Analysis of Blood Specimens for Anticoagulant Rodenticides by LC-HR-MS/MS

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1 INTRODUCTION

A series of compounds structurally related to 4-hydroxycoumarin have been used for many years as rodenticides. All of these compounds work by decreasing circulating levels of vitamin K. This causes a drop in the levels of blood clotting factors, leading to uncontrolled internal bleeding upon receipt of toxic doses of any of these compounds. One of these compounds, warfarin, is also used as a therapeutic anticoagulant for prevention of thrombosis and embolisms.

2 SCOPE

Analyses	☒ Screening ☒ Confirmation ☐ Quantitation
Matrices	Blood
Analytes	Brodifacoum, bromadiolone, coumachlor, coumatetralyl, difenacoum, and warfarin
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

3 PRINCIPLE

Blood samples are protein precipitated with acetonitrile followed by solid-phase extraction. The resulting extract is taken to dryness and analyzed by LC-HR-MS/MS (liquid chromatography with high resolution tandem mass spectrometry) in the positive electrospray ionization mode

4 SPECIMEN CRITERIA

This procedure uses two 1 mL portions of blood.

5 EQUIPMENT

5.1 Equipment

A. Centrifuge
B. Evaporator with nitrogen
C. Pipettes with disposable tips
D. SPE manifold (vacuum or positive pressure)
E. Volumetric flasks (10, 50, and 250 mL)
F. Vortex mixer

5.2 Consumables

A. Bond Elut Certify II solid-phase extraction cartridges
B. Culture tubes with caps
C. Routine laboratory supplies, including: Pasteur pipets, pH paper, graduated cylinders, etc.
D. Screw-top test tubes with caps

5.3 Instruments

A. Thermo LTQ Orbitrap XL Hybrid Ion Trap/Fourier Transform Mass Spectrometer
B. Shimadzu HPLC

5.3.1 Column(s)

15 cm x 2.1 mm x 5 µm d_p Grace Altima C18 (or equivalent)

5.4 Software

Component	Software	Version
Operating System	Microsoft Windows	7 Pro SP 1 / XP Professional
Mass Spectrometer	Foundation	1.0.2 or higher
	Xcalibur	2.1.0 SP1 / 2.0.7
	LTQ Tune Plus	2.5.5
	Shimadzu LC Controller	5.4 / 6.5

5.5 Chemicals/Reagents

5.5.1 Purchased

Chemical or Reagent	Minimum Grade or Purity
Acetic Acid (glacial)	≥Certified ACS
Acetonitrile	≥HPLC
Ethyl Acetate	≥HPLC
Hexane	≥UV grade
Hydrochloric Acid (concentrated)	≥Certified ACS
Methanol	≥HPLC and ≥Optima
Sodium Acetate Trihydrate	≥Reagent
Toluene	≥HPLC
Water	Deionized (DI) and Optima

5.5.2 Prepared

A. 1N Hydrochloric acid

Add 4 mL of concentrated hydrochloric acid to ca. 40 mL of DI water in a graduated cylinder. Mix well and bring to 48 mL with DI water. Store at room temperature in glass; stable for at least 6 months.

B. Sodium Acetate Buffer (0.1 M, pH 7)

To a 250-mL volumetric flask, add 3.4 g sodium acetate trihydrate and 200 mL deionized water. Mix well and adjust to 6.5<pH<7.5 by slow addition of 1 N hydrochloric acid. Bring to volume with deionized water. Store refrigerated in glass. Stable for at least 2 months.

C. Sodium Acetate Buffer (0.1 M, pH 7) with 5% Methanol

Combine 95 mL of 0.1 M sodium acetate buffer with 5 mL methanol and mix well. Store refrigerated in glass; stable for at least 2 months.

D. Wash Solvent (95:5 hexane:ethyl acetate)

Combine 95 mL of hexane with 5 mL of ethyl acetate and mix well. Store in glass at room temperature; stable for at least 2 months.

E. Elution Solvent (75:25:1 hexane:ethyl acetate:acetic acid)

Combine 75 mL of hexane with 25 ml of ethyl acetate and 1 mL of acetic acid and mix well. Store in glass at room temperature; stable for at least 1 month.

F. Methanol:water (1:1)

Combine equal volumes of methanol and DI water and mix well. Store in glass at room temperature; stable for at least 6 months.

G. LC Mobile Phase #1 (0.06% acetic acid in water)

Add 0.3 mL of glacial acetic acid to 500 mL of Optima grade water and mix well. Store in glass at room temperature; stable for a maximum of 2 weeks; do not extend expiration date.

H. LC Mobile Phase #2 (0.06% acetic acid in methanol)

Add 0.3 mL of glacial acetic acid to 500 mL of Optima grade methanol and mix well. Store in glass at room temperature; stable for a maximum of 2 weeks; do not extend expiration date.

