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Multi-residue analysis of eight anticoagulant rodenticides in animal plasma and liver using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry

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ABSTRACT

A sensitive method for the simultaneous quantification of eight anticoagulant rodenticides (brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, difenacoum, difethialone, flocoumafen and warfarin) in animal plasma and liver using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry (LC-HESI-MS/MS) is described. The sample preparation includes a liquid–liquid extraction with acetone. The compound 7-acetoxy-6-(2,3-dibromopropyl)-4,8-dimethylcoumarin is used as an internal standard. Chromatographic separation was achieved using a Nucleodur C18 gravity column. Good linearity was observed up to 750 ng mL^{-1} for chlorophacinone and up to 500 ng mL^{-1} for the other compounds in plasma. In liver, good linearity was seen up to 500 ng g^{-1} for brodifacoum, chlorophacinone, difenacoum and difethialone and up to 750 ng g^{-1} for the other compounds. Depending on the compound, a level of 1 or 5 ng mL^{-1} could be quantified fulfilling the criteria for accuracy and precision and was therefore set as limit of quantification of the method in plasma. In liver, the limit of quantification was set at 250 ng g^{-1} for coumatetralyl and warfarin and at 100 ng g^{-1} for the other compounds. In plasma, the limit of detection varied from 0.07 ng mL^{-1} for flocoumafen to 3.21 ng mL^{-1} for brodifacoum. In liver, the limit of detection varied from 0.37 ng g^{-1} for warfarin to 4.64 ng g^{-1} for chlorophacinone. The method was shown to be of use in a pharmacokinetic study after single oral administration to mice and in the confirmation of suspected poisoning cases in domestic animals.

1. Introduction

Anticoagulant pesticides are substances that are widely used in agricultural and urban rodent control [1]. 4-Hydroxycoumarins and indandione derivates are most frequently used as the active constituents of these pesticides. Anticoagulant rodenticides are usually commercially available in the form of cereal-based baits, pastes, tracking powders or wax blocks [2]. Baits are often coloured with blue, green or red dye in order to reduce the palatability to non-target animals [1]. Second-generation anticoagulants like brodifacoum and flocoumafen are more toxic than the first-generation products in the sense that a single feeding may be lethal.

The main cause for accidental poisoning of domestic animals and to lesser extent wild animals is direct consumption of anticoagulant baits. Secondary poisoning through consumption of rats and mice killed with anticoagulants may be seen in dogs and cats in urban situations but is more likely to occur in farm situations [3]. Predators (mainly foxes and buzzards) are frequently exposed to anticoagulant compounds via contaminated preys. Although in the European Union the use of pesticides is clearly defined by Directive EC 91/414, illegal poisoning of wild animals still occurs. The reasons for illegal poisoning are diverse. Compared to game species, predators are usually considered a nuisance and any potential conflict between wildlife and human beings (hunting, predation...) may result in illegal poisoning [4].

Anticoagulant rodenticides are vitamin K antagonists. The main site of their action is the liver, where several of the blood coagulation precursors undergo vitamin K dependent posttranslational processing before they are released into the circulation. The point of action appears to be the inhibition of vitamin K_1 epoxide reductase.

The liver is the main organ for accumulation and storage of rodenticide anticoagulants making it the most diagnostically useful tissue. However, since liver biopsies are not common practice in clinically affected animals, less invasive procedures like measurement of packed cell volume (haematocrit), clotting parameters (prothrombin time, activated partial tromboplastin time and plasma fibrinogen concentration) and residue analysis in plasma, are necessary for the diagnosis of rodenticide poisoning. The

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determination of anticoagulant rodenticides in liver tissue can be useful in post-mortem diagnosis and for forensic purposes [5].

Most of the analytical methods for the determination of anticoagulant rodenticides are based on high-performance liquid chromatography (HPLC) with UV or fluorescence detection [6–11]. Other techniques, such as gas chromatography coupled with mass spectrometry (GC-MS) [12,13], thin-layer chromatography (TLC) [5] and immunoassay [14] are also described for the analysis of vitamin K-antagonists in biological matrices. An MS/MS method with collision-activated dissociation (CAD) was utilized to distinguish unequivocally the indandione rodenticides diphacinone and chlorophacinone in animal whole blood and liver [15]. Recently, several liquid chromatography coupled to electrospray ionization mass spectrometry methods (LC-ESI-MS) have been described for the determination of multiple coumarin and indandione anticoagulant rodenticides in diverse matrices. Jin and Chen [16] described the rapid determination of the three anticoagulant rodenticides bromadiolone, flocoumafen and brodifacoum in whole blood by LC-ESI-MS. HLPC-MS/MS assays were developed for the determination of flocoumafen and brodifacoum [17], coumatetralyl [18] and bromadiolone [19], at trace levels in whole blood which can be of use in the diagnosis of poisoned human beings. Marek and Koskinen [20] on the other hand described LC-ESI-MS/MS as a method to confirm and quantify seven anticoagulant rodenticides in diverse matrices such as animal feed stocks, baits and liquid matrices.

However, to the authors' knowledge, a multi-residue LC-MS/MS method for the simultaneous quantification in plasma and liver of the eight anticoagulant rodenticides freely available on the Belgian market has not been described yet. The use of an LC-MS/MS method has several advantages compared to other more established methods like HPLC-UV or fluorescence detection. The use of HPLC-UV is encountered by the low detection sensitivity. Although fluorescence detection offers enhanced sensitivity, it still requires an extensive sample clean-up. Mass spectrometry on the other hand presents both sensitivity and unambiguous identification of the compound. This identification in particular is important in clinical circumstances since this helps to establish the dose and the length of vitamin K_1 therapy in cases of intoxication. Therefore a new developed LC-MS/MS method should provide confirmation of multiple analytes but should also be sufficiently sensitive to allow quantification at trace levels in the presented matrices.

This paper describes a multi-residue LC-HESI-MS/MS method for the determination of eight anticoagulant rodenticides (brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, difenacoum, difethialone, flocoumafen and warfarin) in plasma and liver using a simple extraction procedure. These eight tested anticoagulant rodenticides are freely available on the Belgian market as commercial preparations for the control of rodent pests. The method was used to study the pharmacokinetic properties in mice after single oral administration of an anticoagulant rodenticide. The developed method is currently used for the determination and the confirmation of suspected anticoagulant rodenticide toxicosis in animals such as dogs and cats.

