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Article

A Simplified LC-MS/MS Method for the Determination of Anticoagulant Rodenticides in Emergency Department Patients with Unexplained Coagulopathy

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Abstract

Anticoagulant rodenticide (AR) poisoning may cause prolonged and potentially life-threatening coagulopathy, while confirmation of exposure can be challenging when the exposure history is unclear. We developed and validated a simplified liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of five ARs and warfarin in human plasma and evaluated its clinical applicability in a patient with unexplained coagulopathy. The method used 50 µL of plasma with a simple protein-precipitation procedure followed by LC-MS/MS analysis. The validated quantitative range was 10–1000 ng/mL, with a lower limit of quantification of 10 ng/mL, while all analytes fulfilled the predefined qualitative identification criteria at 5 ng/mL. The method demonstrated acceptable accuracy, precision, selectivity, and matrix-effect performance. In the clinical case, bromadiolone was identified, and two plasma measurements showed a declining concentration profile, with an estimated apparent elimination half-life of 10.4 days. Based on this estimate, the plasma bromadiolone concentration was predicted to decline below 10 ng/mL at approximately day 42. This estimated concentration trajectory, together with the patient's clinical course and serial coagulation findings, supported the decision to discontinue oral vitamin K1 on day 42. Subsequent INR measurements remained normal on days 45 and 49. Although 10 ng/mL served as a practical reference in this case, it should not be used as the sole criterion for vitamin K1 discontinuation. This simplified LC-MS/MS method provides a practical approach for identifying and quantifying AR exposure in

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patients with otherwise unexplained coagulopathy and may support clinical assessment and management.

Keywords: anticoagulant rodenticides; bromadiolone; LC-MS/MS; vitamin K1; toxicokinetics

1. Introduction

Abnormal coagulation findings in emergency department (ED) patients may be associated with increased mortality [1]. Major bleeding requires prompt management, including hemodynamic stabilization, replacement of blood loss, correction of coagulopathy, and control of the bleeding source [2,3]. However, correction of coagulation abnormalities does not obviate the need to determine their underlying cause through clinical assessment and directed laboratory evaluation [4].

The differential diagnosis of unexplained prothrombin time (PT)/international normalized ratio (INR) and activated partial thromboplastin time (aPTT) prolongation includes coagulation-factor deficiencies or inhibitors, liver dysfunction, disseminated intravascular coagulation, and exposure to anticoagulants or other toxicants [4,5]. Because factor VII has the shortest half-life among the vitamin K (VK)-dependent coagulation factors, impairment of the VK cycle typically prolongs PT/INR before aPTT [6–8]. Laboratory evidence of anticoagulant rodenticide (AR)-induced coagulopathy may appear within 8–48 h after acute exposure, whereas clinically evident bleeding most often occurs 3–9 days later [9–11].

Further evaluation of unexplained coagulopathy may include mixing studies, specific coagulation-factor assays, and other specialized hemostatic tests [5,12]. Correction of prolonged PT and aPTT after mixing with normal plasma supports coagulation-factor deficiency rather than the presence of an inhibitor [13,14], whereas correction after VK administration supports a VK-dependent coagulopathy [7]. However, specific toxicological testing for long-acting VK antagonists (VKAs) is not routinely available [15]. Recurrent prolongation of PT/INR and aPTT after initial correction with VK and coagulation-factor replacement should therefore raise suspicion for exposure to a long-acting VKA, particularly an AR [13,16].

Warfarin and ARs inhibit VK epoxide reductase complex subunit 1 (VKORC1)-mediated VK recycling, thereby reducing the functional activity of VK-dependent coagulation factors [15,17,18]. Pharmacological doses of VK1 (phytonadione) can restore VK-dependent coagulation through alternative VK reduction pathways [19,20]. Five ARs currently registered for rodent control in Taiwan—brodifacoum, bromadiolone, coumatetralyl, difenacoum, and flocoumafen—were selected for the analytical panel based on Taiwanese environmental-agent and pesticide registration databases accessed in August 2026 [21,22]. Warfarin was included because, although historically used as a first-generation rodenticide, it is also a therapeutic VKA that produces a similar VK-dependent coagulopathy [13]. The chemical structures of the target compounds and warfarin-d5 are shown in Figure 1.

Management of clinically significant AR poisoning includes VK1 administration, with fresh frozen plasma (FFP) or four-factor prothrombin complex concentrate (PCC) used for rapid coagulation-factor replacement in patients with severe or life-threatening bleeding [15,23,24]. Diagnosis may nevertheless be difficult when AR exposure is unknown, concealed, or initially denied [25]. PT/INR and aPTT reflect the pharmacodynamic consequences of AR-induced coagulopathy but cannot identify the causative AR or quantify its residual plasma concentration [15]. Direct measurement of AR concentrations can confirm

exposure [15], while serial quantitative measurements may provide additional information on individual elimination kinetics during clinical management [15,25,26].

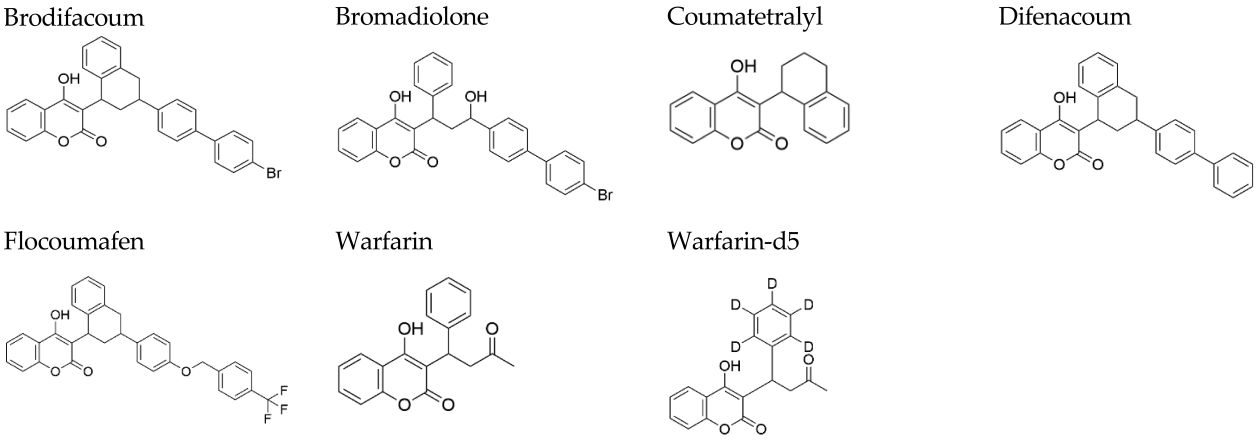


Figure 1. Chemical structures of five ARs, warfarin, and warfarin-d5 (IS).