5.6 Standards/Controls

5.6.1 Purchased

A. Rodenticides Solid Standards

Brodifacoum, bromadiolone, coumachlor, coumatetralyl, and difenacoum: Obtained as powders from approved vendors. Stability and storage conditions determined by manufacturer.

B. Warfarin Stock Solution (1 mg/mL in methanol)

Obtained from Cerilliant or another approved vendor. Stability and storage conditions determined by manufacturer.

C. Negative Control Blood

Blood is purchased from Utak, Cliniqa, or another approved vendor. Storage and stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.

5.6.2 Prepared

A. Brodifacoum Stock Solution (1 mg/mL)

Weigh 10 mg of brodifacoum into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0ºC. Stable for at least 1 year.

B. Bromadiolone Stock Solution (1 mg/mL)

Weigh 10 mg of bromadiolone into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0ºC. Stable for at least 1 year.

C. Coumachlor Stock Solution (1 mg/mL)

Weigh 10 mg of coumachlor into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0ºC. Stable for at least 1 year.

D. Coumatetralyl Stock Solution (1 mg/mL)

Weigh 10 mg of coumatetralyl into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0ºC. Stable for at least 1 year.

E. Difenacoum Stock Solution (1 mg/mL)

Weigh 10 mg of difenacoum into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0ºC. Stable for at least 1 year.

F. Rodenticides Working Solution (1 µg/mL of each component)

Combine 50 µL each of the warfarin, brodifacoum, bromadiolone, coumachlor, coumatetralyl, and difenacoum stock solutions in a 50 mL volumetric flask, fill to the mark with methanol, and mix well. Store refrigerated in glass. Stable for at least 3 months.

G. Rodenticide LC-HR-MS/MS Performance Mix (0.05 µg/mL)

Mix 50 µL of the rodenticides working solution with 950 µL of the rodenticides LC mobile phase #2. Prepare fresh daily.

H. Positive Control Blood

Prepared at 25 ng/mL by spiking 1 mL of Negative Control Blood with 25 µL of the Rodenticides Working Solution and at 100 ng/mL by spiking 1 mL of Negative Control Blood with 100 µL of the Rodenticides Working Solution. Positive Control Blood samples will be extracted and analyzed with every blood assay. Additionally, when sample volume permits, a 1 mL portion of the case specimen to be analyzed will be fortified with 25 µL of the rodenticides working solution to demonstrate recovery from that specific specimen.

6 PROCEDURE

	Step	Note	Reference/Lot
	A. Samples		
	1. To labeled 16 x 100 mm tubes add:		
☐	i. 1 mL of biological fluid		
☐	ii. 0.2 mL of deionized water		
	B. Controls		
☐	1. Prepare Negative Control(s)		
☐	2. Prepare Positive Control(s) ☐ i. 25 ng/mL: Add 25 µL Rodenticides Working Solution to Negative Control(s) ☐ ii. 100 ng/mL: Add 100 µL Rodenticides Working Solution to Negative Control(s) ☐ iii. Optional 25 ng/mL case specimen control: Add 25 µL Rodenticides to case specimen(s)		
	C. Protein Precipitation		
☐	1. Add 4 mL of acetonitrile drop wise while vortexing sample.		
☐	2. Vortex thoroughly for a minimum of 3 minutes.		
☐	3. Centrifuge samples for 15 minutes at 3000 rpm		
☐	4. Transfer the supernatant to a clean 150 mm test tube		
☐	5. Concentrate to about 2 mL under a slow stream of nitrogen at approximately 50°C.		
	D. Buffer		
☐	1. Add 6 mL of 100mM sodium acetate buffer (pH 7)		
☐	2. Vortex		
	E. Extraction (SPE)		
	1. Condition cartridges (1 mL/min, sorbent bed is not dried)		
☐	i. Add 2 mL methanol		
☐	ii. Add 2 mL 100mM sodium acetate buffer with 5% methanol.		
☐	2. Load samples (1 mL/min)		

	3. Wash cartridges (1 mL/min)		
☐	i. Add 1 mL 100mM sodium acetate buffer		
☐	ii. Dry column under full vacuum for 1 minute		
☐	iii. Add 2 mL of Wash Solvent		
☐	iv. Add 5 mL of methanol:water (1:1)		
☐	4. Dry cartridge under full vacuum for 1 minute		
	5. Elute (1 mL/min)		
☐	i. Add 2 mL Elution Solvent		
☐	ii. Collect eluent in 12 x 75 mm tubes		
☐	6. Evaporate to dryness under nitrogen at 50°C.		
	F. Reconstitute		
☐	1. Add 100 µL of Mobile Phase #2 to 12 x 75 mm tubes		
☐	2. Vortex and transfer to ALS vial.		
☐	**G. Instrumental Analysis** 1. LC/MS: analyze 10 µL i. Analyze LC/MS Performance Standard prior to batch analysis		
	ii. Mobile Phase 1 (aqueous)		
	iii. Mobile Phase 2 (organic)		
	iv. LC Column		