2. Experimental

2.1. Chemicals and standards

Analytical standards of bromadiolone, chlorophacinone, coumatetralyl and warfarin were obtained from Riedel-de Häen (Seelze, Germany), brodifacoum and difenacoum from Sorex-Syngenta (Cheshire, England), flocoumafen from BASF (Bad Durkheim, Germany) and difethialone from Merck Santé S.A.S. (Lyon, France). The internal standard (IS) 7-acetoxy-6-(2,3-dibromopropyl)-4,8-dimethylcoumarin was purchased from Sigma–Aldrich (Bornem, Belgium). The choice of the IS was based on the method of Grobosch et al. [21], since this product is not commercially available as rodent pesticide and will therefore not interfere in a multi-residue method used in clinical cases of toxicosis. Solvents of HPLC grade were used for LC-HESI-MS/MS analysis and were obtained from VWR International bvba (Haasrode, Belgium) and Acros (Geel, Belgium). The other solvents used, acetone and diethyl ether were of analytical grade (VWR). Ammonium formate was purchased from Sigma.

A stock solution mixture of 100 $\mu g\,mL^{-1}$ of the eight analytical standards was prepared in HPLC grade methanol. A working solution mixture of 10 $\mu g\,mL^{-1}$ was prepared by dilution with methanol. The internal standard stock solution of 1 $mg\,mL^{-1}$ was prepared in methanol and subsequent dilution with methanol resulted in a working solution of 10 $\mu g\,mL^{-1}$. All stock and working solutions were stored at −20 °C.

2.2. Biological samples

Known anticoagulant rodenticide-free plasma and liver samples were obtained from mice which did not receive any medication. Incurred plasma and liver samples were obtained from healthy mice—both male and female—which received a single oral dose of the analytical standard of one of the studied rodenticides. Blood samples were taken from anesthetized animals at different time points after administration and plasma was frozen at −20 °C until analysis. Immediately after blood collection, the mice were euthanized through cervical dislocation after which the liver was prelevated. The liver samples were minced and frozen at −20 °C until analysis.

Table 1
LC-HESI-MS/MS analysis parameters

Anticoagulant rodenticide	Precursor ion (Da)	Product ion (Da)	Collision energy (V)	Tube lens (V)
Brodifacoum	$[M-H]^-$ 521.0	**134.9**	36	116
		142.9	56	
		186.9	37	
Bromadiolone	$[M-H]^-$ 525.0	180.9	31	113
		219.0	45	
		249.9	41	
Chlorophacinone	$[M-H]^-$ 373.0	116.0	44	90
		144.9	20	
		200.9	20	
Coumatetralyl	$[M-H]^-$ 291.1	**140.9**	29	86
		142.9	39	
		247.0	19	
Difenacoum	$[M-H]^-$ 443.1	134.9	35	110
		142.9	55	
		293.0	32	
Difethialone	$[M-H]^-$ 536.9	**150.9**	37	119
		202.9	37	
		370.9	37	
Flocoumafen	$[M-H]^-$ 541.1	160.9	42	120
		289.0	28	
		382.0	25	
Internal standard	$[M+H]^+$ 432.9	214.9	47	103
		230.9	36	
		390.8	20	
Warfarin	$[M-H]^-$ 307.1	**160.9**	17	82
		116.9	25	
		250.0	25	

Product ions in bold are used for quantitation.