Although multi-analyte liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods for AR determination have been reported, many were developed for whole blood or require multistep sample preparation, including liquid–liquid extraction and, in some methods, solvent evaporation and reconstitution [27–29]. A simplified plasma-based method targeting ARs relevant to rodent control in Taiwan could therefore facilitate clinical toxicological assessment. Accordingly, we developed and validated a targeted LC-MS/MS method for the simultaneous quantification of brodifacoum, bromadiolone, coumatetralyl, difenacoum, flocoumafen, and warfarin in human plasma. The method was subsequently applied to a patient with unexplained coagulopathy and bleeding to demonstrate its utility in confirming AR exposure and to explore its potential role as an adjunct to clinical management.

2. Case Report

A 50-year-old man with hypertension and diabetes mellitus developed gingival bleeding, hematuria, and bloody stool 3 days before presentation to the referring hospital. Initial laboratory investigations showed severe anemia (hemoglobin, 5.4 g/dL), a normal platelet count, markedly prolonged PT (INR > 6), and prolonged aPTT (100 s). Hemoglobin, PT, and aPTT had been normal 1 month earlier. Because of unexplained coagulopathy and the absence of an identifiable bleeding source on endoscopy, he was transferred to our hospital the following day. Presentation to the referring hospital was designated day 0, and transfer to our hospital was designated day 1. Before transfer, he had received packed red blood cells (PRBCs), FFP, intravenous VK1, tranexamic acid, and a proton pump inhibitor.

On arrival, he was alert, with stable vital signs. Serial laboratory findings and treatment are summarized in Table 1. Mixing studies corrected both PT and aPTT, suggesting a coagulation factor deficiency rather than an inhibitor. Abdominal ultrasonography demonstrated fatty liver without evidence of cirrhosis or advanced liver disease. The markedly prolonged PT/INR and aPTT, normal platelet count, correction on mixing studies, absence of advanced liver disease, and response to coagulation-factor replacement and empirical VK1 raised suspicion of warfarin or AR exposure. The patient initially denied exposure to any suspicious agents.

A plasma sample collected on day 4 was analyzed by LC-MS/MS and demonstrated a bromadiolone concentration of 122.5 ng/mL. After repeated history taking, the patient admitted ingesting bromadiolone 11 days before day 0, although the amount remained unknown. Repeat plasma analysis on day 17 demonstrated a bromadiolone concentration

of 51.6 ng/mL. Intravenous VK1 was transitioned to oral VK1 (10 mg twice daily) on day 4. The patient was discharged without recurrent major bleeding, and oral VK1 was continued through day 42 and then discontinued during outpatient follow-up.

Table 1. Serial laboratory findings and clinical management of the index case.

Clinical Day *	0	1	2	3	4	7	14	17	35	42	45	49
Laboratory data (reference range)												
Blood routine:												
White blood cell counts ($3.9–10.6 \times 10^3/\mu L$)	15.63	9.6	8.59	8.41		5.28	5.06					
Neutrophil, %	77.1	77.1		81.6		70.8	69.9					
Lymphocyte, %	16	15		10		15.9	17.6					
Monocyte, %	6.8	6.8		6.4		6.8	8.9					
Hemoglobin (13.5–17.5 g/dL)	5.4	6.4	8.1	8.2		8.9	9.7	9.5				
Platelet ($150–400 \times 10^3/\mu L$)	348	244	229	215		358	409					
Serum chemistry:												
Sodium (136–145 mEq/L)	138	142	145	140		141	137	139				
Potassium (3.5–5.1 mEq/L)	3.1	3.1	2.9	3.2		3.9	2.9	3.5				
Blood urea nitrogen (5–25 mg/dL)	28	25	17	11		12	7	6				
Creatinine (0.7–1.4 mg/dL)	1.57	1.21	1.03	0.8		0.67	0.7	0.63				
Glucose, mg/dL	177	132					67					
Alanine aminotransferase (10–50 U/L)	13	11				14	13	13				
Aspartate aminotransferase (<41 U/L)	11											
Total bilirubin (0.2–1.2 mg/dL)		1.25	1.46	1.54		1.69	1.13	0.89				
Direct bilirubin (0–0.5 mg/dL)			0.48	0.55		0.75	0.44					
Lactate dehydrogenase (120–240 U/L)		144										
High-sensitivity C-reactive protein (≤0.5 mg/dL)	0.036	3.565										
Lactate (4.5–19.8 mg/dL)		10.6										
Coagulation profiles:												
INR (0.85–1.15)	>6	2.26	1.94	2.15		1.39	1.28	1.21	1.06		1.07	1.1
Activated partial thromboplastin time (24.3–32.7 s)	100	43.8	35	42.3		35.6	34.8	32.9	26.5		29.3	29
Mixing PT (0 min, 2 h; s)		12.7, 12.8			12.4, 12.5							
Mixing aPTT (0 min, 2 h; s)		27.3, 32.3										
Fibrinogen (200–400 mg/dL)		329										
D-dimer (<0.55 mg FEU/L)		0.23										
FDP (<5 μg/mL)		<2.5										
Toxicant analysis:												
Bromadiolone (ng/mL)					122.5			51.6				
Management (including transfusion)												
Packed red blood cells (150 mL/unit, units)	6	2	2									
Fresh frozen plasma (100 mL/unit, units)	4	6	6									
Vitamin K1 dose and route (iv or po)	10 mg iv qd	10 mg iv qd	10 mg iv qd	10 mg iv qd	10 mg po bid	10 mg po bid	10 mg po bid	10 mg po bid	10 mg po bid	10 mg po bid		

* Day 0 was defined as the day of presentation to the referring hospital.

3. Materials and Methods

3.1. Chemicals and Reagents

Brodifacoum (purity, 98.96%) was purchased from Chiron (Trondheim, Norway). Bromadiolone (purity, 95.22%) and difenacoum (purity, 99.01%) were purchased from Dr. Ehrenstorfer GmbH, LGC Standards (Augsburg, Germany), whereas coumatetralyl (purity, 98%) was purchased from Chem Service (West Chester, PA, USA). Flocoumafen (purity, 100%) and warfarin (purity, ≥98%) were purchased from AccuStandard (New Haven, CT, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The isotopically labeled internal standard (IS), warfarin-d5 (purity, ≥99%), was purchased from Cayman Chemical (Ann Arbor, MI, USA). All reference standards were used before their assigned reanalysis dates.

