the delayed data submission.)*
	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	20 September 2005, revised 6 December 2006
Evaluation of applicant's justification	The Rapporteur Member State agrees with the Applicant's justification for non-submission of data. The very high toxicity of Difethialone makes it difficult to design a meaningful carcinogenicity/chronic toxicity study. In addition, Difethialone has shown a lack of genotoxic properties in the performed studies and the mechanism of toxicity is fairly well documented. As anticoagulant rodenticides share the same mechanism of toxicity, a read-across from Warfarin is a reasonable approach for the assessment of long-term toxicity of Difethialone in humans. There is no evidence of carcinogenicity from long-term use of Warfarin. However, bone protein depletion in women has been associated with long-term Warfarin therapy and could therefore be a hazard from long-term exposure to other anticoagulants.
Conclusion	Acceptable
Remarks	-

Table 6.5-1

Rodenticide	**Terminal Half-life***	**Species**
Brodifacoum	130 days	Rat (liver)
Brodifacoum	282 days+	Rat (liver)
Bromadiolone	318 days+	Rat (liver)
Difenacoum	120 days	Rat (liver)
Difethialone	126 days	Rat (liver)
Diphacinone	~8 days	Rat
Flocoumafen	220 days +	Rat (liver)
Warfarin	42 hours	Human (plasma)

* After WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995)
+ LiphaTech (unpublished 1986)

Table 6.5-2

Rodenticide	**Water solubility mg/L 20°C* (+ = 25°C)**
Brodifacoum	<10
Bromadialone	19
Chlorophacinone	100
Coumachlor	0.5
Coumatetralyl	425
Difenacoum	<10
Difethialone	0.39+
Diphacinone	0.3
Flocoumafen	1.1 (22°C)
Pindone	18+
Warfarin	insoluble

* After WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995)

Table 6.5-3

Rodenticide	**Acute oral (LD_{50} mg/kg) in species*:**						
	Rat	**Guinea-pig**	**Rabbit**	**Dog**	**Cat**	**Sheep**	**Pig**
Brodifacoum	0.26	2.78	0.29	0.25-3.56	~25	>25	0.5-2
Bromadiolone	>0.56-<0.84	2.8	1.0	10[+]	>25[+]	-	3
Difenacoum	1.8	50	2	~50	100	100	80-100
Difethialone	0.56	-	0.75	11.8[@]	>16[@]	-	2-3[@]
Diphacinone	3.0	-	35	3-7.5	14.7	-	150
Flocoumafen	0.46	>10	0.7	0.075-0.25	>10	>5	~60
Warfarin	58.0	-	800	20-50	6-40	-	1-5

* After WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995)
Bromadiolone rat data: LiphaTech (unpublished 1987)

[+] MTD

[@] LiphaTech data

Table 6.5-4

	Number per group required to detect excess of*:					
Background incidence:	*1%*	*5%*	*10%*	*15%*	*20%*	*25%*
0%	1051	206	100	65	47	37
1%	2729	270	115	71	51	39
5%	9101	514	173	95	63	46
10%	16294	788	237	122	77	54

* alpha 5%, power 90%. ONE sided test

Section A 6.6.4 **Annex Point IIA VI.6.6.4**	**In-vivo mutagenicity (bone marrow)** *In vivo* mouse micronucleus test	
	11 REFERENCE	**Official use only**
11.1 Reference	1. Xxxxxx, X. (XXXXx) Dose rangefinding acute toxicity study on difethialone technical grade. Xxxxxxxx Xxxxxxxxxxxxxx, XXX, laboratory report no. XXXXXXXXXXXX Report date XX Xxxxxxxxxx XXXX (unpublished). **A6.6.4-01. See point 3.1.2.4** 2. Xxxxxx, X. (XXXXx) Mutagenicity test on difethialone technical grade in an *in vivo* mammalian micronucleus assay. Xxxxxxxx Xxxxxxxxxxxxxx, XXX, laboratory report no. XXXXXXXXXXXX Report date XX Xxxxxxxxxx XXXX (unpublished). **A6.6.4-02**.	
11.2 Data protection	Yes	
11.2.1 Data owner	LiphaTech S.A.S.	
11.2.2 Companies with letter of access	None	
11.2.3 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.	
	12 GUIDELINES AND QUALITY ASSURANCE	
12.1 Guideline study	Yes. EPA 84-2b. The study design was in accordance with EC Method B.12. using a repeated administration regimen with sampling at 24 hours after dosing.	
12.2 GLP	Yes	
12.3 Deviations	No	
	13 MATERIALS AND METHODS	
13.1 Test material	As given in section 2. Difethialone technical grade.	
13.1.1 Lot/Batch number	XXX XXXX	
13.1.2 Specification	As given in section 2.	
13.1.2.1 Description	Off-white powder	
13.1.2.2 Purity	XX.XX% active substance	
13.1.2.3 Stability	Not stated in report	
13.1.2.4 Maximum tolerable dose	The preliminary study (Reference 1, point 1.1) investigated a range of dose concentrations administered by intraperitoneal injection on three consecutive days. Groups of five male and five female mice (approximately 8 weeks old in a bodyweight range of 28.7 to 35.9g (males) and 22.2 to 31.1g (females)) were dosed at 0.1, 1, 5, 10, 15, 20 or 40 mg/kg bw. All males and all females dosed at 1 mg/kg bw or above died (except for one female dosed at 10 mg/kg bw). Deaths occurred between day 3 and day 11. There were no deaths in the 0.1 mg/kg group. The maximum tolerable dose established in this study was 0.1 mg/kg bw.	X
13.2 Test Animals		

Section A 6.6.4 **Annex Point IIA VI.6.6.4**	**In-vivo mutagenicity (bone marrow)** *In vivo* mouse micronucleus test	
13.2.1 Species	Mouse	
13.2.2 Strain	ICR	
13.2.3 Source	Harlan Sprague-Dawley, Inc. Frederick, MD, USA	
13.2.4 Sex	Male and female (see 13.2.6)	
13.2.5 Age/weight at study initiation	Approximately 8 weeks old at time of dosing. Male bodyweight range 28.5 to 37.3g and female range 21.9 to 31.8g.	
13.2.6 Number of animals per group	15 males and 15 females in the dose group. 5 males and 5 females were selected for the micronucleus bioassay.	
13.2.7 Control animals	Yes. 5 males and 5 females dosed with vehicle alone (corn oil, 10 mL/kg bw) and 5 males and 5 females dosed with positive control (cyclophosphamide, 80 mg/kg bw).	
13.3 Administration/ Exposure	Intraperitoneal	
13.3.1 Number of applications	3 In the preliminary investigation, death occurred at all dose levels greater than 0.1 mg/kg bw. However the onset of mortality was to most cases delayed to between 4 and 9 days after three consecutive daily single administrations. Since the mortality pattern was erratic and effects were not apparent shortly after completion of dosing, it was decided to select a dose regimen that would exacerbate the toxic insult and at same time increase the group size to ensure adequate numbers of animals were available for the micronucleus assay.	
13.3.2 Interval between applications	24 h	
13.3.3 Postexposure period	24 h after treatment for the micronucleus assay. The surviving additional animals were observed for up to 7 days after dosing and were then killed.	
13.3.4 Vehicle	Corn oil	
13.3.5 Concentration in vehicle	2 mg/mL; 0.2%	
13.3.6 Total volume applied	10 mL/kg	
13.3.7 dose applied	20 mg/kg bw	X
13.3.8 Substance used as Positive Control	Cyclophosphamide (monohydrate) 80 mg/kg.	
13.3.9 Controls	Vehicle control (corn oil)	

