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Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration

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The first aim of the study was to investigate the pharmacokinetics of eight anticoagulant rodenticides (brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, difenacoum, difethialone, flocoumafen and warfarin) in plasma and liver of the mouse after single oral administration. Eight groups of mice dosed orally with a different anticoagulant rodenticide in a dose equal to one-half the lethal dose 50 (LD_{50}), were killed at various times up to 21 days after administration. The eight anticoagulant rodenticides were assayed in plasma and liver by an LC-ESI-MS/MS method. Depending on the compound, the limit of quantification was set at 1 or 5 ng/mL in plasma. In liver, the limit of quantification was set at 250 ng/g for coumatetralyl and warfarin and at 100 ng/g for the other compounds. The elimination half-lives in plasma for first-generation rodenticides were shorter than those for second-generation rodenticides. Coumatetralyl, a first-generation product, had a plasma elimination half-life of 0.52 days. Brodifacoum, a second-generation product, showed a plasma elimination half-life of 91.7 days. The elimination half-lives in liver varied from 15.8 days for coumatetralyl to 307.4 days for brodifacoum. The second aim of the study was to illustrate the applicability of the developed method in a clinical case of a dog suspected of rodenticide poisoning.

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INTRODUCTION

Anticoagulant rodenticides are substances that are widely used to control infestations of rats and mice (Berny *et al.*, 1995). 4-Hydroxycoumarins and indandione derivates are usually the active constituents of these pesticides. Most rodent baits contain a very small concentration of the active substance, varying within each product and generally ranging from 0.005 to 0.25% (Campbell & Chapman, 2000).

Anticoagulant rodenticides are categorized as either first-generation anticoagulants like chlorophacinone, coumatetralyl and warfarin or as second-generation anticoagulants such as brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen, which are more efficacious due to the greater affinity to binding sites in the liver and consequently greater accumulation and persistence (Parmar *et al.*, 1987; Huckle *et al.*, 1988).

Anticoagulant rodenticides continue to be a major cause for morbidity and mortality for companion animals (Kohn *et al.*, 2003). Dogs seem to be overwhelmingly affected by anticoagulant poisoning compared to cats. The Belgian Poison Centre receives yearly around 400 enquiries dealing with exposures to anticoagulant rodenticides in animals, of which 80% concern dogs. Other nontarget animals like wild animals (foxes, birds of prey, etc.) can also be intoxicated because of direct ingestion of baits or to a lesser extent, ingestion of poisoned rodents (Fauconnet *et al.*, 1997).

The anticoagulant rodenticides are easily absorbed from the gastrointestinal tract and diminish synthesis of the vitamin K_1-dependent clotting factors (II, VII, IX, X) by inhibiting hepatic vitamin K_1 epoxide reductase activity. The toxicokinetics of anticoagulant rodenticides have been studied in several animal species such as dogs, rats and quails. Nevertheless, little information has been found on the toxicokinetics in mice. Most of the studies performed on mice were field trials to determine the efficacy and palatability of anticoagulant rodenticides containing baits in control programs (El-Bahrawy & Morsy, 1990; Gill, 1992).

Anticoagulant poisoning is manifested by severe haemorrhage, with massive bleeding and poor coagulation. External bleeding may be observed, but is usually internal. The initial

clinical signs are associated with blood loss such as anaemia, pale mucous membranes, weakness and tachycardia (DuVall *et al.*, 1989; Murphy & Gerkin, 1989; Berny, 2007). In clinical circumstances, clotting assays consisting of measurements of the prothrombin time, activated partial tromboplastin time and plasma fibrinogen concentration are common tools in the diagnosis of a suspected intoxication.

Vitamin K_1 (phytomenadione) is the antidote for treating dogs exposed to anticoagulant rodenticides. The length of vitamin K_1 treatment depends on the dose and kinetics of the specific anticoagulant rodenticide involved. The type of anticoagulant derivative is a poor basis for a treatment protocol and the identity of the product is rarely known when the patient is presented (Robben *et al.*, 1997). However, the identification of the type of anticoagulant rodenticide, in addition to the clinical and pathological signs is required for diagnosing intoxication especially in cases of malicious poisoning of companion animals. Unfortunately residues of rodenticides found in tissues from animal casualties are usually very low and hence sensitive analytical techniques are required for the chemical diagnosis of poisoning (Hunter *et al.*, 1988). To identify the pesticide responsible for a poisoning incident, the tissue of choice is the one with the largest residue of the original unchanged pesticide (parent compound). Liver is commonly used because it is known to accumulate anticoagulants for days or weeks and it is easily sampled to confirm anticoagulant poisoning in dead animals.