Ammonium acetate (ACS reagent; purity, ≥98%) was purchased from Honeywell (Muskegon, MI, USA). Methanol (HPLC grade; purity, ≥99.9%) was purchased from Macron Fine Chemicals (Avantor, Radnor, PA, USA). Ultrapure water was produced using Elix 10 and Milli-Q water purification systems (Merck Millipore, Darmstadt, Germany). Residual heparinized plasma specimens obtained from patients without suspected AR poisoning were used as blank plasma for method validation.

3.2. Preparation of Calibration Standards, QC Samples, and the IS Working Solution

Two independent stock solutions of each analyte were separately prepared from the same reference standard in methanol at 100 μg/mL, one for calibration standards and the other for QC samples, and stored at −20 °C. For each analytical run, separate mixed calibration and QC working solutions were freshly prepared by diluting their respective stock solutions with methanol. These working solutions were then separately spiked into pooled blank human plasma to prepare calibration standards and QC samples at the desired concentrations. Matrix-matched calibration was employed to minimize the impact of matrix-related differences on quantitative accuracy [30]. Each calibration standard and QC sample contained all six target analytes. Calibration standards were prepared at 10, 50, 200, 500, 750, and 1000 ng/mL, whereas QC samples were prepared at 10, 30, 500, and 800 ng/mL. The IS working solution containing warfarin-d5 (100 ng/mL) was freshly prepared in methanol. All calibration standards, QC samples, and the IS working solution were used on the day of preparation.

Warfarin-d5 was selected as a common IS because it is a stable isotope-labeled analogue structurally related to the coumarin-type compounds in the analytical panel and is not expected to occur endogenously in clinical samples. Its use as a common IS for the simultaneous LC-MS/MS quantification of multiple ARs in human blood has also been reported [28].

3.3. Sample Preparation

Clinical blood samples were collected in heparinized Vacutainer tubes and centrifuged at 5000 rpm for 5 min at 4 °C to obtain plasma. Blank and clinical plasma samples were stored at −20 °C until analysis. Frozen plasma samples were thawed at room temperature and vortex-mixed before sample preparation.

For protein precipitation, 50 μL of plasma was mixed with 450 μL of methanolic IS working solution containing warfarin-d5 (100 ng/mL), resulting in a final IS concentration of 90 ng/mL. The mixture was vortex-mixed for 30 s and centrifuged at 14,000 rpm for 10 min. The supernatant was filtered through a 0.2-μm polytetrafluoroethylene (PTFE) syringe filter into an autosampler vial for LC-MS/MS analysis.

3.4. LC-MS/MS Conditions

Liquid chromatography was performed using an ExionLC™ system coupled to a QTRAP® 6500+ mass spectrometer (SCIEX, Marlborough, MA, USA). Chromatographic separation was achieved on a Poroshell EC-C18 column (2.1 × 100 mm, 1.9 μm; Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of 5 mM ammonium acetate in water (A) and methanol (B), delivered at a flow rate of 0.3 mL/min. The column temperature was maintained at 40 °C, and the injection volume was 3 μL. The gradient was initiated at 40% B, held for 1.0 min, increased linearly to 99% B at 5.0 min, maintained at 99% B until 7.3 min, returned to 40% B at 7.5 min, and re-equilibrated at 40% B until 10.0 min.

The mass spectrometer was operated in positive and negative electrospray ionization modes with polarity switching in multiple reaction monitoring (MRM) mode. Compound-specific quantifier and qualifier MRM transitions and optimized declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) are

summarized in Table 2. The source temperature was 500 °C, and the ion spray voltage was 5000 V in positive mode and −4000 V in negative mode. Data acquisition and processing were performed using SCIEX OS software (version 4.0; SCIEX, Marlborough, MA, USA). Under these chromatographic conditions, bromadiolone exhibited split peaks that may reflect stereoisomers [31]. The areas of both peaks were summed for quantification of total bromadiolone [32,33], without assigning individual peaks to specific stereoisomers. The same integration procedure was applied to calibration standards, QC samples, and clinical specimens.

Table 2. Retention times, MRM transitions, and optimized mass spectrometric parameters for ARs, warfarin, and warfarin-d5.

Name	Retention Time (min)	Polarity	Precursor Ion	Product Ion (Quantifier)			Product Ion (Qualifier)				Ion Optics Parameters	
			m/z	*m/z*	CE (eV)	CXP (V)	*m/z*	Ion Ratio % (Ion Ratio Tolerances)	CE (eV)	CXP (V)	DP (V)	EP (V)
Brodifacoum	5.32	+ve	523	335	32	25	178	41 (30.8–51.3)	48	15	40	10
Bromadiolone	4.79	−ve	525	250	−50	−10	93	17 (11.9–22.1)	−70	−10	−90	−10
Coumatetralyl	2.94	−ve	291	141	−36	−15	247	61 (48.8–73.2)	−30	−15	−40	−10
Difenacoum	4.96	+ve	445	257	28	17.5	179	87 (69.6–104.4)	42	15	50	10
Flocoumafen	5.19	−ve	541	161	−46	−15	382	88 (70.4–105.6)	−38	−12.5	−60	−10
Warfarin	2.91	−ve	307	161	−28	−15	250	77 (61.6–92.4)	−32	−17.5	−40	−10
Warfarin-d5	2.88	−ve	312	161	−26	−15	255	77 (61.6–92.4)	−32	−17.5	−20	−10

CE: collision energy; CXP: collision cell exit potential; DP: declustering potential; EP: entrance potential; +ve: positive; −ve: negative.

3.5. Method Validation

A fit-for-purpose analytical validation was performed using selected principles and acceptance criteria from ANSI/ASB Standard 036, CLSI EP06 and C62, and the ICH M10 guideline [34–37]. Validation parameters included selectivity, calibration performance and analytical range, qualitative detection level, accuracy, precision, and matrix effects.

Selectivity was evaluated using blank plasma from six independent individuals. Interfering responses at the retention times (RTs) of the analytes and IS were required to be ≤20% of the corresponding analyte response at the lower limit of quantification (LLOQ) and ≤5% of the IS response, respectively [36].

Analyte identification required detection of both compound-specific quantifier and qualifier MRM transitions, an RT deviation within ±2.5% of the corresponding matrix-matched calibration standards [37], and qualifier-to-quantifier ion-ratio agreement within relative tolerances of ±20%, ±25%, ±30%, and ±50% for ion ratios of >50%, >20–50%, >10–20%, and ≤10%, respectively [38]. These criteria were used for the identification of target analytes in plasma samples throughout the analytical procedure. Structurally related compounds, potential metabolites, concomitant substances, and haemolysed or lipaemic plasma were not systematically evaluated as separate interference challenges.