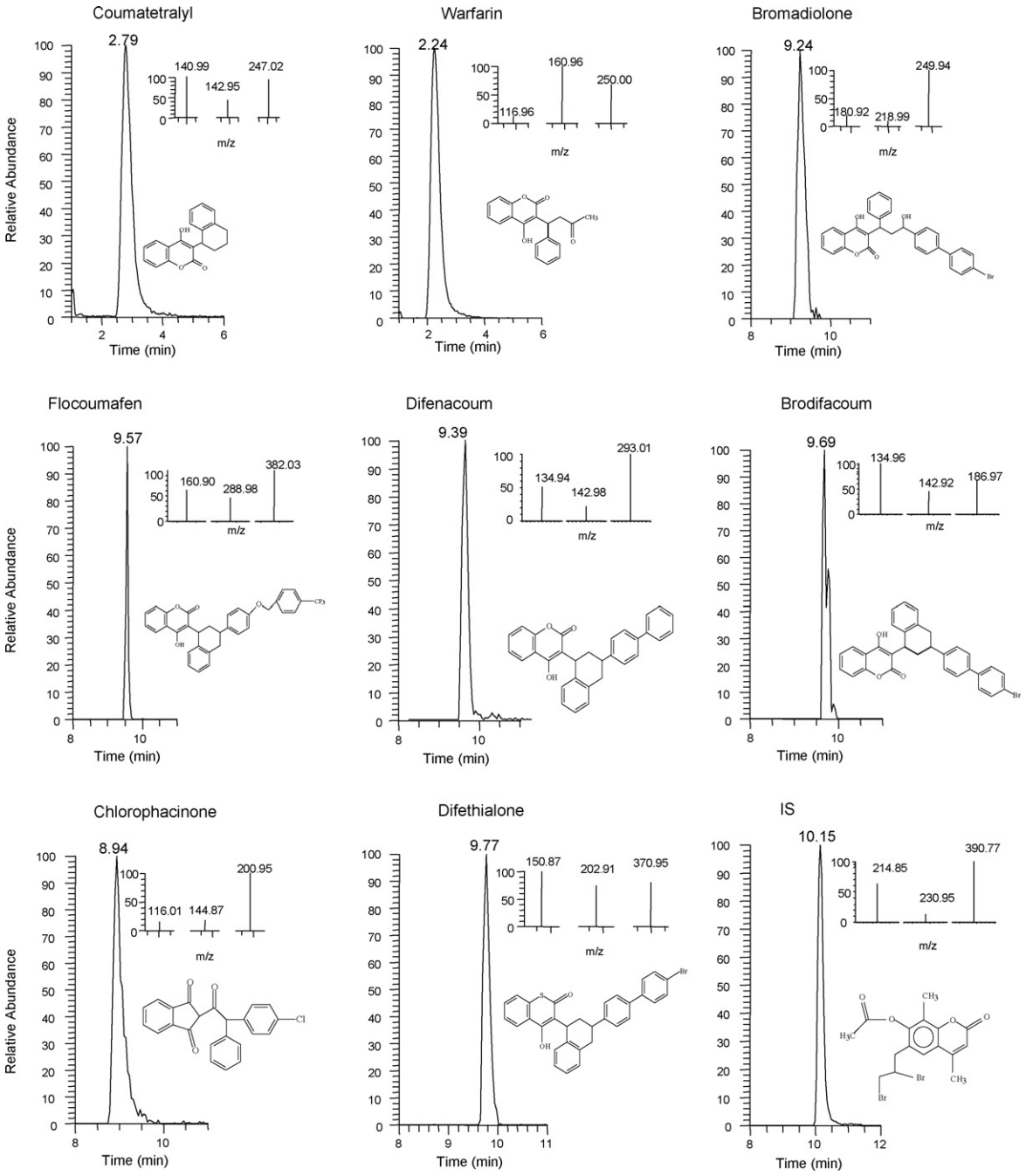

Fig. 1. Chemical structures, MS/MS spectra and mass chromatograms of the internal standard and the eight anticoagulant rodenticides in plasma spiked at 10 ng mL^{-1}. The mass chromatograms of the product ions used for quantification, i.e. those mentioned in bold in Table 1, are shown.

2.3. Sample preparation

The extraction procedure was based on a previously published method for liver and plasma [5]. These authors developed a High-Performance Thin-Layer Chromatographic Method for the simultaneous determination of 8 anticoagulant rodenticides (brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, coumachlor, difethialone, difenacoum and warfarin) in liver and plasma samples.

Samples were prepared by weighing 0.5 g of homogenized liver tissue or pipetting 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 (2500 rpm at 4 °C) the supernatant was transferred to a glass tube. Again 4 mL of acetone were added to the remaining residue, extracted for 10 min and centrifuged for 10 min (4000 rpm at 4 °C). Both supernatant fractions were combined and 1 mL of diethyl ether was added to further eliminate proteins. After centrifugation for 10 min at 4000 rpm, 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 (30 A/70 B), briefly vortexed and 10 μL were injected onto the LC-HESI-MS/MS instrument.

Table 2
Results of the linearity, accuracy and intra-day precision, LOD and LOQ for the eight analytes in plasma and liver

Analyte	Linearity				Accuracy (%) (precision, (R.S.D., %)) ($n = 6$)				LOD	
	Plasma		Liver		Plasma		Liver		Plasma	Liver
	Conc (ng mL^{-1})	Result	Conc (ng g^{-1})	Result	Conc (ng mL^{-1})	Result	Conc (ng g^{-1})	Result	ng mL^{-1}	ng g^{-1}
Brodifacoum	5–50	$r = 0.9987$, $g = 3.85$	10–500	$r = 0.9986$, $g = 8.77$	5 (LOQ)	+5.9 (3.1)	100 (LOQ)	+5.8 (10.6)	3.21	0.41
							250	+5.3 (8.5)		
	10–500	$r = 0.9998$, $g = 2.55$			500	+7.6 (4.1)	500	−2.9 (10.4)		
Bromadiolone	1–10	$r = 0.9910$, $g = 11.83$	10–750	$r = 0.9995$, $g = 5.76$	1 (LOQ)	−24.5 (29.2)	100 (LOQ)	+6.4 (5.9)	0.09	0.48
					10	−20.0 (4.3)	250	+4.1 (9.3)		
	10–500	$r = 0.9976$, $g = 7.84$			250	+7.5 (6.8)	500	+8.1 (10.3)		
Chlorophacinone	5–50	$r = 0.9987$, $g = 4.90$	10–500	$r = 0.9997$, $g = 4.07$	5 (LOQ)	−9.6 (21.2)	100 (LOQ)	+8.8 (6.8)	0.45	4.64
					20	+7.1 (1.2)	250	−2.4 (11.7)		
	50–750	$r = 0.9995$, $g = 2.10$			250	−3.0 (4.4)	500	−4.2 (4.6)		
					500	+1.4 (5.8)				
Coumatetralyl	1–10	$r = 0.9982$, $g = 5.79$	25–750	$r = 0.9975$, $g = 5.78$	1 (LOQ)	+10.0 (19.7)			0.17	1.43
					10	−14.4 (6.9)	250 (LOQ)	+2.9 (7.9)		
	10–500	$r = 0.9997$, $g = 4.76$			250	+3.4 (4.8)	500	−0.8 (9.8)		
					500	−2.9 (9.9)				
Difenacoum	1–10	$r = 0.9988$, $g = 3.73$	10–500	$r = 0.9988$, $g = 11.79$	1 (LOQ)	+19.9 (6.6)	100 (LOQ)	+7.9 (6.4)	0.45	1.23
					10	−14.3 (17.0)	250	+9.7 (6.1)		
	10–500	$r = 0.9991$, $g = 4.98$			500	+3.9 (3.9)	500	+7.6 (9.5)		
Difethialone	5–50	$r = 0.9987$, $g = 5.99$	10–500	$r = 0.9994$, $g = 6.84$	5 (LOQ)	+2.4 (10.8)	100 (LOQ)	+7.7 (6.3)	0.46	0.71
					20	+9.5 (10.7)	250	+9.4 (11.1)		
	10–500	$r = 0.9977$, $g = 8.21$			500	+3.1 (6.6)	500	+5.8 (8.0)		
Flocoumafen	1–10	$r = 0.9955$, $g = 4.77$	25–750	$r = 0.9990$, $g = 5.85$	1 (LOQ)	+14.5 (6.2)	100 (LOQ)	−5.7 (14.1)	0.07	1.11
					10	−13.6 (13.9)	250	+0.3 (10.1)		
	10–500	$r = 0.9978$, $g = 7.09$			100	−5.5 (12.0)	500	+8.0 (7.3)		
Warfarin	1–10	$r = 0.9993$, $g = 2.87$	10–750	$r = 0.9983$, $g = 8.38$	1 (LOQ)	−3.3 (19.8)		+7.3 (8.4)	0.21	0.37
					10	−8.6 (10.0)	250 (LOQ)			
	10–500	$r = 0.9998$, $g = 3.09$			250	+3.8 (4.7)	500	+2.8 (11.0)		
					500	−7.7 (4.0)				

r: correlation coefficient, g: goodness-of-fit coefficient, R.S.D.: 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.