A number of techniques, such as gas chromatography coupled with mass spectrometry (GC-MS) (Duffield *et al.*, 1979; Ray *et al.*, 1989), thin-layer chromatography (TLC) (Berny *et al.*, 1995) and immunoassay (Mount *et al.*, 1988) are described for the analysis of vitamin K-antagonists in biological matrices. However, chromatographic analysis of coumarin and indandione anticoagulants presents specific difficulties. The low detection sensitivity hampers the use of HPLC-UV. Fluorescence detection offers enhanced sensitivity but requires extensive sample clean-up. Mass spectrometry presents both sensitivity and unambiguous identification of the compound. This identification has clinical importance since this helps to establish the dose and length of vitamin K_1 therapy in cases of intoxication. A developed LC-MS/MS method should provide confirmation of multiple analytes but should also be sufficiently sensitive to allow identification at trace concentrations in the desired matrices. Recently, LC-ESI-MS has been used for the simultaneous determination of frequently used coumarin and indandione anticoagulant rodenticides in matrices like animal feed stockages, beverages (Marek & Koskinen, 2007) and whole blood (Jin & Chen, 2006).

The aim of this study was twofold. The first aim was to investigate the pharmacokinetics of eight anticoagulant rodenticides (brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, difenacoum, difethialone, flocoumafen and warfarin) in plasma and liver of the mouse after single oral administration using a newly developed LC-ESI-MS/MS method. The eight tested anticoagulant rodenticides are freely available on the Belgian market as commercial preparations for the control of rodent pests. This part of the study gives information on the persistence of these products in mice, which may be important when small animals such as birds of prey ingest poisoned mice. Accidental or, in some cases, intentional exposure of pet animals to these substances can cause a severe poisoning which can result in severe haemorrhage and death if no appropriate antidote is administered. The second aim was to test the applicability of the developed LC-ESI-MS/MS method in clinical cases of anticoagulant rodenticides poisoning.

MATERIALS AND METHODS

Animals

The experiment was carried out on outbred mice, RjHan:NMRI (Laboratoire Elevage Janvier, Le Genest-ST-Isle, France). The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2006/096). In order to study the residue depletion of eight products in liver, the same guideline as for the residue depletion studies of veterinary drugs were used (Anonymous, 2005). Based on this guideline, four mice were killed on each time point. Since eight products were tested and six time points were included to determine the absorption and elimination profiles, 192 mice were included in the experiment. Eight mice were added as blank to fine-tune the LC-MS/MS method. The mice had an average bodyweight of 32.2 ± 1.93 g, males and females equally divided. Male mice were individually caged; female mice were housed in pairs. All animals were maintained on commercial rodent feed. Food and tap water were available *ad libitum* during the whole trial. The cages were equipped with bedding material, paper tubes and tissues. The mice were allowed a 7-day acclimatization period prior to the study.

Drugs and reagents

Analytical standards of bromadiolone, chlorophacinone, coumatetralyl and warfarin were collected from Riedel-de Häen (Seelze, Germany), brodifacoum and difenacoum from Sorex-Syngenta (Cheshire, UK), flocoumafen from BASF (Bad Durkheim, Germany) and difethialone from Merck Santé S.A.S (Lyon, France). The internal standard 7-acetoxy-6-(2,3-dibromopropyl)-4,8-dimethylcoumarin was purchased from Sigma-Aldrich (Bornem, Belgium).

Solvents of HPLC grade were used for LC-MS/MS analysis and were collected from VWR International bvba (Haasrode, Belgium) and Acros (Geel, Belgium). The other solvents used, acetone and diethyl ether were of analytical grade (VWR). Ammoniumformate was purchased from Sigma. Ethanol 96 vol.% was collected from Riedel-de Häen.

Experimental protocol

Dosage regimens

The mice were randomly allocated to the studied anticoagulant rodenticides, resulting in eight study groups of each 24 mice (12

Table 1. Lethal dose 50 (LD_{50}) and dose of each anticoagulant rodenticide administered to the mice (μg/mouse)

Anticoagulant rodenticide	LD_{50} (mg/kg bw)	Dose administered (μg/mouse)
Brodifacoum	0.4	6.44
Bromadiolone	1.75	28.18
Chlorophacinone	20.5	336
Coumatetralyl	<1000	8000
Difenacoum	0.8	12.88
Difethialone	1.29	20.77
Flocoumafen	0.8	12.88
Warfarin	374	5985