Section A 6.6.4 **Annex Point IIA VI.6.6.4**	**In-vivo mutagenicity (bone marrow)** *In vivo* mouse micronucleus test		
13.4 Examinations			
13.4.1 Clinical signs	Yes. The clinical condition of all animals was observed up to point of removal of animals for the micronucleus assay. The remaining animals were observed for up to 7 days after dosing when all survivors were terminated due to moribundity.		
13.4.2 Tissue	Bone marrow		
	Number of animals:	5 males and 5 females from each treatment group	
	Number of cells:	1000	
	Time points:	24 h after treatment. EC test guidelines state that no standard treatment can be recommended for the mouse micronucleus test (Directive 2000/32/EC; L136/52 para 1.5.2) but indicate options for a single dose or repeated dose regimen. The study reported here followed the recommendations for repeated administration except for the collection of a second bone marrow sample between 36 and 48 hours after the last dosing occasion. The use of a single sampling endpoint was justified on basis the findings of Heddle *et al*. 1991.[1]	X
	Type of cells	erythrocytes in bone marrow	
	Parameters:	polychromatic/normochromatic erythrocyte ratio	
13.5 Further remarks	Prepared slides were scored for micronuclei and the PCE:NCE cell ratio. The frequency and percent micronucleated cells was reported. Laboratory background frequency for micronuclei in the mouse strain used is 0.0 to 0.4%. Micronuclei were identified using the criteria of Schmid (1976). A positive response was concluded if there was a statistically significant increase in treatment group PCE's in comparison with the vehicle control.		
	14 RESULTS AND DISCUSSION		
14.1 Clinical signs	From the test group of 15 males and 15 females, five males and one female died prior to the 24-hour harvest point. Ante-mortem signs were typical of an anti-coagulant rodenticide. Animals were commonly languid and occasionally prostrate with bleeding apparent, normally from the area of eartag attachment. Signs of poor grooming behaviour and pallor were noted and one animal had a large haematoma in the lower abdominal area by six days after the last dose administration. All surviving animals were terminated seven days after dosing due to poor clinical condition.		
14.2 Haematology / Tissue examination	Difethialone did not induce an increase in micronucleated polychromatic erythrocytes in comparison with the vehicle control. The positive control did significantly increase the number of micronucleated cells in both sexes confirming the sensitivity of the methods used. The results are summarised in Table A6.6.4-1.		

[1] Heddle, J.A., Cimino, M.O., Hayashi, M., Romagna, F., Shelby, M.D., Tucker, J.D., Vanparys, Ph., and MacGregor, J.T.: Micronuclei as an Index of Cytogenetic damage: Past, Present and Future. Env. Mol. Mutagen., **18**:277-291, 1991.

Section A 6.6.4 **Annex Point IIA VI.6.6.4**	**In-vivo mutagenicity (bone marrow)** *In vivo* mouse micronucleus test	
14.3 Genotoxicity	No	
	15 APPLICANT'S SUMMARY AND CONCLUSION	
15.1 Materials and methods	The study was designed in accordance with EPA test guideline 84-2b to evaluate the potential for difethialone to induce micronuclei in bone marrow polychromatic erythrocytes. Fifteen ICR mice of each sex were allocated to the treatment group and further groups of five mice of each sex were dosed with the vehicle or positive controls (corn oil and cyclophosphamide respectively). A single dose level, 20 mg/kg bw, was selected based on rangefinding investigations. The dose was administered by intraperitoneal injection for three consecutive days. The large group size was included to take account of anticipated significant mortality levels and to allow for sufficient animals to survive to provide bone marrow samples. Bone marrow was harvested from five males and five females of each group, 24 hours after the last dose was injected. The remaining mice were retained for a seven day post-dosing observation period to check on toxicity and clinical signs. A thousand PCEs were evaluated for each marrow smear and the number of micronucleated polychromatic erythrocytes recorded and the ratio of PCE:NCE was calculated.	
15.2 Results and discussion	Among the animals included in the study to investigate potential toxic effects, ante-mortem signs were typical of an anti-coagulant rodenticide. Animals were commonly languid and occasionally prostrate with bleeding apparent, normally from the area of eartag attachment. Signs of poor grooming behaviour and pallor were noted and one animal had a large haematoma in the lower abdominal area (6 days after the last dose administration). All surviving animals were terminated seven days after dosing due to poor clinical condition. Five males and five females were used in the micronucleus assay. Difethialone did not induce an increase in micronucleated polychromatic erythrocytes in comparison with the vehicle control. The positive control did significantly increase the number of micronucleated cells in both sexes confirming the sensitivity of the methods used.	
15.3 Conclusion	Difethialone did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes and was considered negative in the mouse micronucleus test.	
15.3.1 Reliability	2	
15.3.2 Deficiencies	No	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	2 February 2005, Revised December 2006	

Section A 6.6.4 Annex Point IIA VI.6.6.4	In-vivo mutagenicity (bone marrow) *In vivo* mouse micronucleus test	
Materials and Methods	Agree with the applicant´s summary and conclusion. **Comment (3.1.2.4):** It should have been stated that in the 0.1 mg/kg dose group no signs of toxicity was noted. **Comment (3.3.7):** The study appears to have suffered from high mortality due to the requirement to administer a sufficiently high dose to cause a measurable toxic effect. The rational for using a dose Difethialone of 20 mg/kg bw is not evident. Amongst the criteria to be taken into consideration when determining the highest amount of the test substance to be used are the cytotoxicity and the solubility in the final treatment mixture. It is stated that in this study they found a tolerable dose at 0.1 mg/kg bw, and there is no information on the PCE:NCE from the range finding study. The EU test guideline states that the dose should be the maximum tolerated dose or the one that produce some cytotoxicity to the bone marrow. From the present study summary report it is not evident that these criteria have been applied. ***Comment (3.4.2):*** The study design was in accordance with EC Method B12 using a repeated administration regimen with sampling at 24 hours after dosing. According to the guideline there exists no standard treatment schedule. Samples from extended dose regimens are acceptable as long as a positive effect has been demonstrated or, for a negative study, as long as toxicity has been demonstrated or the limit dose has been used and dosing continued until the time of sampling.	
Results and discussion	Agree with applicant´s version.	
Conclusion	Agree with applicant´s version.	
Reliability	3 **Comment:** The rational for using a high exposure dose of 20 mg/kg bw was not sufficiently discussed /justified.	
Acceptability	Acceptable	
Remarks		

Table A 6.6.4-1: Mortality table for micronucleus test preliminary investigation in-vivo

Dose mg/kg	Number of mortalities m = male; f = female									Total mortality x dead/x treated	
	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	male	female
0.1										0/5	0/5
1	1m		1m1f	2m	2f	1m	1f	1f		5/5	5/5
5		2m	1f	2m	2f	1m1f	1f			5/5	5/5
10		1f	1m		3m1f	1m1f	1f			5/5	4/5
15		2f	3m1f	1m	1f	1m	1f			5/5	5/5
20		1f		3m	2m	2f			2f	5/5	5/5
40	1m	2f	1f	1m	2m2f	1m				5/5	5/5

Table A 6.6.4-2: Table for micronucleus test *in-vivo*

		control group		Difethialone treated group		Positive control group	
Number of cells evaluated per animal		**1000**		**1000**		**1000**	
Sampling time (h)		**24 h**		**24 h**		**24 h**	
		M	**F**	**M**	**F**	**M**	**F**
Mean percent erythrocytes	**Polychromatic with micronuclei**	0.06	0.08	0.10	0.04	2.88*	2.20*
Ratio of erythrocytes	**Polychromatic with micronuclei / normochromatic**	0.84	0.95	0.53	0.56	0.35	0.61

* Significantly greater than controls, p>0.05

Section A6.7-01 Annex Point 6.7	Carcinogenicity in rats	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [x]** **Scientifically unjustified [x]**	
Limited exposure [ ]	**Other justification [ ]**	

Detailed justification:

Waiver for carcinogenicity/toxicity studies in rodents on Difethialone.

The following is a series of rationales to waive the requirement to perform carcinogenicity/chronic toxicity studies on the anticoagulant rodenticide active substance Difethialone under the Biocidal Products Directive 98/8/EEC.

16 INTRODUCTION.

The Biocidal Products Directive (98/8/EEC 'the Directive') requires long-term testing in rodents as part of the suite of toxicology tests in order to assess the possible adverse consequences of chronic exposure (i.e., chronic toxicity and carcinogenicity) to the biocidal active substance Difethialone.