Each analytical sequence included a blank sample, a zero sample containing the IS, and six matrix-matched non-zero calibration standards ranging from 10 to 1000 ng/mL [36]. Calibration curves were constructed using the six non-zero calibration standards, excluding the blank and zero samples [36]. Analyte-to-IS peak-area ratios were regressed against nominal analyte concentrations using a linear model with 1/x weighting. Back-calculated concentrations were required to be within ±15% of the nominal concentrations, except at the LLOQ, where ±20% was accepted. All six non-zero calibration levels were required to meet these criteria [36].

A concentration of 5 ng/mL, corresponding to one-half of the validated LLOQ, was administratively selected as the qualitative detection level and was not intended to represent the lowest analytically detectable concentration. It was evaluated using the decision-point approach described in Section 8.7.5 of ANSI/ASB Standard 036 [35]. Three independent blank plasma sources were fortified at 5 ng/mL and analyzed in each of three analytical runs (n = 9 per analyte). All measurements at 5 ng/mL were required to fulfill the predefined analyte identification criteria described above.

The upper limit of quantification (ULOQ) of 1000 ng/mL was selected based on concentration ranges reported in previous human AR poisoning cases [27,39]. Concentrations exceeding this range would require a separately validated dilution procedure before quantitative reporting.

Accuracy and precision were evaluated at the LLOQ and low-, medium-, and high-QC levels (10, 30, 500, and 800 ng/mL, respectively). Within-run precision was assessed using five replicates per concentration level on three separate analytical days, with within-run coefficients of variation (CVs) calculated separately for each day. Inter-day precision was calculated from all 15 measurements across the three analytical days. Accuracy was expressed as the mean measured concentration relative to the nominal concentration, and precision as the CV (standard deviation/mean × 100%). Mean accuracy was required to be within 85–115% of the nominal concentration, except at the LLOQ, where 80–120% was accepted. Precision was required to be ≤15% CV, except at the LLOQ, where ≤20% was accepted [35,36].

Matrix effects were evaluated using an accuracy- and precision-based approach across independent plasma sources, following the principles described in ICH M10 Section 3.2.3 [36,40]. Low- and high-QC samples were independently prepared from six individual sources of blank human plasma, without pooling, with three replicates analyzed at each concentration level for each source [36]. For each plasma source, mean accuracy was required to be within 85–115% of the nominal concentration and precision to be ≤15% CV [36].

4. Results

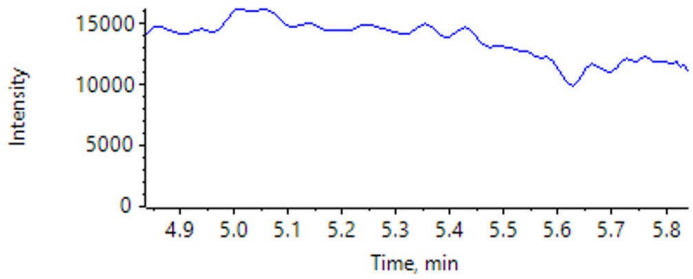
4.1. MRM Transitions and Selectivity

The optimized RTs, precursor ions, quantifier and qualifier ions, and compound-specific mass spectrometric parameters are summarized in Table 2. No significant endogenous interference was observed at the RTs of the analytes or the IS in any of the six independent blank plasma samples. Representative chromatograms of blank plasma and plasma spiked at 10 ng/mL are shown in Figure 2, together with the bromadiolone chromatogram from the patient sample collected on day 4.

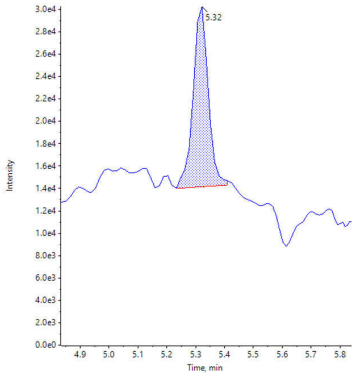
4.2. Calibration Performance and Analytical Range

The validated calibration range was 10–1000 ng/mL for all five ARs and warfarin, with an LLOQ of 10 ng/mL and a ULOQ of 1000 ng/mL. All calibration standards met the predefined acceptance criteria, with coefficients of determination (R^2) > 0.998 for all analytes (Figure 3). All analytes also fulfilled the predefined qualitative identification criteria at 5 ng/mL in all nine measurements.

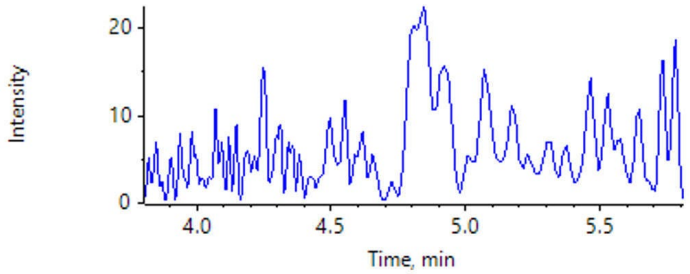
A1. Brodifacoum (*m*/*z* 523 → 335)



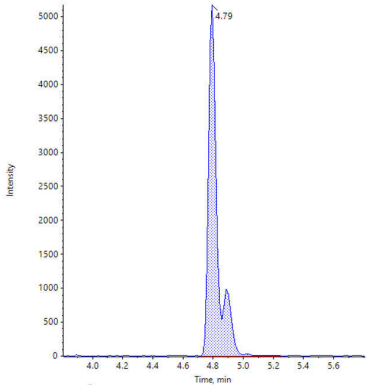
B1. Brodifacoum (*m*/*z* 523 → 335)



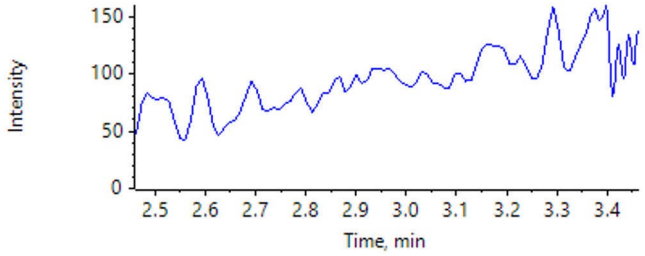
A2. Bromadiolone (*m*/*z* 525 → 250)



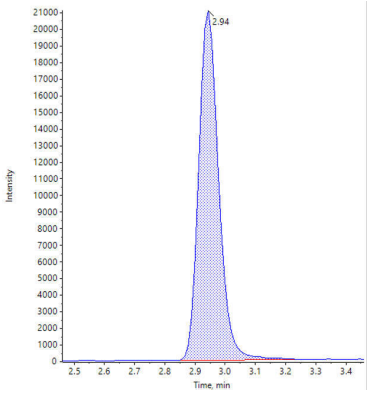
B2. Bromadiolone (*m*/*z* 525 → 250)