males, 12 females). The mice of each group were given a single dose of the analytical standard of one of the studied rodenticides. The chosen dose was a compromise between ensuring mice were exposed to a sufficient amount of each substance to be able to detect the products in plasma and limiting the lethal effects of the treatment. Consequently, a single dose equivalent to one-half the lethal dose 50 (LD_{50}) for mice was selected and administered, taking into account the average bodyweight of the mice (Table 1). For coumatetralyl, an LD_{50} value of more than 1000 mg/kg bw is reported. For this compound a dose corresponding to 250 mg/kg bw was chosen. The number of spontaneous deaths that occurred per day and per tested active substance was recorded over the subsequent 21 days. Because of the low solubility of the tested products in water, the analytical standards of brodifacoum, bromadiolone, difenacoum, difethialone and flocoumafen were each dissolved in ethanol and further diluted in water, resulting in an ethanol percentage varying from 2 to 6% in the final solution, corresponding to 246.6 to 739.8 mg/kg bw ethanol. Compared with the LD_{50} of ethanol for mice (9500 mg/kg bw), it can be concluded that this percentage ethanol was not toxic for the mice. Chlorophacinone, coumatetralyl and warfarin however, were administered as a suspension in polyethylene glycol due to the large amount of analytical standard that was needed for dosing. The oral toxicity of polyethylene glycol is low (21 000–34 000 mg/kg bw for the rat) and therefore the ingestion of a small quantity (300 μL) corresponding to 9750 mg/kg bw did not cause toxicity.

To assure uniform dosing, each animal was gavaged via a gastric tube after being anesthetized with isoflurane 5% (Isoflo®; Abbot Lab, Louvain-la-Neuve, Belgium). A volume of 300 or 500 μL was administered to each mouse, depending on the compound. An overview of the LD_{50} in mice and the dose of each anticoagulant rodenticide administered is given in Table 1.

Sample collection

At 1, 2, 3, 5, 7 and 21 days after dosing, blood and liver samples were collected from repeatedly 4 mice of each group. Blood samples were collected via terminal blood collection by cardiac puncture using a 26½ gauge needle. The mice were previously anesthetized through an intraperitoneal injection with ketamin (Anesketin®, 100 mg/mL; Eurovet, Heusden-Zolder, Belgium) and xylazin (Xyl-M®, 20 mg/mL, VMD, Arendonk, Belgium). Blood was aspired in a syringe containing 10 μL of heparin 50 000 IU/mL (Leo Pharma, Wilrijk, Belgium), obtaining approximately 1 mL of blood. The heparinized blood was centrifuged at 2000 *g* at 4 °C for 15 min. The supernatant was transferred to an Eppendorf tube. After the blood collection the mice were killed through cervical dislocation after which the liver was prelevated. All plasma and liver samples were stored at −20 °C until assayed.

Sample analysis

A stock solution mixture of 100 μg/mL of the eight analytical standards was prepared in HPLC grade methanol. A working solution mixture of 10 μg/mL was prepared by dilution with methanol. The internal standard stock solution of 1 mg/mL was prepared in methanol and subsequent dilution with methanol resulted in a working solution of 10 μg/mL. All stock and working solutions were stored at −20 °C.

The extraction procedure was based on a previously published method (Berny *et al.*, 1995). Samples were prepared by weighing 0.5 g of homogenized liver tissue or 100 μL of plasma in a capped 50-mL polypropylene centrifuge tube, followed by the addition of 50 μL of the internal standard working solution. After vortexing briefly, 5 mL of acetone were added and the sample was extracted for 10 min. After 10 min of centrifugation (1050 *g* at 4 °C) the supernatant was transferred to a glass tube. Again 4 mL of acetone was added to the remaining residue, extracted for 10 min and centrifuged for 10 min (2700 *g* at 4 °C). Both supernatant fractions were combined and 1 mL of diethyl ether was added to further eliminate proteins. After centrifugation (10 min at 2700 *g*) 2.0 mL of supernatant were evaporated to dryness under a nitrogen flow at 40 °C. The residue was redissolved in 200 μL of mobile phase (30A/70B), briefly vortexed and 10 μL were injected onto the LC-ESI-MS/MS instrument.

Chromatographic analysis was performed on a Nucleodur C18 Gravity column (125 × 2.0 mm i.d., 3 μm) from Macherey-Nagel (Düren, Germany), using 5 mM ammoniumformate in HPLC – water (pH 9) and 5 mM ammoniumformate in methanol as mobile phase (30A/70B). Gradient elution was performed at a flow rate of 200 μL/min. Samples were analysed in the SRM-mode on a TSQ triple quadrupole mass spectrometer (Thermo-Electron Corporation, San Jose, CA, USA).

Method validation parameters

The developed method was validated using spiked blank plasma and liver. The validation parameters were in accordance with the recommendations defined by the EU and with criteria in the literature (Anonymous, 2002, 2005). The following set of parameters were included: linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ).