It is a unique feature of the rodenticides that the test species used in long-term toxicity and carcinogenicity studies is also the target species, and that the active substances are lethal in the target species at very low levels. This gives rise to several questions: Is it relevant to consider the possible use of long term rodent studies to predict possible effects of rodenticides in humans. Is it scientifically feasible? Can the data be derived using other species? Given that at one rodenticide molecule has been used for over forty years in human medicine, are there data in the human that are more relevant than animal data would be? Are there other data that demonstrate the potential, or lack of potential, carcinogenic properties of active substances used as rodenticides?

The Directive states in Article 8 (5) that "*information which is not necessary owing to the nature of the biocidal product or of its proposed uses need not be supplied. The same applies where it is not scientifically necessary or technically possible to supply the information. In such cases, a justification, acceptable to the competent authority must be submitted…*". A more detailed waiver concept is given in the TNsG on data requirements.

The TNsG gives the strong recommendation "*to minimise testing on vertebrate animals or to avoid unnecessary suffering of experimental animals the data should not be generated*".

The TNsG recommendations were further refined in an Addendum to the TNsG entitled Refined waiving concept for rodenticides (TMII03-item9a-CA-Jun03-Doc9-TNsG.doc). These include:

The study is technically not possible to perform,

Use of other data,

Data evaluated with regard to agricultural use

Read-across from data on related substances

Evaluation of acceptable human data,

The study is not scientifically necessary

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The choice of species is not appropriate

The study is not necessary owing to limited exposure and toxicity profile.

The Notifier has prepared a scientific justification based on this guidance to waive the requirement for these studies. Before the waiving arguments are given, it will be useful to review the way the coagulation system works in mammals and the mechanism by which the anticoagulant rodenticides function.

17 FUNCTION

Anticoagulant rodenticides such as Difethialone function by inhibiting the ability of the blood to clot at the site of a haemorrhage, by blocking the regeneration of vitamin K in the liver.

Blood clots form when the soluble protein fibrinogen, normally present in the blood, is converted by the enzyme thrombin to the insoluble fibrous protein fibrin, which binds platelets and blood cells to form a solid mass referred to as a blood clot, sealing the site of the haemorrhage and preventing further blood loss. Fibrinogen is present in the blood, but thrombin is not. Thrombin factor IIa in the scheme below) is formed at the site of injury from prothrombin (factor II), which is present in the blood. Conversion of prothrombin to thrombin occurs via the coagulation cascade, in which the blood clotting factors are employed. Without these blood factors clotting cannot take place, and the haemorrhage will not be controlled by clot formation. If the blood vessel is large and/or serves a vital organ, the haemorrhage will be fatal. The synthesis of a number of blood coagulation factors (factors II [prothrombin], VII [proconvertin] IX [Christmas factor], X [Stuart-Prower factor] and the coagulation inhibiting proteins C and S) is dependent upon vitamin K, which acts as a co-enzyme.

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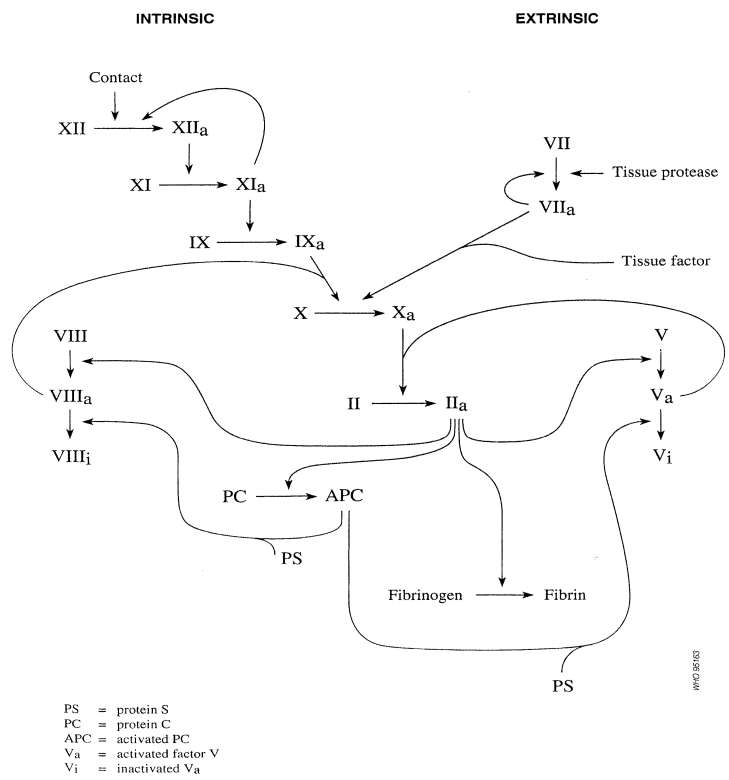
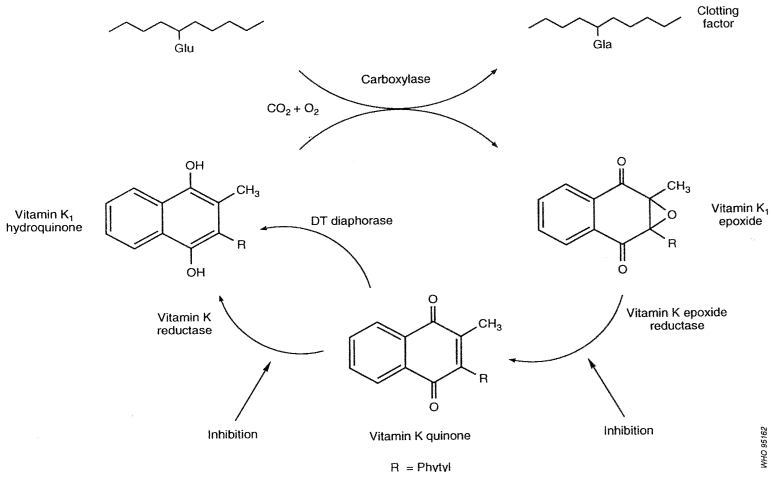


Vitamin K hydroquinone is the active co-enzyme, and its oxidation to vitamin K 2,3-epoxide provides the energy required for the carboxylation reaction where glutamate (Glu) in the precursor is converted to γ-carboxyglutamate (Gla) to make the activated clotting factor.



The anticoagulant rodenticide active substances such as Difethialone work by blocking the regeneration of vitamin K 2,3-epoxide to vitamin K hydroquinone. The Glu→Gla conversion does not take place.

The action is cumulative, increasing levels of the anticoagulant leading to increased clotting times, such that in the event of a significant haemorrhage, death occurs. The amount of vitamin K in the body is finite, and progressive blocking of the regeneration of vitamin K will lead to an increasing probability of a fatal haemorrhage. In general terms, progressive intake of anticoagulants results in death. The active

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substances are highly toxic and bioaccumulative. The oral LD_{50} of Difethialone is 0.56 mg/kg. Rodenticide baits generally contain 25 ppm Difethialone and are fatal after one meal.

18 TECHNICAL FEASIBILITY

Carcinogenicity/toxicity studies seek to determine the consequences of long-term (near life-span) exposure to the active substance by the daily, dietary administration for two years of (typically) three increasing doses to groups of rats or mice, and observing their effects in comparison to a similar group of untreated animals (the control group).

18.1 Dose-setting and the Maximum Tolerated Dose

In order to demonstrate the validity of long-term carcinogenicity/toxicity study, the highest dose should induce some form of toxicity. This toxic effect is not necessarily carcinogenicity *per se* but should be a difference from the control group that can be demonstrated experimentally (e.g. reduced body-weight gain, altered enzyme levels, changes in function of an organ exhibited by either weight change or histopathology). This measurable indicator of toxicity should be present in the high dose level, ideally at a level that does not affect the animals sufficiently to affect survival adversely over the length of the study. This high dose level referred to as the Maximum Tolerated Dose (MTD) and, conventionally, should not cause more than 10% mortality above that observed in the control group.