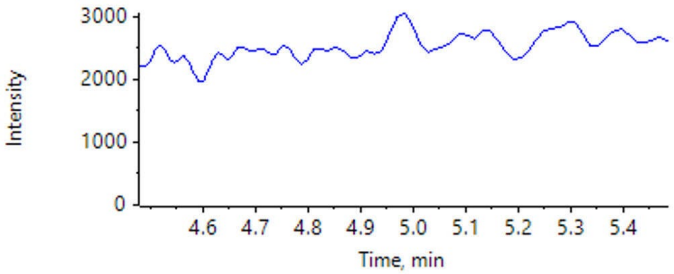
A3. Coumatetralyl (*m*/*z* 291 → 141)



B3. Coumatetralyl (*m*/*z* 291 → 141)



A4. Difenacoum (*m*/*z* 445 → 257)



B4. Difenacoum (*m*/*z* 445 → 257)

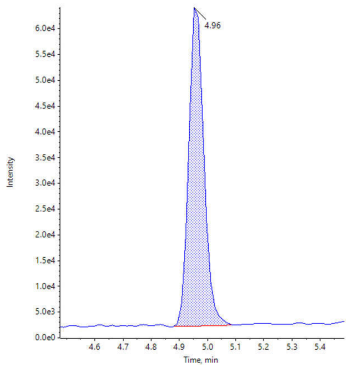
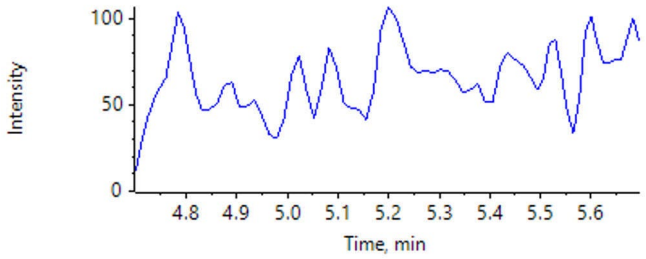
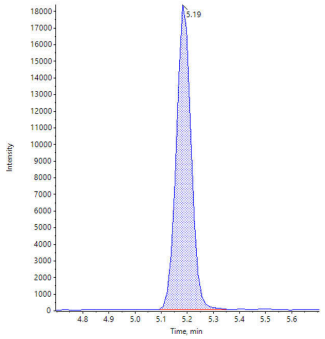


Figure 2. *Cont.*

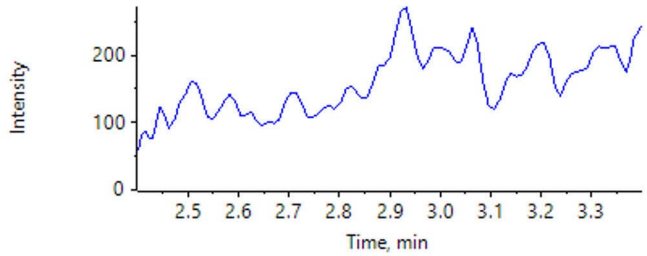
A5. Flocoumafen (*m*/*z* 541 → 161)



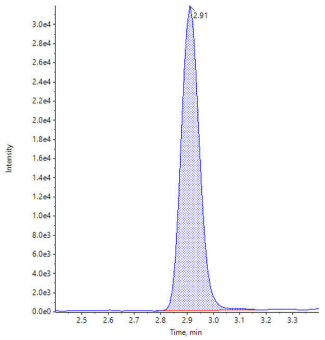
B5. Flocoumafen (*m*/*z* 541 → 161)



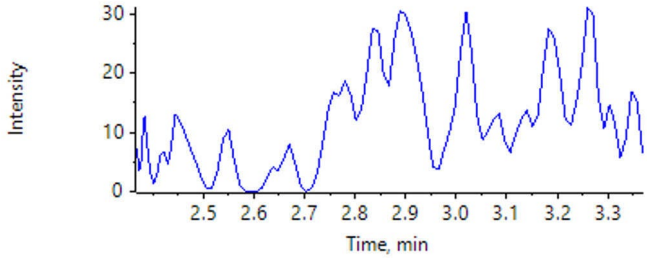
A6. Warfarin (*m*/*z* 307 → 161)



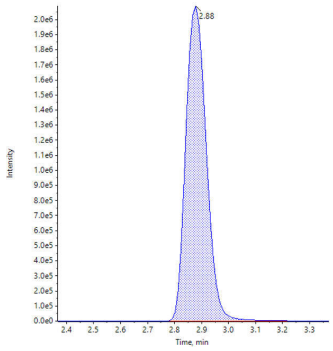
B6. Warfarin (*m*/*z* 307 → 161)



A7. Warfarin-d5 (*m*/*z* 312 → 161)



B7. Warfarin-d5 (*m*/*z* 312 → 161)



C. Patient plasma: Bromadiolone, 122.5 ng/mL (day 4)

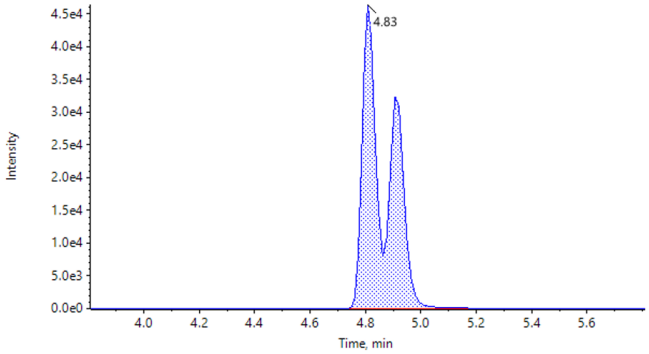


Figure 2. Representative LC-MS/MS chromatograms of ARs, warfarin, and warfarin-d5. Blank human plasma (**A1**–**A7**), plasma spiked with each indicated compound at 10 ng/mL (**B1**–**B7**), and patient plasma containing bromadiolone at 122.5 ng/mL on day 4 (**C**) are shown. The split bromadiolone peaks may reflect stereoisomers; the reported concentration was calculated from the combined peak areas. The *y*-axis scale was adjusted independently for optimal visualization.