Pharmacokinetic analysis of data

Pharmacokinetic data from each group of mice were analysed using a computerized program (WinNonlin® v5.01; Pharsight, Mountain View, CA, USA). Pharmacokinetic calculations were performed on the mean results per group, using a naïve pooled

data approach (Ette & Williams, 2004). The plasma and liver concentrations versus time data were subjected to noncompartmental analysis. The total area under the curve was calculated using the linear trapezoidal method and adding the estimated terminal portion of the curve ($AUC_{0 \rightarrow \infty}$). Other pharmacokinetic parameters evaluated were: the elimination half-life ($t_{½(el)}$), the elimination rate constant (k_{el}), the apparent volume of distribution (V_d) and the clearance (Cl). For V_d and Cl, the values were expressed as V_d/F and Cl/F since the bioavailability (F) is not known in the literature.

Applicability

The developed method was used for the confirmation of suspected poisoning cases in domestic animals. A liver sample was analysed from a dog that was lethargic for several days and suffered from abdominal pain. The dog received a blood transfusion and was treated with vitamin K_1 but unfortunately died the day after. Autopsy revealed several bleedings in the thorax and gastrointestinal system, a pale liver and a contracted spleen.

RESULTS

Method validation

The results are summarized in Table 2. For the calibration curves good linearity was observed up to 500 ng/mL for all compounds, except for chlorophacinone where a concentration of 750 ng/mL was reached in plasma. In liver, good linearity was seen up to 500 ng/g for brodifacoum, chlorophacinone, difenacoum and difethialone and up to 750 ng/g for the other compounds. The accuracy fell within the range of -20 to +10% for concentrations ≥ 10 ng/mL or ng/g, within -30 to +10% for concentrations > 1 and < 10 ng/mL or ng/g and within -50 to +20% for concentrations ≤ 1 ng/mL or ng/g, and the precision also fell within the maximum RSD values (Anonymous, 2002, 2005). The results of the LOD and LOQ are also summarized in Table 2. Depending on the compound, a concentration of 1 or 5 ng/mL could be quantified fulfilling the criteria for accuracy and precision and was therefore set as LOQ of the method in plasma. In liver, the LOQ was set at 250 ng/g for coumatetralyl and warfarin and at 100 ng/g for the other compounds.

Toxicity of the eight anticoagulant rodenticides in male and female mice

None of the mice died spontaneously after a single dose of brodifacoum, flocoumafen or difenacoum. For the other compounds, between day 1 and day 7, three mice died after administration of warfarin, one mouse died of difethialone, seven mice died of chlorophacinone, four mice died of coumatetralyl and one mouse died of bromadiolone. Multiple haemorrhages throughout the body with unclotted blood in the chest and abdominal cavities were generally seen during postmortem examination of these animals. Between day 7 and the end of the experiment (21 days), no further deaths were recorded.

Pharmacokinetic analysis

The semi-logarithmic plots of the plasma concentration-time curves of all compounds are shown in Fig. 1, the liver concentration–time curves after oral administration are shown

Table 2. Results of the method validation for each anticoagulant rodenticide in plasma and liver

	Linearity				Accuracy (%) [Precision, (RSD, %)] (n = 6)				LOD	
	Plasma		Liver		Plasma		Liver			
Analyte	Conc (ng/mL)	Result (r)	Conc (ng/g)	Result	Conc (ng/mL)	Result	Conc (ng/g)	Result	Plasma ng/mL	Liver ng/g
Brodifacoum	5–50	0.9987	10–500	0.9986	5 (LOQ)	+ 5.9 (3.1)	100 (LOQ)	+5.8 (10.6)	3.21	0.41
	10–500	0.9998			500	+ 7.6 (4.1)	250	+5.3 (8.5)		
Bromadiolone	1–10	0.9910	10–750	0.9995	1 (LOQ)	−24.5 (29.2)	100 (LOQ)	+6.4 (5.9)	0.09	0.48
	10–500	0.9976			250	+7.5 (6.8)	250	+4.1 (9.3)		
Chlorophacinone	5–50	0.9987	10–500	0.9997	5 (LOQ)	−9.6 (21.2)	100 (LOQ)	+8.8 (6.8)	0.45	4.64
	50–750	0.9995			500	+1.4 (5.8)	250	−2.4 (11.7)		
Coumatetralyl	1–10	0.9982	25–750	0.9975	1 (LOQ)	+10.0 (19.7)	250 (LOQ)	+2.9 (7.9)	0.17	1.43
	10–500	0.9997			500	−2.9 (9.9)	500	−0.8 (9.8)		
Difenacoum	1–10	0.9988	10–500	0.9988	1 (LOQ)	+19.9 (6.6)	100 (LOQ)	+7.9 (6.4)	0.45	1.23
	10–500	0.9991			500	+ 3.9 (3.9)	250	+9.7 (6.1)		
Difethialone	5–50	0.9987	10–500	0.9994	5 (LOQ)	+2.4 (10.8)	100 (LOQ)	+7.7 (6.3)	0.46	0.71
	5–50	0.9987			500	+ 3.1 (6.6)	250	+9.4 (11.1)		
Flocoumafen	1–10	0.9955	25–750	0.9990	1 (LOQ)	+14.5 (6.2)	100 (LOQ)	−5.7 (14.1)	0.07	1.11
	10–500	0.9978			100	−5.5 (12.0)	250	+0.3 (10.1)		
Warfarin	1–10	0.9993	10–750	0.9983	1 (LOQ)	−3.3 (19.8)	250 (LOQ)	+7.3 (8.4)	0.21	0.37
	10–500	0.9998			500	−7.7 (4.0)	500	+2.8 (11.0)		