Studies without an MTD are considered invalid by many regulatory authorities. The intention is to administer sufficient test material such that the animal has to respond to the chemical burden i.e. it is placed under toxic stress. The implication is that if the animal does not respond to the stress by showing increased incidence of tumours, then the chemical is considered unlikely to be carcinogenic in man. Secondly, if the animal is not stressed sufficiently to show MTD response, it has not been stressed sufficiently to demonstrate the potential to cause increased incidence of tumours.

A difficulty in the administration of an MTD in a two-year study is caused by the fact that the anticoagulants are not excreted rapidly. Terminal half-lives in the liver are relevant, as the liver is the site of vitamin K regeneration, and these half-lives are very long. See Table 6.7-1.

Warfarin has the lowest half-life at 42 hours in human plasma. Human liver data are not available (because liver biopsy is too hazardous for routine investigation in humans), but the liver half life is predicted to be several days, where ‘several’ is probably greater than ten but less than one hundred). Absorbed doses accumulate, and lethality occurs when a threshold dose is exceeded. This may occur after one or two large doses, or several smaller doses.

It is feasible to conduct short-term animal studies with these substances because it is possible to ensure that the accumulated dose does not exceed lethal levels. However, the LD_{50} of these molecules is very low and, since the level for low lethality (e.g. LD_{10}) will be lower still, the amount to be administered daily over a two year study, in order to deliver (but not to exceed) an LD_{10}, would technically impossible to achieve. For example, for bromadiolone, the LD_{50} in rats is >0.56 mg/kg but < 0.84 mg/kg. A reasonable estimate of the LD_{10} (a value that would theoretically induce 10% mortality allowed in a long-term rodent study) is 0.6 mg per animal during the study. Using excretion data for

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bromadiolone, and computer software it can be shown that over the 730 days of a typical rat carc/tox study, to reach the LD_{10} by termination would require daily doses (at food intake of 25 g/rat/day) of 0.2 ppm. This is not a feasible level of dietary inclusion.

18.2 Route of Administration of the Test Substance

Dietary admixture is the only practical long-term route for administration of the test substance. It is not feasible accurately to prepare homogenous rodent test diets (to the standards required by GLP and Guidelines) at the very low concentrations needed for the MTD (i.e. 0.2 ppm as shown above). Even lower concentrations would be required for the other dose levels and these would approach the analytical method limit of detection of 0.02 ppm. It may be argued that a regulator would not expect accurate formulations, but that a study should be performed anyway. However, if inhomogeneous diet were administered, some rats would be given a feed ration that contained too much active substance, which could simply be fatal to that entire cage of five rats. Even if the rats were housed singly, the risk of fatality over a two-year period would be too great to anticipate enough animals surviving to the end of the study to provide meaningful data.

An alternative to dietary administration is the use of oral gavage. However, handling for gavage can lead to minor haemorrhage in the nasal passages (shown as brown facial staining), and the act of introducing the plastic or rubber gavage tube or steel cannula may cause minor haemorrhage in the buccal cavity and oesophagus. The use of this procedure daily for two years is considered unfeasible for an anticoagulant. Injection is also not worth considering for similar reasons. The active substances are mostly only sparingly soluble in water, so that administration in drinking water is not feasible. See Table 6.7-2

Similarly, inhalation is not feasible. Whole body exposure would lead to oral intake from grooming, resulting in death, and nose-only administration is not feasible because the increased handling and restraint of the test animals would promote the likelihood of haemorrhage. Dermal administration is also not feasible: rats need to be shaved frequently to expose the skin. Shaving is inevitably associated with minor cuts and haemorrhage.

18.3 Choice of species

Rodents are used in safety testing because they are small (easy to handle and house), readily available (large numbers can be bred in captivity), and they have a relatively short life span (studies are of shorter duration than with longer-lived species). In the case of rodenticides, designed to kill the wild form of the test species at low doses, long-term testing of the target species is inherently difficult. It is logical to see if there are alternative species, suitable for long-term tests that are less sensitive to these active substances. A comparison of LD_{50} values in other mammals shows that for each active substance the range of tolerance between species is generally one order of magnitude, and all are very low in absolute terms. See Table 6.7-3.

It has been shown above that a dose intended to achieve LD_{10} in two years for Bromadialone would be equivalent to 0.2ppm in the diet. A slightly less sensitive species such as the dog would need a dose of 2 ppm (by simple pro-rata increase of the dose in proportion to the ratio of LD_{50}s) to reach LD_{10}. Dietary concentrations of 2 ppm are still very difficult to achieve accurately.

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Carcinogenicity in rats

There are also practical considerations in performing carcinogenicity studies in large animals such as dogs, pigs or cats. In theory, a carcinogenicity study should be performed over the life span of an animal. This is two years in the rat, but is seven to ten years in the dog and pig, and ten to fifteen years in the cat. Studies of one year duration are performed on pesticides in the dog, but these are considered extensions of the 90-day subchronic study, rather than chronic studies. Dogs are amenable to laboratory housing over lengthy periods; cats are not. They require frequent handling if they are not to revert to feral behaviour and they do not respond well to being caged.

There is also the statistical power of such a study. The EC Guidelines for carcinogenicity (B.32, B.33, Directive 87/302/EEC) recommend 100 rodents per group (50 male and 50 female), with at least three treated groups plus one control. One year dog studies are typically performed with four males and four females per group.

The following statistical proof (from Quantics Consulting, 2004, based on 'The design and analysis of long term animal experiments', Gart JJ, Krewski D, Lee PN, Tarone RE, Wahrendorf J.1986. IARC Scientific Publications no 79. IARC, Lyon) shows that unless there are approximately 50 animals per group, it would not be possible to detect excess tumour incidences of less than 20%.

If there are N animals in each of four treatment groups: control and 3 doses.

Per organ at post mortem examination, the number of animals with at least one tumour in that organ is counted. Incidence in that group is percentage of animals with at least one tumour.

Each treated group is compared with the control group in turn. See Table 6.7-4.

It can be seen that with a background incidence of 5%, at least 46 animals would be needed per group to detect an excess of 25% (i.e. total incidence of 30%) in the treated group. Such studies are not feasible in larger (non-rodent) mammals.

In addition, there would be virtually no background control tumour incidence data on the species chosen, as such studies are rarely if ever performed in the larger mammals.

European legislation militates against the use of animals in unnecessary experimentation; the use of large mammals in such studies, particularly cats and dogs, would be considered unethical in most jurisdictions.

18.4 Antidotal treatment

Studies are presented in the dossier which administer vitamin K as an 'antidote'. These studies variously show that it is possible to use vitamin K in the treatment of low single doses of anticoagulants.

For Difethialone, rats were given diet containing the active substance at 25 ppm for either 24, 48 or 72 hours, followed by daily administration of vitamin K. The animals treated for 48 and 72 hours all died, despite the antidote, but the animals treated for 24 hours survived (reference A 6.10-03). In the dog, animals were given a single oral dose of 40 mg/kg, and vitamin K was administered as soon as prothrombin time was elevated to 2 to 4 times above background. Vitamin K administration was continued for 30 days. All dogs survived (reference A 6.10-04).

The anticoagulant active substances are highly lipophilic. They have been shown to accumulate in the liver. The inhibition of the

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regeneration of vitamin K occurs by blocking, i.e. competitive binding of the active substance and the vitamin K reductase enxyme (see above) to form a lipophilic complex, which will accumulate in the liver in the same manner as the active substance. Long term co-administration of vitamin K as an antidote, would result in the accumulation in the liver of the lipophilic complex; not the active substance. As there would be no free active substance present the test would not be valid.