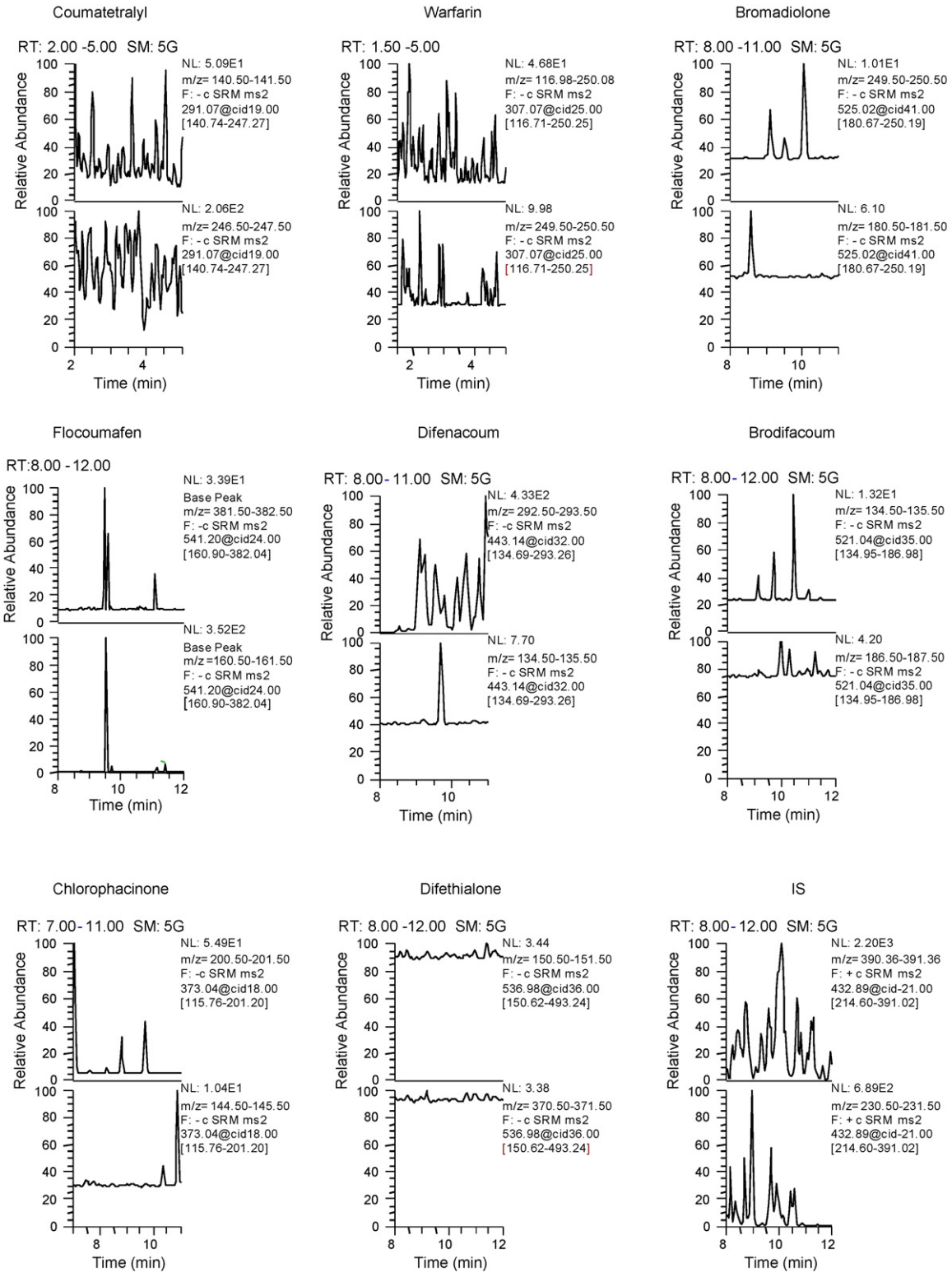

Fig. 2. Mass chromatograms of an extracted blank plasma sample. The upper and lower trace for each analyte represent the product ion used for quantification and the second most abundant product ion, respectively.

2.4. Chromatography and mass spectrometry

The LC-MS/MS analyses were performed using a Surveyor Plus LC system consisting of a vacuum degasser, a quaternary MS pump, an autosampler with cooling device and a TSQ Quantum triple quadrupole mass spectrometer equipped with a Heated Electrospray Ionization (HESI) source operating in the negative mode (all from Thermo Finnigan, San Jose, USA), run by XCALIBUR software (version 2.0 SUR1).

Chromatographic separation was achieved using a Nucleodur C18 Gravity column (125 mm × 2.0 mm i.d., 3 μm) from Macherey-Nagel (Düren, Germany). The mobile phase A was a solution of 5 mM