r, correlation coefficient, RSD, relative standard deviation (the ratio between standard deviation and mean found concentration), accuracy (difference between mean found concentration and spiked concentration); LOD, limit of detection (signal-to-noise ratio of 3/1); LOQ, limit of quantification.

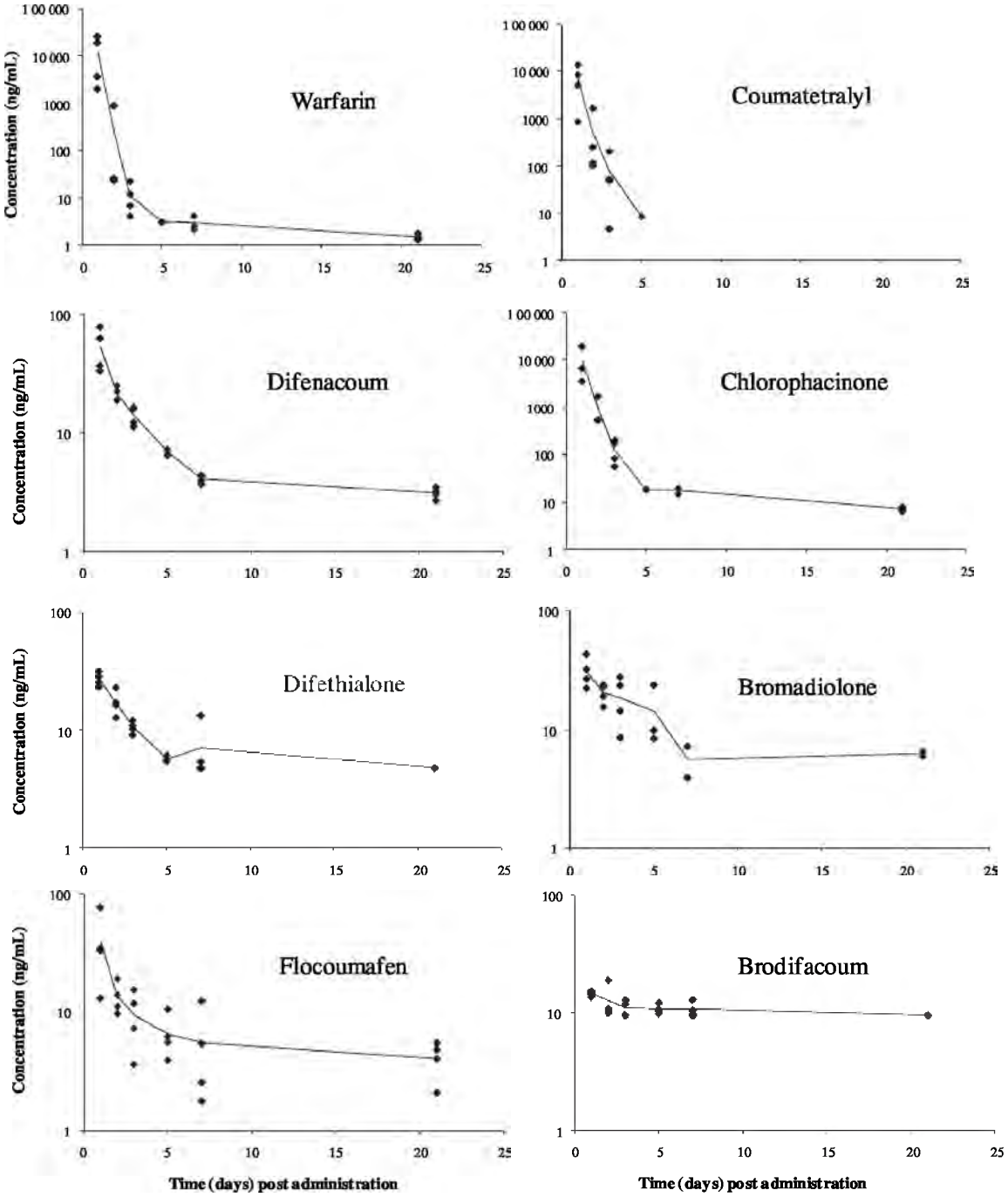

Fig. 1. Plasma concentrations-time profiles of eight anticoagulant rodenticides after single oral administration in mice.

in Fig. 2. For most of the studied compounds, a clear biphasic pattern was seen in the concentration-time profiles.