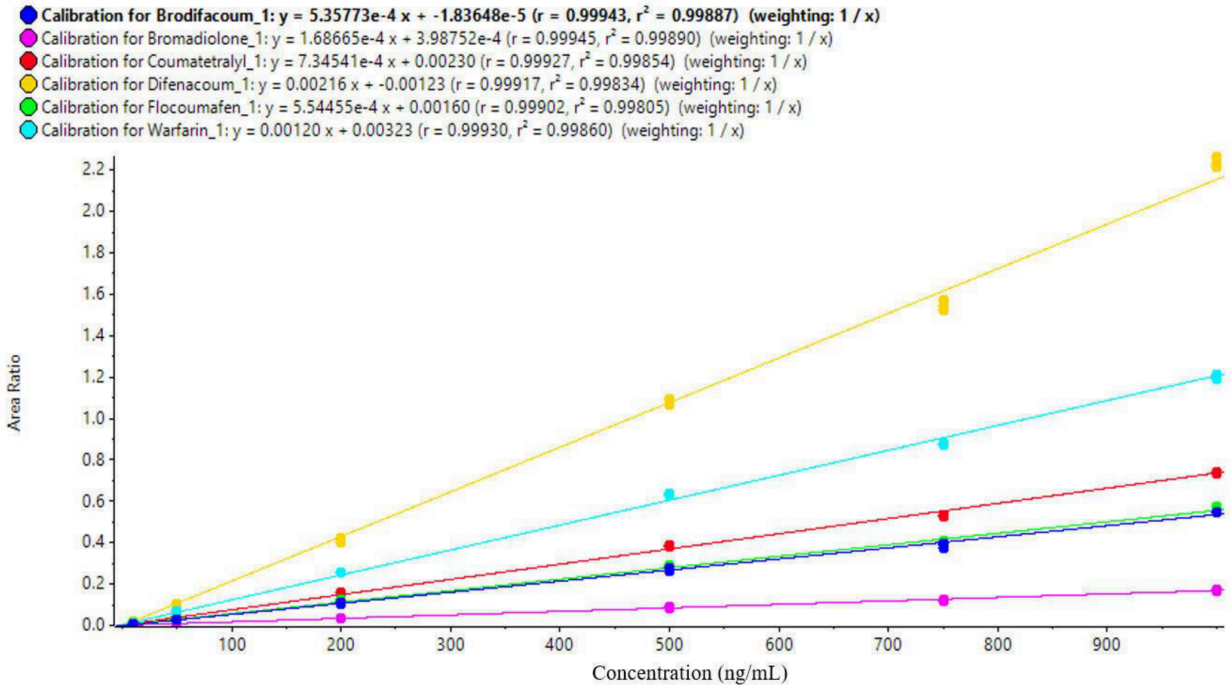


Figure 3. Calibration curves of the five ARs and warfarin.

4.3. Accuracy and Precision

Within-run accuracy and precision were evaluated using five replicates at each QC level on three separate analytical days. Across the LLOQ and low-, medium-, and high-QC levels, within-run accuracy ranged from 85.8% to 110.2%, with CVs ranging from 0.2% to 6.4%.

Inter-day accuracy and precision were calculated from all 15 measurements at each QC level. Across all QC levels, inter-day accuracy ranged from 93.9% to 106.6%, with CVs ranging from 2.1% to 10.5% (Table 3). All results met the predefined acceptance criteria.

Table 3. Accuracy and precision of ARs and warfarin measured by LC-MS/MS.

Compound	Nominal Concentration (ng/mL)	Day 1 (*n* = 5)			Day 2 (*n* = 5)			Day 3 (*n* = 5)			Inter-Day (*n* =15)		
		Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)
Brodifacoum	10	9.9	98.8	3.2	10.2	102.2	3.4	9.6	95.6	3.3	9.9	98.9	4.2
	30	28.6	95.3	3.8	29.1	97.0	3.6	28.5	94.9	2.2	28.7	95.8	3.2
	500	464.6	92.9	1.1	506.4	101.3	1.4	479.0	95.8	6.1	483.3	96.7	5.0
	800	781.4	97.7	2.4	841.6	105.2	1.0	746.8	93.4	1.6	789.9	98.7	5.4
Bromadiolone	10	8.6	85.8	2.1	10.2	101.8	1.1	9.4	94.0	3.0	9.4	93.9	7.5
	30	28.8	96.1	2.6	30.3	100.9	1.3	30.0	99.9	2.6	29.7	99.0	3.0
	500	486.6	97.3	1.4	509.6	101.9	3.0	497.1	99.4	5.1	497.8	99.6	3.8
	800	779.6	97.5	2.5	822.8	102.9	1.7	738.2	92.3	1.0	780.2	97.5	4.8
Coumatetralyl	10	9.1	90.8	1.2	10.9	108.6	1.4	11.0	110.2	0.8	10.3	103.2	8.9
	30	31.3	104.3	1.7	32.5	108.4	1.5	32.2	107.2	0.7	32.0	106.6	2.1
	500	513.8	102.8	0.7	529.6	105.9	1.0	473.5	94.7	0.8	505.7	101.1	4.9
	800	811.6	101.4	0.9	847.3	105.9	1.2	731.5	91.4	0.2	796.8	99.6	6.3
Difenacoum	10	10.1	101.2	2.6	9.7	97.4	1.6	10.0	99.8	1.3	9.9	99.5	2.4
	30	28.0	93.2	0.5	29.2	97.5	2.7	28.7	95.7	0.7	28.6	95.4	2.4
	500	470.0	94.0	1.1	508.7	101.7	3.0	488.9	97.8	6.4	489.2	97.8	5.1
	800	793.1	99.1	1.9	876.1	109.5	1.0	753.3	94.2	2.3	807.5	100.9	6.8
Flocoumafen	10	8.6	86.2	1.5	10.7	107.4	1.9	10.7	107.2	2.8	10.0	100.3	10.5
	30	29.8	99.2	2.8	32.0	106.7	1.4	30.9	103.0	1.9	30.9	103.0	3.6
	500	491.3	98.3	2.6	514.5	102.9	2.9	501.8	100.4	4.1	502.5	100.5	3.6
	800	807.0	100.9	1.3	830.8	103.8	1.8	748.6	93.6	1.9	795.5	99.4	4.8
Warfarin	10	8.8	88.0	0.8	10.8	107.6	0.8	10.7	107.2	1.2	10.1	100.9	9.4
	30	30.1	100.2	0.7	32.1	106.9	1.9	31.1	103.7	0.5	31.1	103.6	3.0
	500	500.8	100.2	1.1	521.8	104.4	0.8	469.0	93.8	0.9	497.2	99.4	4.6
	800	775.6	96.9	0.7	816.7	102.1	1.3	734.3	91.8	0.7	775.5	96.9	4.6

4.4. Matrix Effect

Matrix effects were evaluated using six independent plasma sources. At the low- and high-QC levels, accuracy ranged from 88.0% to 109.9% and from 87.1% to 107.2%, respectively, with corresponding CVs of 0.0–7.1% and 0.0–3.1% (Table 4). All results met the predefined acceptance criteria.