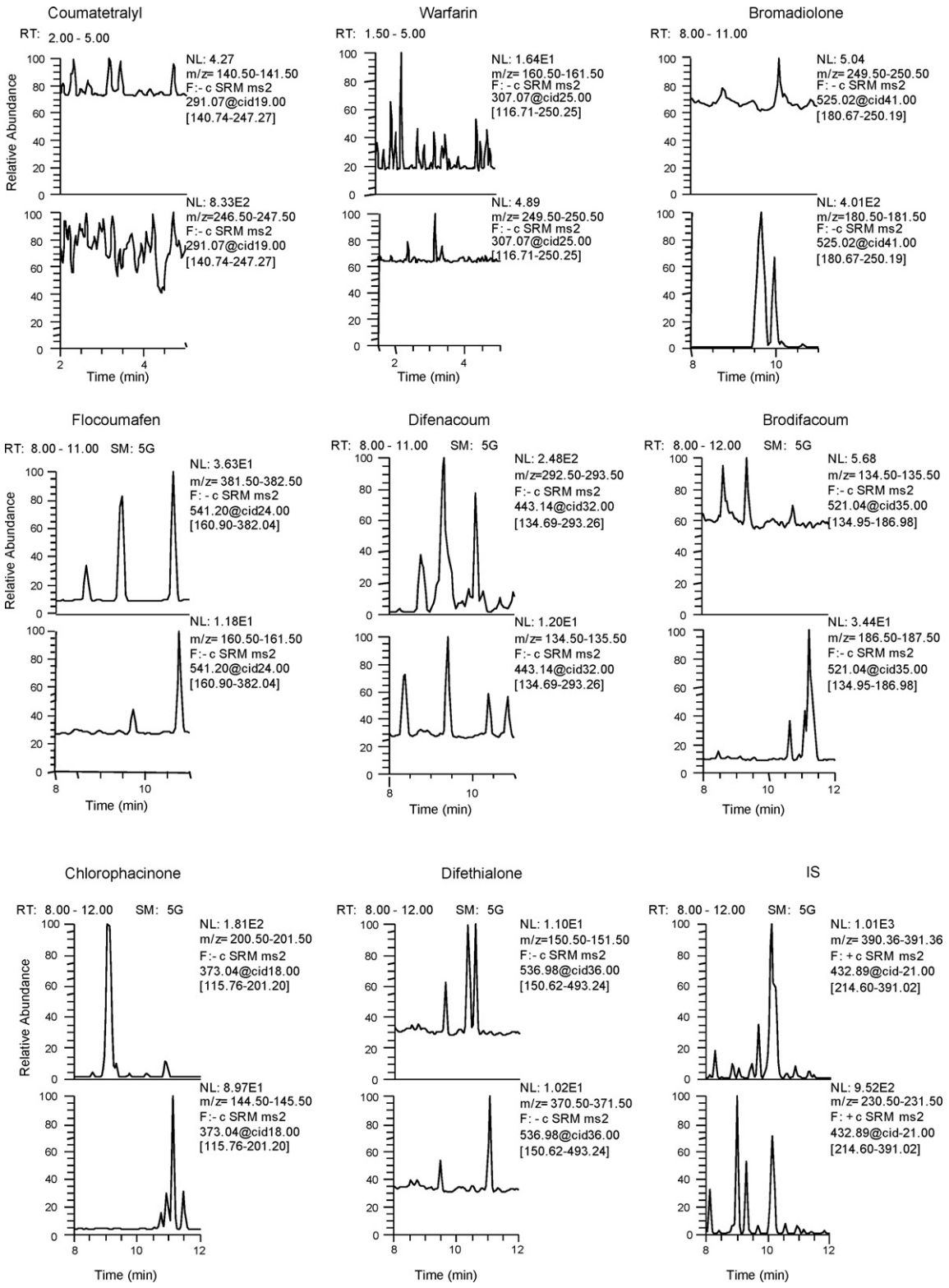

Fig. 3. Mass chromatograms of an extracted blank liver sample. The upper and lower trace for each analyte represent the product ion used for quantification and the second most abundant product ion, respectively.

ammonium formate in HPLC—water (adjusted to pH 9 using 10% ammonia in water), while solvent B consisted of 5 mM ammonium formate in methanol. Mobile phase was delivered to the LC column at a flow rate of 0.2 mL min^{-1}. A gradient elution was performed, i.e. 0–4 min: 50% A, 4–6 min: 10% A, 6–14 min: 10% A, 14–14.5 min: 50% A and 14.5–20 min: 50% A.

Operating conditions for the HESI source used in the negative ionization mode were optimized by constantly adding the individual compounds each at a concentration of 1 μg mL^{-1}, to the HPLC flow by a syringe pump via a T connector.

The following tune parameters were obtained for detection: spray voltage, 3.0 kV; sheath gas flow-rate, 40 (arbitrary units);