The pharmacokinetic parameters of brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, difenacoum, difethialone, flocoumafen and warfarin in plasma and liver are reported in Table 3.

Applicability

Analysis of liver tissue from a dog with the developed LC-MS/MS method indicated the presence of brodifacoum. A brodifacoum concentration was measured in the range of 4–5 μg/g. For quantification, the product ion at *m/z* 135 was used for brodifacoum. The ion ratios for ions at *m/z* 143/135 and 187/135 were used as identification criteria and fell in the range set by the EU (Anonymous, 2002).

DISCUSSION

Wide variations exist in the literature for LD_{50} values of anticoagulant rodenticides. A particular reason for these

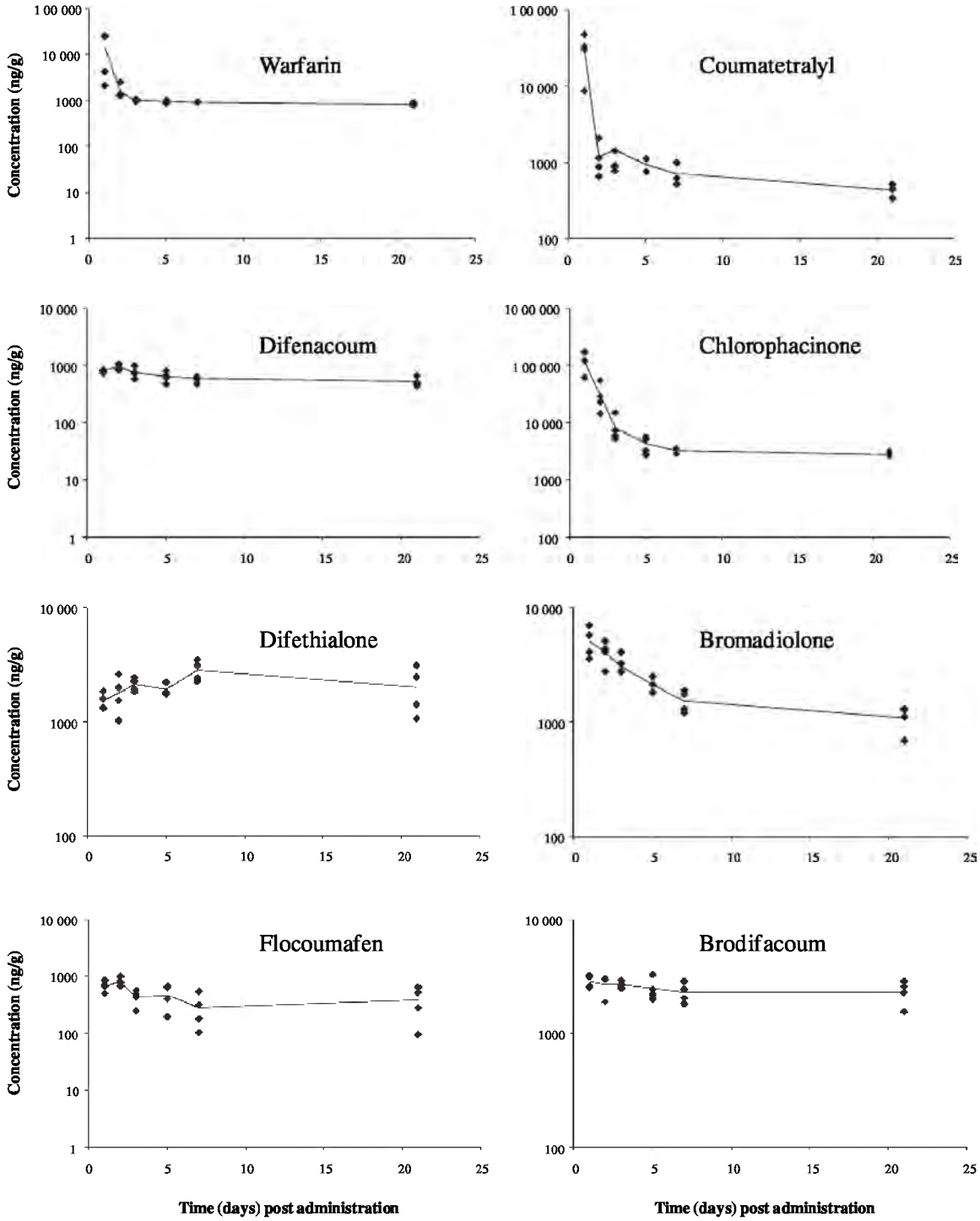

Fig. 2. Liver concentration-time profiles of eight anticoagulant rodenticides after single oral administration in mice.