Table 4. Matrix effects of ARs and warfarin in plasma QC samples.

Compound	Nominal Concentration (ng/mL)	Source 1 (n = 3)			Source 2 (n = 3)			Source 3 (n = 3)			Source 4 (n = 3)			Source 5 (n = 3)			Source 6 (n = 3)		
		Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)
Brodifacoum	30	27.1	90.2	1.2	26.8	89.2	1.7	28.0	93.2	5.3	28.0	93.4	2.3	30.1	100.4	2.2	26.4	88.0	2.4
	800	729.8	91.2	2.8	789.7	98.7	0.0	696.9	87.1	1.9	703.4	87.9	1.7	698.6	87.3	0.6	717.0	89.6	1.2
Bromadiolone	30	26.8	89.3	1.9	27.8	92.6	3.4	27.6	92.1	3.0	26.4	88.1	1.4	33.0	109.9	1.7	27.6	92.0	2.3
	800	748.2	93.5	1.8	831.5	103.9	0.9	781.0	97.6	1.5	761.3	95.2	2.1	731.1	91.4	1.3	777.1	97.1	1.7
Coumatetralyl	30	29.0	96.8	0.4	30.2	100.6	0.5	31.5	104.9	1.5	30.3	101.1	0.8	29.7	98.9	0.8	31.0	103.4	1.3
	800	787.7	98.5	0.8	821.6	102.7	0.2	788.2	98.5	0.8	731.4	91.4	0.5	742.9	92.9	0.3	790.4	98.8	1.1
Difenacoum	30	26.5	88.4	1.3	27.3	91.0	1.9	27.0	90.1	0.8	26.5	88.3	1.7	32.0	106.6	1.1	27.2	90.8	1.7
	800	759.0	94.9	1.8	829.8	103.7	0.6	775.7	97.0	1.7	752.3	94.0	2.1	714.9	89.4	1.6	723.6	90.5	0.3
Flocoumafen	30	29.5	98.3	0.3	30.3	101.0	7.1	31.3	104.2	1.8	31.2	103.9	1.8	30.5	101.6	0.8	31.4	104.7	1.6
	800	849.2	106.1	1.6	857.7	107.2	2.1	839.9	105.0	3.1	786.3	98.3	0.9	802.4	100.3	0.8	843.6	105.5	0.3
Warfarin	30	29.6	98.6	0.5	30.7	102.4	0.5	31.4	104.8	0.5	30.6	101.9	1.6	30.2	100.7	0.9	31.6	105.3	0.0
	800	809.3	101.2	1.0	852.1	106.5	1.5	821.6	102.7	1.1	776.6	97.1	0.4	792.4	99.1	1.0	841.5	105.2	0.9

5. Discussion

The present LC-MS/MS method demonstrated satisfactory analytical performance for the simultaneous quantification of the five ARs and warfarin in human plasma. The validated analytical range, selectivity, accuracy, precision, and matrix-effect performance supported its intended use for clinical quantitative analysis, including bromadiolone concentration within ranges previously reported in clinical studies [16,39,41]. The objective was to establish a clinically applicable quantitative assay rather than to maximize analytical sensitivity. Accordingly, the LLOQ was defined as the lowest concentration meeting the predefined quantitative validation criteria rather than simply the lowest concentration at which an analyte signal could be detected [35,36].

Plasma was selected because a previous study reported a mean plasma-to-whole-blood bromadiolone concentration ratio of approximately 1.7 [42]. AR determination has also been reported in serum, urine, and whole blood [43–46]. However, serum preparation may be problematic in severe AR-induced coagulopathy because adequate clot formation is required for separation [47], while human data on urinary AR excretion remain limited [46,48]. Studies using serial AR concentrations for toxicokinetic assessment or treatment monitoring have predominantly used plasma or serum [16,26,39,41,49], supporting the clinical relevance of plasma for the present application.

Several LC-MS/MS methods for AR determination in human specimens use multistep extraction, often followed by solvent evaporation and reconstitution [27–29,31,39,43,50–57]. Although these procedures can improve sample clean-up and analyte enrichment, they increase sample handling. Simplified approaches using protein precipitation with low-fold dilution have also been reported [42,48] and may be described as modified dilute-and-shoot (DnS) methods [58]. Because diluted biological samples remain susceptible to matrix effects [59], matrix-matched calibration was used, and matrix effects were evaluated across six independent plasma sources according to the ICH M10 approach. The present workflow minimized sample handling while maintaining acceptable analytical performance. In our clinical setting, AR testing is generally performed for individual patient samples rather than large batches. A typical analytical sequence, including blank/zero samples, six calibration standards, QC samples, and the patient sample, requires approximately 2–3 h from sample preparation to result review. The simplified sample-preparation procedure may facilitate clinical application in suspected AR poisoning.

Beyond confirming exposure, quantitative AR measurements may also provide adjunctive information during VK1 therapy. For brodifacoum poisoning, a plasma or serum concentration of <10 ng/mL has been proposed as a practical reference for VK1 discontinuation [26,60,61], and a similar concentration has been suggested for bromadiolone poisoning [16,39,41]. Serial measurements may also facilitate estimation of individual elimination kinetics and provide additional information when considering treatment duration [26,41]. These reported bromadiolone concentrations were not resolved by stereoisomer.

In the present case, two plasma bromadiolone concentrations measured on hospital days 4 and 17 were used to estimate the elimination rate constant using a first-order elimination model [39,62]. The estimated elimination rate constant was 0.0665 day^{-1}, corresponding to an estimated apparent elimination half-life of approximately 10.4 days. The plasma bromadiolone concentration was predicted to decline below the working reference concentration of 10 ng/mL at approximately hospital day 42. This estimated concentration trajectory provided additional information for clinical assessment during VK1 therapy. VK1 was continued through day 42 and discontinued thereafter based on the overall clinical course and serial coagulation findings, with PT/INR remaining within the normal range on days 45 and 49.

Reported elimination half-lives of bromadiolone vary substantially among animal species and human cases [27,39,42,43,62]. Although bromadiolone and brodifacoum are structurally related second-generation ARs, their toxicokinetic profiles should not be assumed to be equivalent. In mice, reported plasma elimination half-lives were 33.3 days for bromadiolone and 91.7 days for brodifacoum [62,63]. In humans, reported bromadiolone half-lives range from approximately 2 to 24 days, depending on the study and the phase of elimination evaluated [27,39,42,43]. Such variability may reflect exposure dose, saturation kinetics, interindividual differences, and the relative proportions of cis- and trans-isomers, which were not determined in the present study [17,61,64].