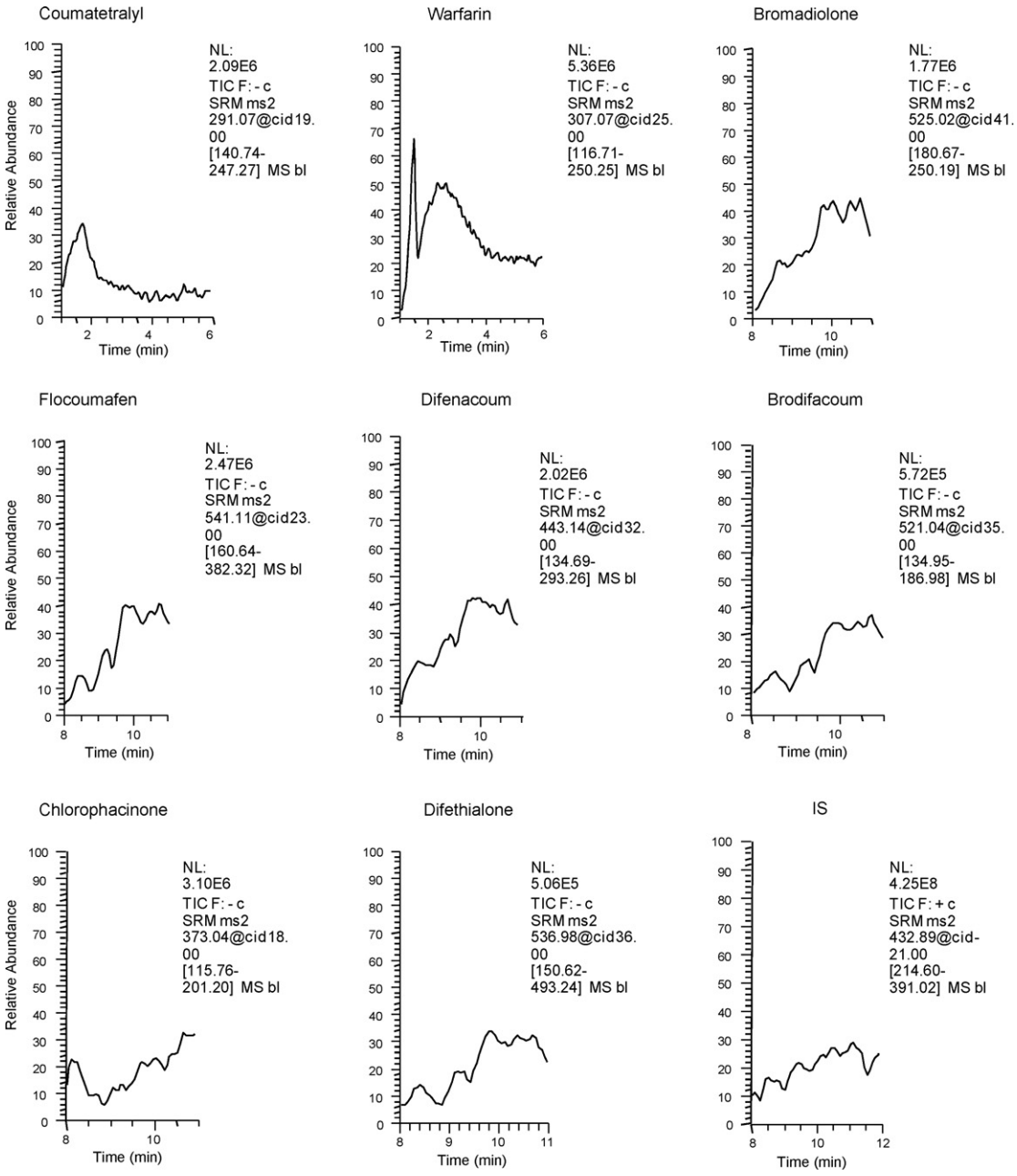

Fig. 4. Mass chromatograms for the ion suppression study in a liver.

auxiliary gas flow-rate, 35 (arbitrary units); ion sweep gas pressure, 1 (arbitrary units), capillary temperature, 350 °C, HESI vaporizer temperature, 300 °C. For each compound ion-dependent parameters were automatically tuned by the system and all three precursor ion–production ion transitions were stored in separate scan events.

2.5. Method validation

The presented method for the quantification of the eight anticoagulant rodenticides was validated by a set of parameters which are in compliance with the recommendations as defined in European guidelines and in the literature [22–24].

2.5.1. Linearity

The linearity was determined on calibration curves which were created using spiked blank plasma and liver samples.

In plasma, the linearity was tested in a low (1–10 ng mL^{-1} and 5–50 ng mL^{-1}) and a high (10–500 ng mL^{-1} or 50–750 ng mL^{-1} for chlorophacinone) concentration range.

In liver, the linearity was determined on concentration ranges of 10–500 ng g^{-1} for brodifacoum, chlorophacinone, difenacoum and difethialone, 25–750 ng g^{-1} for coumatetralyl and flocoumafen and 10–750 ng g^{-1} for bromadiolone and warfarin.

The acceptance criterion was a correlation coefficient $r \geq 0.99$ and a goodness-of-fit coefficient $g \leq 10\%$ or $\leq 20\%$ for calibration curves with the majority of calibrators above 10 ng mL^{-1} or below 10 ng mL^{-1}, respectively.

2.5.2. Accuracy

The accuracy was evaluated by analyzing on the same day six independently spiked blank plasma or liver samples at the same spike level. For plasma, these levels depended on the individual

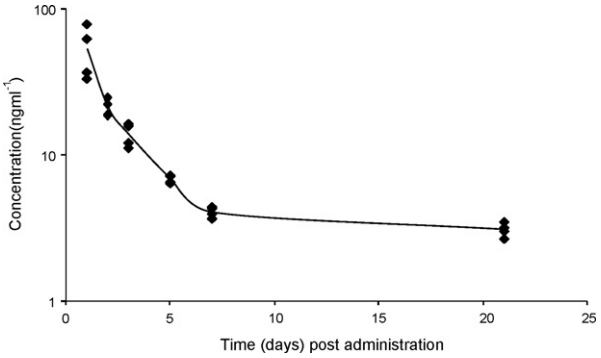

Fig. 5. Plasma concentration–time profile of difenacoum in the mouse after administration of a single oral dose of 0.4 mg kg^{-1} bw.

anticoagulant rodenticide. In liver, levels of 100, 250 and 500 ng g^{-1} were tested, except for coumatetralyl and warfarin where the levels were 250 and 500 ng g^{-1}.

The accuracy, expressed as the difference between the mean found concentration and the spiked concentration (in %), had to be within −50 to +20% for levels ≤1 ng mL^{-1} or ng g^{-1}, from −30 to +10% for levels >1 and <10 ng mL^{-1} or ng g^{-1} and from −20 to +10% for levels ≥10 ng mL^{-1} or ng g^{-1}.

2.5.3. Precision

The intra-day precision was expressed as the relative standard deviation (R.S.D., in %), being the ratio between the standard deviation (S.D.) and the mean found concentration. The R.S.D. (%) had to fall within the values calculated according to two thirds of the Horwitz equation: $\text{R.S.D.}_{\max} = 2/3 \times 2^{(1-0.5\log C)}$, with C being the analyte concentration in g mL^{-1} or g g^{-1}. The precision was determined on the same samples as for the accuracy evaluation.

2.5.4. Limit of quantification

The limit of quantification (LOQ) was defined as the lowest concentration for which the method is validated with an accuracy and precision that fall within the ranges recommended by the EU [23].

2.5.5. Limit of detection

The limit of detection (LOD) was defined as the lowest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. In this study the criterion of the signal-to-noise (S/N) ratio of 3/1 was used.

2.5.6. Specificity

The specificity was evaluated with respect to endogenous interferences by extracting and analyzing blank samples with the above-mentioned method. Known rodenticides-free plasma and liver samples were obtained from animals that were not exposed to anticoagulant rodenticides.

2.5.7. Ion suppression

For the study of the ion suppression a blank plasma and blank liver sample was injected onto the LC-MS instrument. A standard solution containing all compounds was continuously infused through a T-coupling device into the LC eluate. This allowed to visualize sections in the chromatogram where ion suppression occurs.