variables is the use of single or repeated (5-day) doses. LD_{50}-doses also vary according to animal strain and sex, but both have not always been indicated in reported data (Ashton *et al.*, 1987). Reported oral LD_{50} values for mice show variations from less than 1 mg/kg bw for second-generation rodenticides to 374 mg/kg bw for warfarin (Tomlin, 1997). The toxicity of warfarin varies according to species and whether exposure was a single or multiple dose.Warfarin is a first-generation anticoagulant so for most animals the baits will need to be ingested regularly over several days before any of the clinical signs of poisoning will occur.

In this study, each anticoagulant rodenticide was administered at a dose of one half the LD_{50}, based on available LD_{50} data for mice. For coumatetralyl, an LD_{50} value of more than 1000 mg/kg bw is reported and therefore a dose corresponding to 250 mg/kg bw was chosen. To the author's knowledge, no

Table 3. Pharmacokinetic parameters in plasma and liver after single oral administration of the anticoagulant rodenticides in mice at a dose of half the LD_{50} values

Pharmacokinetic Parameter	Units	BDF	BDL	CHF	CTE	DIF	DFT	FLO	WAR
Plasma									
$AUC_{0 \rightarrow \infty}$	day·ng/mL	1474.9	511.3	11015.7	7709.0	252.3	427.3	310.1	12884.0
$t_{1/2(el)}$	days	91.7	33.3	11.7	0.52	20.4	38.9	26.6	14.9
V_d/F	L/kg	17.94	82.33	15.69	24.11	46.70	86.29	49.42	312.56
Cl/F	L/kg·day	0.14	1.71	0.93	32.43	1.59	1.54	1.29	14.51
k_{el}	1/day	0.008	0.021	0.059	1.345	0.034	0.018	0.026	0.046
Liver									
$t_{1/2(el)}$	day	307.4	28.1	35.4	15.8	61.8	28.5	93.8	66.8
k_{el}	1/day	0.002	0.025	0.020	0.043	0.011	0.024	0.007	0.010

BDF, brodifacoum; BDL, bromadiolone; CHF, chlorophacinone; CTE, coumatetralyl; DIF, difenacoum; DFT, difethialone; FLO, flocoumafen; WAR, warfarin.

literature is available about the pharmacokinetics of chlorophacinone in mice. In the literature different LD_{50} values for chlorophacinone were described for rats, varying from 6.26 to 20.5 mg/kg bw (Jackson & Ashton, 1992; Petterino & Paolo, 2001). The single oral dose of 10.25 mg/kg bw administered to the mice corresponded with half the LD_{50} value as described by Jackson and Ashton (1992). This high dosage could possibly explain the number of spontaneous deaths seen in this group (7 out of 24 mice).

For most of the studied compounds, a clear biphasic pattern was seen in the plasma concentration-time profiles. For all the studied compounds, the highest plasma concentrations were seen at day 1 after the single oral administration. The plasma concentrations declined fast, except for brodifacoum where the plasma concentrations remained relatively constant over the 21 days. This is also described in dogs, receiving a total oral dose of 1.1 mg/kg brodifacoum. The serum concentrations were highest during the 3 days after dosing and were still detectable until 24 days postadministration (Woody *et al.*, 1992). Coumatetralyl, a first-generation anticoagulant rodenticide, was rapidly eliminated from the plasma which was indicated by a high total body clearance Cl/F of 32.43 L/kg·day and resulted in plasma concentrations below the LOD at day 7 and at day 21.

It should be mentioned that the noncompartmental modelling approach to perform the pharmacokinetic analysis is not optimal. Due to the sparse data points, the use of a nonlinear mixed effect model would have been more appropriate. However, this would have implicated the collection of at least two samples per animal, which was not possible in this design since blood samples were taken by intracardial aspiration followed by euthanasia of the mice.

The hypothesis that anticoagulant rodenticides tend to accumulate rapidly in the liver, was confirmed by the high liver concentrations seen at day 1 and remaining relatively high during the total duration of the experiment, as can be seen in Fig. 2. This tendency is described in the literature for most of the anticoagulant rodenticides included in this study and for several animal species, and implicates that the calculated terminal half-life is much longer than the sampling time duration. Nevertheless, the calculated half-lives of elimination in this study allow comparing the values reported in other animal species.