18.5 Absence of carcinogenic risk

The anticoagulant action is the sole pharmacological action of the materials. The mode of action has been described in detail. It is difficult to demonstrate that this is the sole mode of action, as administration is acutely lethal, but it is supported by the available short-term toxicology data and an investigation into possible pharmacological or neurotoxicological effects (see IIIA 6.9-01). The absence of any other toxic effect indicates that the probability of a physiological effect (such as chronic irritation of gut walls leading to hyperplasia, or adaptive proliferation of liver or kidney cells in response to increased workload) leading to non-genotoxic carcinogenesis is low. Indeed the very long half-lives and accumulation within the liver indicate that the liver is unable actively to excrete the active substances, further indicating that a proliferative or adaptive response is unlikely in that organ. The 90-day rat study showed no indications of any adverse hyperplasia or hypertrophy in the target organ, the liver, at near-lethal levels of administration.

The absence of carcinogenic potential is further supported by the fact that mutagenicity studies on the active substances are negative. Given that the materials are not mutagenic/genotoxic, the likely mechanisms of carcinogenicity are limited to those resulting from effects such as hepatic hypertrophy, or irritance, and short-term studies show that there are no responses of that nature. It is reasonable to conclude that the active substances have no carcinogenic potential. This is supported by human data (see below).

19 USE OF OTHER DATA

19.1 Data evaluated with regard to agricultural use

Difethialone is not registered for agricultural uses. All of the available data are presented in the BDP dossier: no other data have been derived specifically to defend agricultural uses.

19.2 Long-term human data

There is long term experience in humans with warfarin, widely used in anti-clotting therapy in humans for over forty years, with no association with increased incidence of cancer.

Warfarin was the first of the anti-vitamin K rodenticides. The anticoagulant rodenticides fall into two categories: inandones, such as chlorophacinone, and hydroxycoumarins such as warfarin, bromadialone and difethialone.

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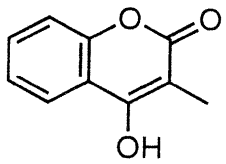
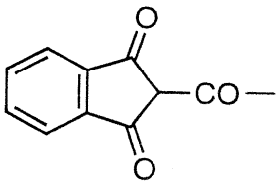
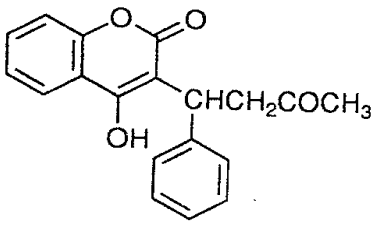
- hydroxycoumarins:



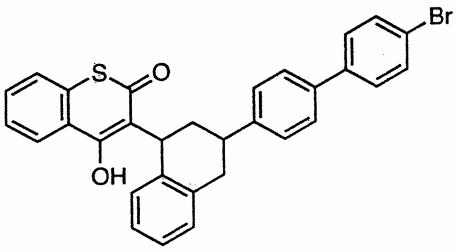
- indandiones:



The molecules all have significant structural similarity to the forms of vitamin K shown in Section 2 above. It can be seen that this structural similarity is responsible for the ability to interfere with i.e. block the enzymes used to regenerate vitamin K. The major differences in the active substances lie in the 'tail', which has varying degrees of lipophilicity. In general, the longer, and more lipophilic the 'tail' the longer the half-life, and more potent the active substance.



Warfarin



Difethialone

It has been established that the molecules are structurally similar, and all have the same mode of action. It is therefore appropriate to use information in humans in one molecule, warfarin, to support the risk assessment of Difethialone. This 'bridging' is an acceptable strategy under the TNG Risk assessment for human health (Section 3.2.2.5 '(Quantitative) structure-activity relationships ((Q)SARs)').

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Carcinogenicity in rats

Warfarin is the most frequently prescribed oral anticoagulant human drug. It is the eleventh most frequently prescribed drug in the USA (EU figures not available), with annual sales of $500 million. It is used in stroke prevention, in treatment of vascular heart disease and deep vein thrombosis. For stroke and heart disease, including patients with prosthetic heart valves, duration is 'lifelong' i.e. the patient takes the drug for the rest of their life. (Horton, J., Bushwick, B.M., Warfarin therapy: Evolving strategies in anticoagulation. American Family Physician, February 1, 1999). Doses employed in humans are typically 3 – 9 mg/person/day (dose equivalent to 0.05 – 0.15 mg/kg/day for a 60 kg human [British National Formulary, March 2002]), with most doses being in the 4 – 6 mg/person/day range (Horton op cit). Treatment is associated with increased risk of bleeding episodes, but long-term use in predominantly elderly humans over forty years has not been associated with any increased risk of tumours. The sole long-term effect is bone protein depletion in female humans after 10-12 years of continuous use (WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995).

The absence of adverse effects in millions of humans following four decades of long term warfarin therapy is considered sufficient evidence that warfarin is not carcinogenic. The structural similarity of Difethialone to warfarin, together with the negative results in the guideline mutagenicity tests, indicates that Difethialone is not carcinogenic.

19.3 Exposure

The predominant use of anticoagulant rodenticides is at bait points, (varying in design for given situations to provide on a case-by-case basis for protection from enviromental factors such as sunlight or moisture, to prevent access to or interference by non-target animals/children/humans or to incorporate more formal physical obstruction e.g, enclosed boxes designed to be 'tamper-proof'), protected such that members of the general public cannot easily gain access to the baits within. This minimises the chances of secondary exposure, and reduces risk.

Where sale to the general public is permitted, block baits (and some pelleted and grain baits) are sold in plastic (LDPE) sachets, such that the user is not directly exposed to the bait. In theory, exposure could occur when partly used baits are cleared up. In this case, exposure should again be minimal, because the user should wear protective equipment (rubber gloves) to guard against rodent-born disease, such as leptospirosis and hepatitis. Amateur use is intermittent, typically occurring at a maximum of three times a year. This does not constitute long term exposure.

In terms of long-term risk, manufacturers regularly monitor the health of personnel, including regular assessment of clotting times. This immediately provides a warning if exposure is occurring, and allow for both vitamin K administration (if necessary to remedy the individual condition) and implementation of measures to prevent further exposure. Pest control operators are advised to wear protective clothing, not only because of the inherent acute toxicity of the active substances, but principally because the wild rodents themselves are significant disease vectors.

20 CONCLUSION

In conclusion, a waiver for long-term rodent studies on anticoagulant rodenticides is scientifically justified, based on lack of

Section A6.7-01 Annex Point 6.7	Carcinogenicity in rats
	mutagenic/genotoxic effects, absence of any other effects that may lead to non-genotoxic carcinogenesis, and the absence of any carcinogenic effects following long-term administration of a closely-related molecule in humans. A waiver of the studies is further supported by the practical difficulties of performing a study, and the low risk of exposure in manufacturing and use. The practical difficulties of long-term administration of anticoagulants are such that an attempt at a study would be certain to fail; knowing this in advance is unethical and contrary to Directive 86/609/EEC. For the Biocidal Products Directive 98/8/EEC, a waiver for the requirement to submit rodent carcinogenicity/toxicity studies under Annex IIA, Section 6.7 is requested.
References	
Undertaking of intended data submission [ ]	*Give date on which the data will be handed in later (Only acceptable if test or study is already being conducted and the responsible CA has agreed on the delayed data submission.)*
	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	20 September 2005, revised 6 December 2006
Evaluation of applicant's justification	The Rapporteur Member State agrees with the Applicant's justification for non-submission of data. The very high toxicity of Difethialone makes it difficult to design a meaningful carcinogenicity/chronic toxicity study. In addition, Difethialone has shown a lack of genotoxic properties in the performed studies and the mechanism of toxicity is fairly well documented. As anticoagulant rodenticides share the same mechanism of toxicity, a read-across from Warfarin is a reasonable approach for the assessment of long-term toxicity of Difethialone in humans. There is no evidence of carcinogenicity from long-term use of Warfarin. However, bone protein depletion in women has been associated with long-term Warfarin therapy and could therefore be a hazard from long-term exposure to other anticoagulants.
Conclusion	Acceptable
Remarks	-

Table 6.7-1

Rodenticide	**Terminal Half-life***	**Species**
Brodifacoum	130 days	Rat (liver)
Brodifacoum	282 days+	Rat (liver)
Bromadiolone	318 days+	Rat (liver)
Difenacoum	120 days	Rat (liver)
Difethialone	126 days	Rat (liver)
Diphacinone	~8 days	Rat
Flocoumafen	220 days +	Rat (liver)
Warfarin	42 hours	Human (plasma)