Although zero-order elimination kinetics have been proposed for estimating treatment duration in brodifacoum poisoning [26], their applicability to bromadiolone remains uncertain. Moreover, kinetic models rely on mathematical extrapolation, and bromadiolone concentrations may not decline monotonically [28]; treatment decisions should therefore not rely solely on model-based predictions. The 10 ng/mL concentration should likewise be regarded as a working reference rather than an absolute therapeutic or safety threshold. In a recent case series, some patients required VK1 despite serum bromadiolone concentrations below 4 ng/mL, although analytical validation of the assay was not reported [65]. Furthermore, serum bromadiolone concentrations did not consistently correlate with INR; for example, a concentration of 7.5 ng/mL was associated with a lower INR than concentrations of 6.17 and 3.9 ng/mL [65]. These findings support interpreting AR concentrations together with clinical findings and serial coagulation profiles rather than in isolation.

Comparable target concentrations for guiding VK1 discontinuation have not been established for other ARs, including coumatetralyl [66], difenacoum [67], and flocoumafen [68]. For poisoning with these compounds, serial coagulation profiles remain the primary basis for treatment decisions, with AR concentrations providing supportive information when available.

The route and dosing regimen of VK1 also warrant consideration. In the present case, an injectable VK1 formulation was administered orally after clinical stabilization, consistent with previous reports [69,70], because VK1 tablets were temporarily unavailable in Taiwan [71]. Although VK1 has a plasma terminal half-life of only 1–2 h [72,73], dosing every 6 h has been proposed for AR poisoning, with reported initial doses ranging from 15 to 300 mg [50,61]. However, successful once- or twice-daily regimens have also been reported [50], and twice-daily dosing in the present case maintained normal coagulation

without recurrent bleeding. This may reflect a pharmacodynamic effect of VK1 that persists beyond its plasma half-life [72–74].

6. Limitations

This study has several limitations. First, clinical application was demonstrated in a single patient, and the estimated elimination kinetics were derived from only two plasma bromadiolone measurements. These estimates are therefore case-specific rather than validated pharmacokinetic parameters. Although the estimated concentration trajectory was temporally consistent with successful VK1 discontinuation, this observation does not validate a concentration-guided discontinuation strategy. Larger clinical studies with serial concentration measurements are needed to evaluate this approach.

Second, the 10 ng/mL concentration should be regarded as a working reference rather than a validated therapeutic or safety threshold, and comparable reference concentrations have not been established for other ARs. Neither the VK epoxide/VK quinol ratio nor active clotting-factor activity was measured, as these proposed pharmacodynamic markers are not routinely available in most clinical laboratories [26]. Therefore, AR concentrations should be interpreted alongside clinical findings and serial coagulation profiles.

Finally, analytical validation was designed for fit-for-purpose clinical AR testing rather than comprehensive high-throughput laboratory implementation. Although matrix effects were evaluated using the accuracy- and precision-based approach described in ICH M10 across six independent plasma sources, absolute matrix factors and ion suppression or enhancement were not directly assessed by post-extraction spiking. Given the limited matrix cleanup associated with protein precipitation, potential column or ion-source contamination and long-term analytical drift cannot be excluded. During clinical sample analysis, RTs were compared with matrix-matched calibration standards, with no deviations exceeding $\pm$2.5% observed. However, systematic long-term robustness testing was not performed. Other assessments outside the scope of this study included long-term frozen-plasma stability, autosampler and processed-sample stability, carry-over, recovery, dilution integrity, reinjection reproducibility, and dedicated interference challenges involving structurally related compounds, metabolites, concomitant substances, haemolysed or lipaemic plasma, and high-concentration analyte-to-analyte cross-interference. In addition, although warfarin-d5 provided satisfactory performance as a common IS and has been used previously [28], analyte-specific stable isotope-labeled ISs may offer improved compensation. Further evaluation of these parameters is warranted before broader routine or high-throughput implementation.

7. Conclusions

In this study, we developed and validated a simplified plasma-based LC-MS/MS method for the simultaneous determination of five ARs currently registered for rodent control in Taiwan and warfarin. The method demonstrated satisfactory analytical performance, with a validated quantitative range of 10–1000 ng/mL, while all analytes fulfilled the predefined qualitative identification criteria at 5 ng/mL. Application to a patient with unexplained coagulopathy confirmed bromadiolone exposure and enabled case-specific estimation of its elimination kinetics. Serial AR concentration measurements may provide useful adjunctive information alongside clinical findings and coagulation profiles during patient management, although further clinical validation is needed to establish their role in guiding VK1 treatment duration. This simplified method provides a practical approach for the clinical toxicological assessment of suspected AR poisoning.

Author Contributions: H.-C.L.: Investigation; S.-M.C.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing—original draft; K.-L.C.: Methodology, Resources; J.-H.L.: Methodology, Validation, Resources; S.-C.C.: Investigation; Y.-C.L.: Investigation; S.-Y.H.: Investigation; S.-R.L.: Investigation; S.-T.L.: Investigation; K.-J.L.: Investigation; H.-W.H.: Investigation; H.-Y.L.: Investigation; P.-Y.L.: Supervision, Writing—review & editing; J.-J.L.: Conceptualization, Supervision, Writing—review & editing; Y.-C.M.: Conceptualization, Methodology, Formal analysis, Supervision, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Taichung Veterans General Hospital (protocol code CE26382C and date of approval 29 June 2026).

Informed Consent Statement: Written informed consent has been obtained from the patient to publish this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding author due to patient privacy considerations, institutional data management regulations, and the storage of raw LC-MS/MS files on laboratory instrument computers in instrument-specific formats.

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Conflicts of Interest: J.-H.L. and K.-L.C. are employed by the company SCIEX. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

aPTT	activated partial thromboplastin time
AR	anticoagulant rodenticide
CE	collision energy
CV	coefficient of variation
CXP	collision cell exit potential
DnS	dilute-and-shoot
DP	declustering potential
ED	emergency department
EP	entrance potential
FFP	fresh frozen plasma
INR	international normalized ratio
IS	internal standard
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LLOQ	lower limit of quantification
MRM	multiple reaction monitoring
PCC	prothrombin complex concentrate
PRBCs	packed red blood cells
PT	prothrombin time
QC	quality control
RT	retention time
ULOQ	upper limit of quantification

VK	vitamin K
VK1	vitamin K1
VKA	vitamin K antagonist
VKORC1	vitamin K epoxide reductase complex subunit 1

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