3. Results and discussion

3.1. Sample preparation

The extraction method was based on the method of Berny et al. [5]. The method presents a generic and straightforward approach that is applicable for the extraction of the group of coumarin and indandione anticoagulants. The extraction is based on the solubility of the compounds in acetone, and is inexpensive and technically easy to perform. Due to the high detection sensitivity of the MS instrument used in this study, the extraction could be performed

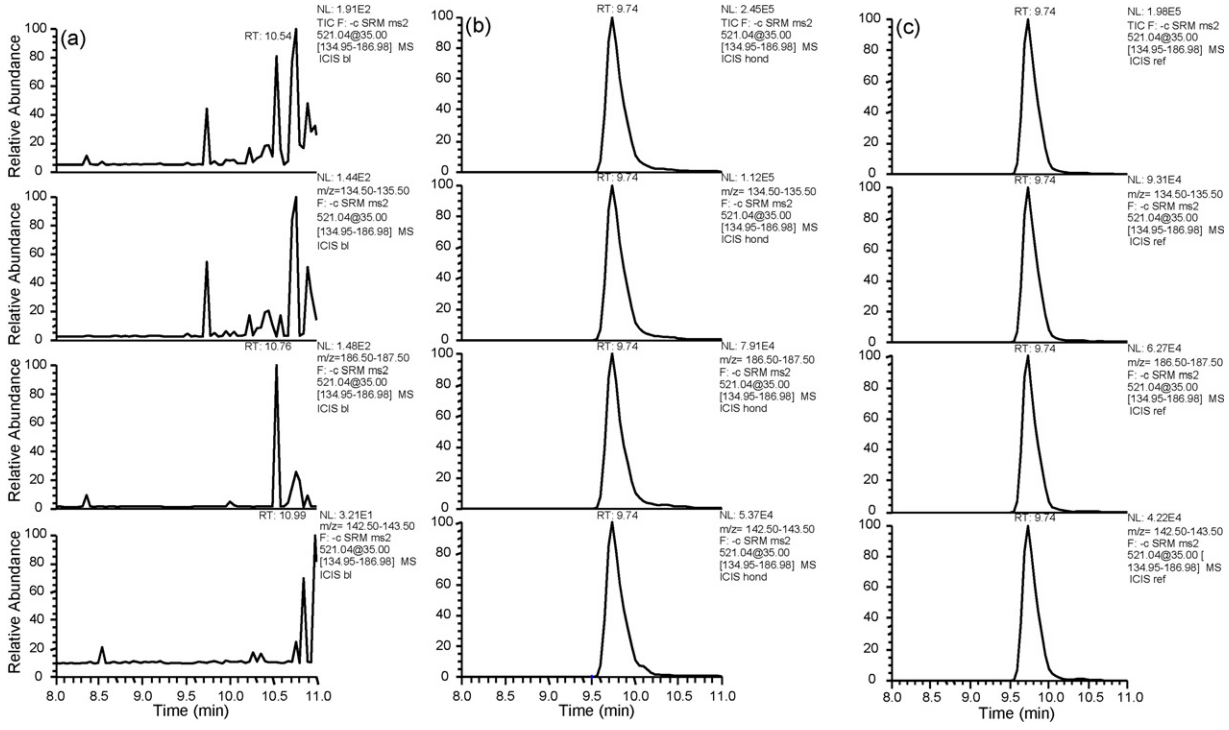

Fig. 6. MS/MS chromatograms for a blank liver sample (a), a liver sample positive for brodifacoum (b) and a blank liver sample spiked at 100 ng g^{-1} (c).

on 0.5 g of liver and 0.5 mL of plasma instead of 1.0 g and 2.0 mL as mentioned in the method of Berny et al. [5]. Although these small sample amounts were used, very low LOD and LOQ values could be obtained. In our method, the LODs were below 5 ng mL^{-1} and 5 ng g^{-1} in plasma and liver, respectively, which are well below the LODs of 200 ng g^{-1} reported by Berny et al. [5]. The latter levels are sufficient to detect the compounds in animal poisoning cases, but may be too high to be of use in the conduct of pharmacokinetic studies where low levels can be expected during the terminal elimination of the compounds.

3.2. Chromatography and mass spectrometry

A previously developed HPLC-UV or fluorescence method was not sensitive enough for routine toxicological analysis, so there was a need for a more sensitive detection system like LC-MS/MS. The HESI source offered specific advantages in comparison to the ESI source, with respect to the ion intensity. In the MS-mode the most prominent ion for all compounds was the deprotonated $[M-H]^-$ molecular ion. In the SRM-mode the $[M-H]^-$ ions were fragmented in Q2 and three precursor-product transitions were performed for each compound. The internal standard was analyzed in the positive ion mode since in negative ion mode no signal could be generated. This was also seen by Grobosch et al. [21], using the same internal standard. The transitions are listed in Table 1, the product ions in bold were used for quantitation. As mentioned in the literature some rodenticides gave carry-over problems on the detection instrument at higher concentrations. This problem could be solved by flushing the injection needle with 3 mL of methanol followed by an additional wash with again 3 mL of methanol between each injection.

Fig. 1 shows the chemical structures, MS/MS spectra and mass chromatograms of the internal standard and the eight anticoagulant rodenticides in plasma spiked at 10 ng mL^{-1}. The mass chromatograms of the product ions used for quantification, i.e. those mentioned in bold in Table 1, are shown.

3.3. Method validation

The results obtained for the method validation are shown in Table 2.

3.3.1. Linearity

In plasma, linear calibration curves were obtained in a concentration range of 1–10 ng mL^{-1} for bromadiolone, coumatetralyl, difenacoum, flocoumafen and warfarin and in a range of 5–50 ng mL^{-1} for brodifacoum, chlorophacinone and difethialone. Good linearity was observed up to 500 ng mL^{-1} and up to 750 ng mL^{-1} for chlorophacinone. The goodness-of-fit coefficients of the individual curves were all ≤11.83% and the correlation coefficients (r) ≥0.9910 for all compounds.

In liver, good linearity was seen up to 500 ng g^{-1} for brodifacoum, chlorophacinone, difenacoum and difethialone and up to 750 ng g^{-1} for the other compounds. The goodness-of-fit coefficients were all ≤11.79% and the correlation coefficients (r) were all ≥0.9975 for all compounds.

3.3.2. Accuracy and precision

The accuracy fell within the range of −20 to +10% for concentrations ≥10 ng mL^{-1} or ng g^{-1}, within the range of −30 to +10% for concentrations >1 and <10 ng mL^{-1} or ng g^{-1} and of −50 to +20% for concentrations ≤1 ng mL^{-1} or ng g^{-1}. The precision, expressed as R.S.D. values, for all matrices and at all levels tested, fell within the maximum R.S.D. values.