* After WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995)
+ LiphaTech (unpublished 1986)

Table 6.7-2

Rodenticide	**Water solubility mg/L 20°C* (+ = 25°C)**
Brodifacoum	<10
Bromadialone	19
Chlorophacinone	100
Coumachlor	0.5
Coumatetralyl	425
Difenacoum	<10
Difethialone	0.39+
Diphacinone	0.3
Flocoumafen	1.1 (22°C)
Pindone	18+
Warfarin	insoluble

* After WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995)

Table 6.7-3

Rodenticide	**Acute oral (LD_{50} mg/kg) in species*:**						
	Rat	**Guinea-pig**	**Rabbit**	**Dog**	**Cat**	**Sheep**	**Pig**
Brodifacoum	0.26	2.78	0.29	0.25-3.56	~25	>25	0.5-2
Bromadiolone	>0.56-<0.84	2.8	1.0	10[+]	>25[+]	-	3
Difenacoum	1.8	50	2	~50	100	100	80-100
Difethialone	0.56	-	0.75	11.8[@]	>16[@]	-	2-3[@]
Diphacinone	3.0	-	35	3-7.5	14.7	-	150
Flocoumafen	0.46	>10	0.7	0.075-0.25	>10	>5	~60
Warfarin	58.0	-	800	20-50	6-40	-	1-5

* After WHO/IPCS Environmental Health Criteria 175 Anticoagulant Rodenticides (WHO Geneva 1995)
Bromadiolone rat data: LiphaTech (unpublished 1987)

[+] MTD

[@] LiphaTech data

Table 6.7-4

	Number per group required to detect excess of*:					
Background incidence:	*1%*	*5%*	*10%*	*15%*	*20%*	*25%*
0%	1051	206	100	65	47	37
1%	2729	270	115	71	51	39
5%	9101	514	173	95	63	46
10%	16294	788	237	122	77	54

* alpha 5%, power 90%. ONE sided test

Section A 6.8.1-02 **Annex Point IIA VI.6.8.1**	**Teratogenicity study** Oral teratology study in rat	
	21 REFERENCE	**Official use only**
21.1 Reference	Xxxxxxxx,XX. (XXXXx). Oral teratology in the rat. xxxxxxxxxxxxxxxxxxxxxxxx, XXXXXXX, Xxxxxx. Report number XXXXXX. Report dated XX Xxxxxxx XXXX (unpublished)	
21.2 Data protection	Yes	
21.2.1 Data owner	LiphaTech S.A.S.	
21.2.2 Companies with letter of access	None	
21.2.3 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.	
	22 GUIDELINES AND QUALITY ASSURANCE	
22.1 Guideline study	Yes. EPA 83-3. Study design was in accordance with EC Method B.31	
22.2 GLP	Yes	
22.3 Deviations	No	
	23 MATERIALS AND METHODS	
23.1 Test material	As given in section 2. XXXXXXX. Supplied as a stock solution containing 1000 ppm Difethialone in PEG 300.	
23.1.1 Lot/Batch number	XXXXXX	
23.1.2 Specification	As given in section 2	
23.1.2.1 Description	No description provided in report	
23.1.2.2 Purity	1.01 g/L	X
23.1.2.3 Stability	No stability information provided in report. Dose formulations were prepared freshly each day.	
23.2 Test Animals	Non-entry field	
23.2.1 Species	Rat	
23.2.2 Strain	ICO OFA Sprague-Dawley	
23.2.3 Source	XXXXXXXXX, XXXXXXXXXX, xxXxxxxxxxX, Xxxxxxxx	
23.2.4 Sex	Time-mated females	
23.2.5 Age/weight at study initiation	Approximately 8 to 10 weeks old	
23.2.6 Number of animals per group	25 females per group, 4 groups, housed individually	
23.2.7 Control animals	Yes	
23.2.8 Mating period	Pregnant females all mated on same day were received at the laboratory on gestation day 0. Day 0 of gestation was taken as day the mucus plug appeared.	
23.3 Administration/ Exposure	Oral	

Section A 6.8.1-02 **Annex Point IIA VI.6.8.1**	**Teratogenicity study** Oral teratology study in rat			
23.3.1 Duration of exposure	12 consecutive administrations from day 6 to day 17			
	rat:	day 6 – 17	post mating	
23.3.2 Postexposure period	All animals were sacrificed on gestation day 20			
23.3.3 Type	Gavage			
23.3.4 Concentration	Gavage 0, 12.5, 25.0 or 50.0 µg/kg bw/day			
23.3.5 Vehicle	PEG 300 diluted in distilled water			
23.3.6 Concentration in vehicle	A stock solution of 1000 ppm Difethialone was prepared in PEG 300. The stock was diluted in distilled water, daily, to prepare the final concentrations for administration. Final concentrations of 0, 2.5, 5.0 and 10.0 µg/mL were prepared.			
23.3.7 Total volume applied	5 mL/kg bw/day			
23.3.8 Controls	Vehicle – PEG 300 diluted in distilled water			
23.4 Examinations				
23.4.1 Body weight	Yes. Animals were weighed on days 0, 6, 11, 17 and 20			
23.4.2 Food consumption	Yes. Recorded for each animal for the periods day 0 to 6; day 6 to 11; day 11 to 17 and day 17 to 20.			
23.4.3 Clinical signs	Yes. Recorded after each daily administration.			
23.4.4 Examination of uterine content	Gravid uterine weight			
	Number of corpora lutea per ovary. Ovaries were removed and samples fixed in formalin but no histopathological examinations were completed. The weight of each dam reaching full term pregnancy was recorded before caesarean section, the weight of the gravid uterus and litter weight were also recorded. For each dam the number of ovarian corpora lutea, number of uterine implantations and number of resorptions were recorded.			
23.4.5 Examination of foetuses				
23.4.5.1 General	Number of live and dead foetuses, number of foetal resorptions, foetal and litter weight and sex ratio.			
23.4.5.2 Soft tissue	External abnormalities examined			
23.4.5.3 Skeletal examination	All live foetuses and viable still births were randomly allocated to two groups after completion of the external examination. Approximately half of each litter was fixed in ethanol for skeletal examination, the remainder were preserved in Bouin's fluid for examination of internal organs following serial sectioning.			
23.5 Further remarks	Data were analysed using Student's t-test.			

Section A 6.8.1-02 **Annex Point IIA VI.6.8.1**		**Teratogenicity study** Oral teratology study in rat	
		24 RESULTS AND DISCUSSION	
24.1	**Maternal toxic Effects**	None of the rats died (see Table 6.8.1-3) No clinical observations of reaction to treatment were observed. One female dosed at 50.0 µg/kg bw/day gave birth to a litter of 13 live pups and one still birth on gestation day 20. Spontaneous occurrence of a shortened gestation perod is occasionally observed in the Sprague-Dawley rat and this was not considered to be an effect of treatment. Food consumption was similar to controls in all treated groups. There was no treatment-related effect on bodyweight for any animals having full term pregnancies. The number of full term pregnancies was 23, 21, 25 and 25 in the control and 12.5, 25 or 50 µg/kg bw/day groups respectively. These gave 295, 274, 347 and 329 foetuses respectively. There was no clear treatment effect on litter or foetus numbers. The mean numbers of corpora lutea and implantation points were similar in each control and treated group. One instance of total resorption occurred in one low dose female with only two implantation points seen at necropsy. This was not considered attributable to treatment with difethialone. The number of uterine resorptions was lower in the high dose group than controls. Early resorptions, prior to day 10, numbered 88 in the control group and 100, 92 and 100 in the three treated groups. The relationship between litter weight and gravid uterus report, for dams sacrificed on day 20, were similar in each group. There were no effects of treatment on fertility or indications of maternal toxicity.	X
24.2	**Teratogenic / embryotoxic effects**	Malformations – indicators of teratogenic effect; defined as abnormalities that could affect foetal viability or normal foetal development (see Table 6.8.1-4). One control foetus was small (2.62g compared with group mean of 3.87g) and had dilatation of the cerebral ventricles. In the low dose group one foetus was small and had dilatation of the cerebral ventricles. A second foetus had multiple malformations including a foreshortened abdomen, acaudia with narrow anal opening, bilateral ovarian ectopia, bilateral renal atrophy, shortening and torsion of the ureters, diaphragmatic ectopia and pulmonary lobe atrophy. In the intermediate dose group, one foetus had agnathia and two had multiple malformations. One of these had pulmonary lobe atrophy, a granular appearance to the lungs, cardiac arterioectopia, a spherical heart and abdominal haemorrhage. The second had a short tail, pancreatic agenesis, splenic hypertrophy, bilateral renal atrophy, cardiac arterioectopia, dilatation of cerebral ventricles and was small (weighed 1.60g). In the high dose group, one foetus was small (2.48g compared to control mean of 3.87g) and had dilatation of the cerebral ventricles. The low incidence and lack of any dose-relationship for these commonly occuring spontaneous malformations indicated they were not attributable to treatment with difethialone.	