Mosterd and Thijssen (1991) reported that brodifacoum rapidly accumulated in the liver and the liver content on day 30 was not different from day 7 after a single oral dose of 0.2 mg/kg bw to rats, which is well in accordance with our results in mice.

After oral administration to rats, bromadiolone seems to disappear quickly from the blood resulting in high concentrations in liver tissues (Kamil, 1987). Ray *et al.* (1989) showed that bromadiolone was less persistent in rodent and canine liver and may be eliminated more rapidly than brodifacoum, which corresponds with our findings. Elimination of radiolabelled bromadiolone from rat liver is reported to be biphasic, consisting of a rapid initial phase lasting from 2 to 8 days after dosing and a slower terminal phase. The elimination half-life was 170 days (Parmar *et al.*, 1987).

Bratt (1987) found that after a single oral dose of radiolabelled difenacoum of 1.2 mg/kg bw to rats, the highest concentration of radioactivity was found in the liver at 24 h after dosing. The elimination from the liver was biphasic with a terminal half-life of elimination of 118 days.

After a single oral dose of flocoumafen of 0.14 mg/kg bw to rats, the absorption into blood was rapid, reaching maximum concentrations of 0.03 to 0.05 μg/mL in plasma within 4 h (Huckle *et al.*, 1989a). Also, a high degree of body retention was found 7 days after repeated oral dosing, approximately half the dose was found in the liver of rats (Huckle *et al.*, 1988).

Robben *et al.* (1997) describe a study where the plasma concentration, plasma half-lives and mean residence times of different superwarfarins were determined in 21 dogs in which a preliminary diagnosis of anticoagulant rodenticide poisoning had been prepared. The plasma $t_{1/2}$ of brodifacoum was calculated taking into account six dogs and varied from 0.9 to 4.7 (mean 2.4) days. The plasma $t_{1/2}$ of difethialone, calculated for two dogs, was 2.2 and 3.2 days respectively. The plasma $t_{1/2}$ of difenacoum was not mentioned.

After oral administration to Japanese quails, flocoumafen was mainly detected bound to the liver microsomal fraction. Its subsequent elimination showed biphasic kinetics with a faster elimination the first 5 days after administration, followed by a second slower phase. It took about 100 days for flocoumafen to disappear from liver tissue in this bird species (Huckle *et al.*,

1989b). In beagle dogs, 8% of an administered flocoumafen dose of 0.4 mg/kg could be found in the liver for 43 weeks (Veenstra *et al.*, 1991).

The half-life in rat liver is reported to be 7–10 days for warfarin, which contrasts with half-lives exceeding 100 days for second-generation anticoagulants (Thijssen, 1995).

In the literature, several assays are described for determination of anticoagulant rodenticides in animal liver tissues and whole blood or plasma samples. These analytical methods usually focus on the quantification of one or more specific compounds (Jin & Chen, 2006, Jin *et al.*, 2007). However, in situations of suspicious animal poisoning, the type of anticoagulant rodenticides used is often not known and the diagnosis has to be prepared based on the anamnesis or the finding of the specific bait in the near surrounding of the poisoned animal. In such cases, the multi-residue method described in this study can be used for the confirmation of anticoagulant poisoning. As an example, a clinical case of a deceased dog poisoned with an unknown rodenticide, was presented here. Liver tissue was collected and analysed with the presented LC-ESI-MS/MS method which clearly indicated the presence of brodifacoum.

CONCLUSIONS

Anticoagulant rodenticides are still used worldwide to control rodent pests. However, poisoning accidents in domestic and farm animals, birds and wildlife may occur in cases of massive intentional or unintentional ingestion. The second-generation anticoagulant rodenticides are more toxic than the first-generation products and are used as single-dose compounds, which mean that they can effectively poison a rat after only one oral uptake.

Our data in mice, as well as published literature, describe the tendency of anticoagulant rodenticides to accumulate in the liver resulting in high liver concentrations for a prolonged period of time. Plasma concentrations on the other hand, tend to decrease rapidly which makes it more difficult to use this matrix as diagnostic material in malicious poisoning cases of companion animals.

The new developed LC-ESI-MS/MS method presented in this study makes it possible to detect simultaneously all anticoagulant rodenticides available on the Belgian market in biological matrices like plasma and liver. The results determined also show that the developed LC-ESI-MS/MS method is sufficiently sensitive to allow quantitation of eight anticoagulant rodenticides at trace concentrations in plasma and liver which can be of importance in the confirmation of poisoning cases in companion animals.

The applicability of the LC-ESI-MS/MS method to investigate suspected poisoning cases in companion animals is illustrated by the identification of brodifacoum in the liver of a poisoned dog.

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