3.3.3. Limit of quantification and limit of detection

The lowest levels that could be quantified fulfilling the criteria for accuracy and precision were, depending on the compound, 1 or 5 ng mL^{-1} in plasma and were therefore set as the LOQs of the method. In liver, the LOQ was set at 100 ng g^{-1} for all compounds, except for coumatetralyl and warfarin where the LOQ was set at 250 ng g^{-1}.

The LODs were determined using the signal-to-noise (S/N) ratio of 3/1 on the same samples used for LOQ determination.

3.3.4. Specificity

Blank plasma and liver samples were extracted and analyzed using the above-mentioned method. The chromatograms were free of interferences at the elution times of the anticoagulant rodenticides, testifying of good specificity of the method, as shown in Figs. 2 and 3 for plasma and liver, respectively. The upper and lower trace in the figures represent the product ion used for quantification and the second most abundant product for each analyte, respectively.

3.3.5. Ion suppression

No ion suppression was noticed at the elution time zones of all eight analytes in either plasma or liver. As an example, the mass chromatograms for the ion suppression experiment in liver is shown in Fig. 4.

3.4. Applicability

The method was used for the analysis of plasma and liver samples from mice that received a single oral administration of an anticoagulant rodenticide in order to study the pharmacokinetics. A representative plasma concentration versus time profile of difenacoum after single oral administration of 0.4 mg kg^{-1} in the mouse is shown in Fig. 5.

The presented method is also used to determine anticoagulant rodenticides in domestic animals in which rodenticide poisoning is suspected. The case presented here deals with a dog that was lethargic for several days and suffered from severe abdominal pain. Although the dog was treated with vitamin K_1 and received a blood transfusion, the animal died the day after. Post-mortem examination showed several bleedings in the thoracal and the abdominal cavities as well as a pale liver and a contracted spleen. Liver tissue was analyzed with the presented LC-MS/MS method indicating the presence of 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 [23]. The chromatograms of a blank liver sample, a liver sample positive for brodifacoum and a blank liver sample spiked at 100 ng g^{-1} are presented in Fig. 6.

4. Conclusions

In this paper we describe, to our knowledge, for the first time the development and validation of a multi-residue LC-HESI-MS/MS method for the quantitative determination of eight anticoagulant rodenticides in biological matrices like plasma and liver. One of the main advantages of the developed LC-MS/MS method is the possibility to detect simultaneously all rodenticides available on the Belgian market. The LODs of the method in plasma and liver make it possible to detect low concentrations which is of particular use in suspected poisoning cases. The method was also successfully applied for the analysis of anticoagulant rodenticides in biological samples, which were taken as part of a pharmacokinetic study in the mouse.

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References

[1] B.E. Watt, A.T. Proudfoot, S.M. Bradberry, J.A. Vale, Toxicol. Rev. 24 (2005) 259.
[2] A. Campbell, M. Chapman, in: A. Campbell, M. Chapman (Eds.), Handbook of Poisoning in Dogs and Cats, first ed., Blackwell Science Ltd., Oxford, 2000, p. 64.
[3] R.E. Marsh, Pest Control 53 (1985) 20.
[4] P.J. Berny, J. Vet. Pharmacol. Ther. 30 (2007) 93.
[5] P.J. Berny, T. Buronfosse, G. Lorgue, J. Anal. Toxicol. 19 (1995) 576.
[6] A.J. Kieboom, C.G. Rammel, Bull. Environ. Contam. Toxicol. 26 (1981) 674.
[7] K. Hunter, E.A. Sharp, A. Newton, J. Chromatogr. 435 (1988) 83.
[8] S.M. O'Bryan, D.J. Constable, J. Anal. Toxicol. 15 (1991) 144.
[9] M.J. Kelly, J. Chambers, A.D. MacNicoll, J. Chromatogr. 620 (1993) 105.
[10] A. Jones, Bull. Environ. Contam. Toxicol. 56 (1996) 8.
[11] F. Guan, A. Ishii, H. Seno, K. Watanabe, T. Kumazawa, O. Suzuki, J. Pharm. Biomed. Anal. 21 (1999) 179.
[12] P. Duffield, A.M. Duffield, M. Kennedy, D.J. Birkett, D.N. Wade, Aust. N. Z. J. Med. 9 (1979) 534.
[13] A.C. Ray, M.J. Murphy, M.D. Duvall, J.C. Reagor, Am. J. Vet. Res. 50 (1989) 546.
[14] M.E. Mount, M.J. Kurth, D.Y. Jackson, J. Immunoassay 9 (1988) 69.
[15] W.E. Braselton, R.D. Neiger, R.H. Poppenga, J. Vet. Diagn. Invest. 4 (1992) 441.
[16] M.C. Jin, X.H. Chen, Rapid Commun. Mass Spectrom. 20 (2006) 2741.
[17] M.C. Jin, X.K. Ouyang, X.H. Chen, J. Appl. Toxicol. 27 (2007) 18.
[18] M.C. Jin, X.K. Ouyang, X.M. Xu, Y.P. Ren, X.H. Chen, Anal. Lett. 40 (2007) 737.
[19] M.C. Jin, Y.P. Ren, X.M. Xu, X.H. Chen, Forensic Sci. Int. 171 (2007) 52.
[20] L.J. Marek, W.C. Koskinen, J. Agric. Food Chem. 55 (2007) 571.
[21] T. Grobosch, B. Angelow, D. Lampe, Toxichem. Krimitech. 72 (2005) 46.
[22] R.J. Heitzman (Ed.), Veterinary Drug Residues, Report Eur. 15127-EN, Commission of the EC, Brussels-Luxembourg, 1994.
[23] Commission Decision of 2002/657/EC Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results, Off. J. Eur. Comm., 17/08/2002, No. L221/8-36.
[24] EMEA/CVMP/573/00-Final. Vol. 8, Notice to Applicants, Veterinary Medicinal Products: Establishment of Maximum Residue Limits (MRLs) for Residues of Veterinary Products in Foodstuff of Animal Origin: Development and validation of a Proposed Regulatory Method.