Section A 6.8.1-02 Annex Point IIA VI.6.8.1	**Teratogenicity study** Oral teratology study in rat	
	Minor abnormalities – including internal and external anatomical changes not classified as malformations and without foreseeable major physiological risk. Commonly observed minor changes such as dilatation and torsion of ureters, haematomas and hydronephrosis were present at a similar incidence in control and treated groups. Other minor abnormalities included: Dilatation of the right auricle for one foetus in the control group. Unilateral renal atrophy for one foetus and dilatation of the right auricle for one foetus in the low dose group. Unilateral renal ectopia for one foetus and concentric brown striations on the right renal medulla for one foetus in the intermediate group. In the high dose group, a large space between upper part of brain and top of skull for one foetus (external hydrocephalus), dilatation of the right auricle for one foetus, unilateral renal ectopia for one foetus and dilatation of the bladder for one foetus. None of these minor changes were considered attributable to treatment with difethialone. Skeletal observations – these have a common background incidence but treatment may affect the normal occurrence (e.g. incomplete or absent ossification, skeletal abnormalities and advanced ossification) (see Table 6.8.1-5). Ossification abnormalities commonly observed had a similar incidence in each of the control and treated groups. Specific changes included right hind paw metatarsal malformation for one control foetus. A fractured right femur for one foetus in the low dose group (probably mechanical damage). There were no notable skeletal malformations in the intermediate group. In the high dose group one foetus had an occipital with slight ossification abnormality. One foetus had right radius ectopia forming a triangle with the humerus and ulna. One foetus presented a ghost outline of the right femur with a shortened thigh. None of these abnormalities were considered to be treatment-related. There was no evidence of treatment related changes in incidence of incomplete, no or advanced ossification.	
	25 APPLICANT'S SUMMARY AND CONCLUSION	

Section A 6.8.1-02 **Annex Point IIA VI.6.8.1**	**Teratogenicity study** Oral teratology study in rat	
25.1 Materials and methods	Groups of twenty-five pregnant female Sprague-Dawley rats were dosed orally by gavage at dose levels of 0, 12.5, 25 or 50 µg/kg bw/day. The doses were administered daily from gestation day 6 to day 17 and animals were terminated on gestation day 20. Records of bodyweight, food consumption and clinical signs were maintained in life and at termination the maternal rats were subject to Caesarean section and examined macroscopically. Terminal investigations also included examination of ovaries to determine the number of corpora lutea and determination of gravid uterine weight. Each uterine horn was examined for live foetuses and still births; implantations and foetal resorptions. Foetuses were weighed and examined for external malformation. They were preserved (50% for skeletal examination and 50% for visceral examination), sexed and examined for malformations, minor abnormalities and skeletal changes.	
25.2 Results and discussion	There were no mortalities and no clinical signs of reaction to treatment following dosing with control or three doses of difethialone (0, 12.5, 25 or 50 µg/kg bw/day). Food consumption and bodyweight changes were similar for treated and control groups. There was no apparent effect on fertility with the number of pregnant females, number of pregnancies carried to term and number of foetuses present all showing little or no difference between treated and control groups. There was no adverse effect on the foetus. The number of live foetuses, average litter weights and sex ratio showed no treatment–related changes. Examination of the entire foetus and of organs and skeleton revealed no treatment–related changes in the incidence of teratogenic indicators.	
25.3 Conclusion	Based on the findings of this study, doses of difethialone given to rats at 12.5, 25 or 50 µg/kg bw/day during the period of organogenesis resulted in no maternal or foetal toxicity and no teratogenic effects on the foetus that could be attributed to treatment.	
25.3.1 LO(A)EL maternal toxic effects	Not applicable. An earlier range-finding study showed maternl haemorrhage and death at 50 or 70 µg/kg bw/day.	
25.3.2 NO(A)EL maternal toxic effects	50 µg/kg bw/day.	X
25.3.3 LO(A)EL embryotoxic / teratogenic effects	Not applicable	
25.3.4 NO(A)EL embryotoxic / teratogenic effects	50 µg/kg bw/day.	X
25.3.5 Reliability	2	
25.3.6 Deficiencies	No	

Section A 6.8.1-02 **Annex Point IIA VI.6.8.1**	**Teratogenicity study** Oral teratology study in rat	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	11 February 2005, revised 6 December 2006	
Materials and Methods	Agree with applicant´s summary and conclusion. **Comment (3.1.2.2):** The number 1.01 g/L reflects the amount of difethialone in the concentrate used to make up the daily doses and not the purity of the active substance.	
Results and discussion	Agree with applicant´s summary and conclusion. **Comments (4.1)** Question from FR: Could you explain where we can find or calculate the number of uterine resorptions indicated in the 8 th paragraph? The following responses to this comment was given by the applicant: Numbers of resorptions quoted in Table A6.8.1-4: The litter data are to be found in pp 73-76 of the .pdf document, but only give total resorptions i.e. the appendices do not distinguish between early and late resorptions. There are no data in the report appendices to support the statement in the text of the report on the percent incidence of so-called premature (before G10) resorptions as a proportion of the total number of resorptions. For the record, the text in the IIIA Summary 6.8.1-02 Section 4.1 should read 'The percentage of early resorptions (as a proportion of total number of resorptions i.e. early + late) was 88 ….' although the value of the statement is questionable as it cannot be verified from the appendix.	
Conclusion	Agree with applicant´s version. **Comment (5.3.2 and 5.3.4):** Since no effects were found when examining the dams and the foetuses the NOAEL values should be $\geq$ 50 µg/kg bw/day.	
Reliability	2	
Acceptability	Acceptable.	
Remarks	Difethialone did not cause any observed teratogenic effects in the oral teratogenicity study in rat. However, the conventional OECD Guideline 414 may have limitations in the detection of possible teratogenic effects of difethialone and other coumarin related compounds e.g. Warfarin as the change to placental nutrition on gestation day 12-14, and partutiration is always associated with significant haemorrhage in the mother. To prevent maternal bleeding and achieve dose levels in animals where a potential developmental toxicity could be observed, the animals should have been co-dosed with Vitamin K. Furthermore, the exposure period in the conventional OECD Guideline 414, normally from gestation day 6-15 (or 6-20) is not optimal for the detection of "Warfarin embryopathy", a syndrome of skeletal anomalies including severe nasal hypolasia and skeletal deformities, since different time schedule exist for bone development in rats and humans. The most Warfarin sensitive stages of rat facial development are probably the last few days of fetal life extending to early postnatal development. Therefore it can not be excluded that difethialone may induce teratogenic effects in rat.	

Table A 6.8.1-3: Table for teratogenic effects (separate data for all dosage groups)

Maternal effects

Parameter	control	12.5 µg/kg	25 µg/kg	50 µg/kg
Number of dams examined	**25**	**25**	**25**	**25**
Clinical findings during application of test substance: No signs observed	+	+	+	+
Mortality of dams *state %*	0	0	0	0
Total resorption	0	1	0	0
Body weight and weight gain *day 0* *day 6* *day 11* *day 17* *day 20* *day 0 to 17 (%)* *day 0 to 20 (%)*	 236 275 304 357 408 30.0 72.7	 229 264 292 347 401 31.1 75.4	 231 271 301 357 412 31.6 78.2	 227 263 296 351 401 33.4 76.6
Food consumption day 0 to 6 day 6 to 11 day 11 to 17 day 17 to 20	 136.7 134.9 177.7 93.4	 136.9 128.4 175.4 91.2	 137.8 136.2 178.0 92.3	 132.1 132.0 177.2 88.5
Pregnancies *pregnancy rate or %*	92%	84%	100%	100%

Table A 6.8.1-4: Table for teratogenic effects

Litter response (Caesarean section data)

Parameter	control	12.5 µg/kg	25 µg/kg	50 µg/kg
Mean no. of corpora lutea	17.2	16.7	16.7	16.2
Mean no. of implantations	14.3	14.0	15.4	13.6
Mean no. of resorptions	1.4	1.0	1.6	0.5
Total number of implantations	328	295	385	341
Total number of resorptions	33	21	38	18
Total number of live foetuses	295	274	347	315
Pre-implantation loss State %	2.1	13.7	7.9	16.0
Post-implantation loss State %	10.1	7.1	9.8	7.3
Total number of litters	23	21	25	25
Live foetuses / litter State ratio	12.3 23 litters	13.0 21 litters	13.9 25 litters	13.2 25 litters
Dead foetuses / litter State ratio	0	0	0	0
Foetus weight (mean) [g]	3.87	3.80	3.87	3.92
Mean gravid uterus weight [g]	75.4	76.2	81.7	77.8
Foetal sex ratio % males	52.9	51.8	43.8	50.0

Table A 6.8.1-5: Table for teratogenic effects

Examination of the foetuses

Parameter	**control**	**12.5 µg/kg**	**25 µg/kg**	**50 µg/kg**
External malformations *[%]/number examined*	**295**	**274**	**347**	**316**
Acaudia	-	0.36	–	-
Short tail	-	-	0.29	-
Bloated stomach	-	0.36	0.29	-
Agnathia	-	-	0.29	-
Haematoma on back	-	-	0.29	-
Haematoma below neck	-	-	-	0.32
Organ abnormalities *[%]/ Number examined*	**149**	**135**	**174**	**157**
Ureteral dilatation, unilateral	16.8	20.7	13.3	16.6
Ureteral dilatation, bilateral	39.6	34.8	37.0	33.1
Torsion, unilateral	27.5	29.6	24.5	28.0
Torsion, bilateral	38.3	34.1	27.8	31.8
Hydronephrosis, unilateral	29.5	25.9	21.3	26.1
Hydronephrosis, bilateral	10.1	11.9	13.2	9.6
Renal atrophy, unilateral		0.7		
Renal atrophy, bilateral		0.7	0.6	
Dilatation of cerebral ventricles	0.7	0.7	0.6	0.6
Cranial haematoma		0.7	0.6	
Space between top of skull and brain (external hydrocephalus)				0.6
Granular appearance to lungs			0.6	
Atrophy of pulmonary lobes		0.7	0.6	
Cardiac arterioectopia			1.1	
Dilatation of right auricle	0.7	0.7		0.6
Spherical heart			0.6	
Ectopia of the diaphragm		0.7		
Abdomen foreshortened		0.7		
Abdominal haemorrhage			0.6	
No pancreas			0.6	
Splenic hypertrophy			0.6	
Renal ectopia, unilateral			0.6	0.6
Concentric brownish striations in renal medulla			0.6	
Dilatation of the urinary bladder				0.6
Ovarian ectopia, bilateral		1.6		
Narrow anal opening		0.7		
Ureteral atrophy, bilateral		0.7		
Ossification abnormalities *[%]/ Number examined*	**146**	**139**	**173**	**159**
Asymmetric sternebrae 2	–	–	0.6	0.6
3	4.1	2.2	6.9	5.0
4	9.6	5.0	8.7	5.7
5	0.7	1.4	1.2	0.6
Ribs				
14 right, 13 left	1.4	0.7	1.2	1.3
14 left, 13 right	0.7	0.7	1.2	0.6
14 pairs	1.4	0.7	--	0.6
Malformation of metatarsals of right hind paw	0.7	--	--	--
Fractured femur	--	0.7	--	--
Occipital ossification abnormality	--	--	--	0.6
Ectopia of the radius	--	--	--	0.6
Ghost outline of the right femur with a reduction in the size of the thigh	--	--	--	0.6

Summary of incomplete ossification *[%] /number examined*		**146**	**139**	**173**	**159**
Sternebrae	*1*	4.8	5.8	4.0	1.3
	2	8.2	9.4	8.7	5.7
	3	9.6	12.2	6.9	6.3
	4	21.2	28.8	22.5	17.0
	5	70.5	61.2	75.7	65.4
	6	28.1	25.2	32.9	26.4
Dorsal vertebrae (centra)	*1*	26.0	28.8	16.8	16.4
	2	2.1	7.2	0.6	1.3
	3		0.7		0.6
	8			0.6	
	9				0.6
	10	2.7	3.6	2.9	3.1
	11	6.8	11.5	15.6	14.5
	12	1.4	6.5	8.7	6.9
	13		2.9	2.3	3.1
Lumbar vertebrae (centra)	*2*				0.6
Bones of the head					
palate				0.6	
presphenoid			0.7	0.6	
basisphenoid		0.7		0.6	
maxillary				0.6	
hyoid				0.6	
nasals		0.7		1.2	0.6
frontals		13.0	9.4	4.6	8.8
parietals		6.8	10.1	5.8	3.1
interparietal		41.1	33.1	34.7	40.3
occipital		48.6	47.5	47.4	53.5
zygomatic			2.9		1.3
squamosa		11.0	12.2	9.8	9.4
tympani bulb				0.6	
Femur					0.6
Right and left ischials		0.7	5.8		1.3
Right and left pubic bones		0.7	5.8		0.6
Right and left forepaws		24.7	30.2	19.7	19.5
Right forepaw			0.7	1.2	1.3
Left forepaw		0.7	0.7	2.3	
Right and left hindpaws		1.4	5.0	0.6	
Right hindpaw					0.6

Summary of absence of ossification *[%] /number examined*		**146**	**139**	**173**	**159**
Stenebrae	*1*		1.4		
	2	1.4	3.6	1.2	0.6
	3	0.7	0.7	0.6	
	4	1.4		0.6	
	5	3.4	14.4	9.2	5.7
	6	2.1	7.2	3.5	2.5
Dorsal vertebrae (centra)			5.8	2.3	1.3
Sacral vertebrae (arches)				0.6	0.6
Sacral vertebrae (arches and centra)			0.7		
Caudal vertebrae (arches)	*1*			1.2	0.6
	2	13.0	24.5	19.7	17.6
	3	93.2	89.2	90.8	92.5
	4	83.6	72.7	82.1	81.8
	5	8.9	13.7	12.1	15.1
Caudal vertebrae (arches and centra)	*1*	0.7	5.0	0.6	
	2	0.7	5.0	1.2	0.6
	3	2.1	7.2	2.3	1.9
	4	16.4	23.7	17.9	17.6
	5	91.1	84.9	87.9	84.3
Mandible				0.6	
Summary of advanced ossification *[%] /number examined*		**146**	**139**	**173**	**159**
Cervical vertebrae (centra)	*1*	18.5	18.0	28.3	25.2
	2	0.7			
	3				1..3
	4	2.1	0.7	0.6	3.1
	5	2.1	2.2	2.9	3.8
	6	1.4	2.2	5.2	6.9
	7	28.8	31.7	38.2	34.0
Phalanges: Right and left paws		35.6	45.3	47.4	47.8
Phalanges: right paw			2.9	0.6	1.3
Point of ossification at junction of left parietal and the squamosa			0.7		
Frontals			0.7		
Parietals			0.7		
Interparietal			0.7		