7 ANALYTICAL PARAMETERS

7.1 Shimadzu HPLC

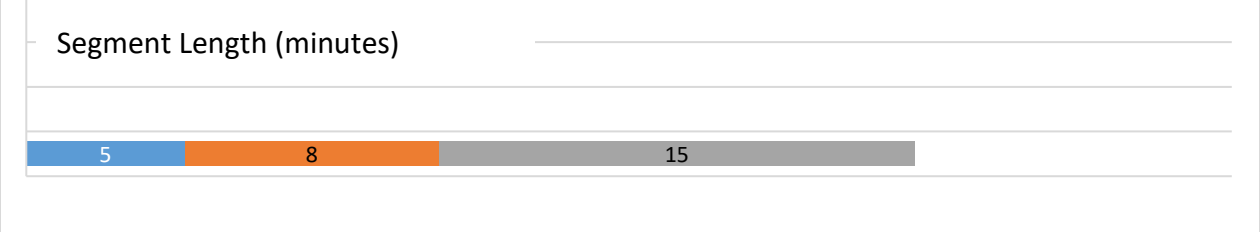
7.1.1 Gradient/Conditions

Time (min)	Mobile Phase %		Flow Rate		
	1-Aqueous	2-Organic	(mL/min)	Column Heater (°C)	40
0	22	78	0.3	Autosampler (°C)	15
3	22	78	0.3	Run Time (min)	28
8	5	95	0.3		
20	5	95	0.3		
21	22	78	0.3		
28	22	78	0.3		

7.2 Thermo LTQ Orbitrap XL

7.2.1 Mass Spectral Parameters

Source Mode: ESI (+)



Seg	Event	Mode	Range (m/z)	Parameters				Analyzer	Resolution
1	1	Full Scan	240-400					FTMS	30000
	2	MS/ MS	Software Control	Mass (m/z)	CE (rel)	IsoW (m/z)	Time (min)	FTMS	7500
				309.112	30	3.0	1.3-4.3		
				293.117	40	3.0	2.0-5.0		
				343.073	30	3.0	2.0-5.0		
2	1	Full Scan	390-580					FTMS	30000

	2	MS/ MS	Software Control	Mass (m/z)	CE (rel)	IsoW (m/z)	Time (min)	FTMS	7500
				509.075	30	3.0	5.0-5.9		
				445.180	30	3.0	8.0-11.5		
				523.090	30	3.0	9.0-12.5		
3	1	Full Scan	240-580					FTMS	30000

NOTE: The precursor ion for bromadiolone (509.08) is the protonated dehydrated pseudomolecular ion. In validation the unfragmented pseudomolecular ion was not reliably detected.

8 DATA ANALYSIS

8.1 Decision Criteria

8.1.1 Performance Mix Suitability

Proper calibration and sensitivity of the LC-HR-MS/MS are demonstrated each day samples are analyzed. The Rodenticide LC-HR-MS/MS Performance Mix (0.05 µg/mL) is used to verify system suitability. Retention times for each analyte should compare favorably with the last performance mix analysis and each analyte should yield correct exact masses (±0.005 Da for full MS, ±0.01 Da for MS/MS, base peak only) for the ions as shown in Table 1. Commercially available standards of bromadiolone are a mixture of orientational isomers, and it is normal for the chromatographic peak for this compound to be asymmetric and exhibit a "shoulder".

Table 1: Exact MS and MSMS fragment masses for anticoagulant rodenticides

Compound	Full MS Mass(es)	MSMS masses (base peak in bold)
Coumatetralyl	293.117	131.085, 163.039, **175.039**
Warfarin	309.112	147.081, **163.039**, 251.071
Coumachlor	343.073, 345.070	**163.039**, 181.042, 285.032
Difenacoum	445.180	189.054, **257.133**, 291.102
Bromadiolone	509.075, 511.073	**251.071**, 277.086, 321.027
Brodifacoum	523.090, 525.088	189.054, 291.102, **335.043**

8.1.2 Analyte Suitability

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control. In most cases, all of the below should be met in order to identify a target analyte within a biological specimen:

8.1.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

8.1.2.1.1 Retention Time

The retention time of the peak should be within ±5% of the retention time obtained from injection of a reference standard or extracted Positive Control of the analyte of interest.

8.1.2.1.2 Signal-to-Noise

To justify the existence of a peak, its signal-to-noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10-fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank

sample injected just prior to that sample. Signal to noise will normally be evaluated based upon extracted ion profiles for the ion(s) of interest, with a ±0.01 m/z extraction window.

8.1.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should favorably compare to that of a reference standard, extracted calibrator, or an extracted Positive Control. See TOX-104 for further guidance.

9 REPORTING

In addition to Level 1 documents, reference TOX-100 and TOX-101 for any reporting considerations.

10 CORRECTIVE MEASURES

Refer to TOX-101 for guidance on action steps in the event of a quality control failure.

11 PERFORMANCE CHARACTERISTICS

11.1 LOD

Analyte	LOD (ng/mL)
Warfarin	10
Coumachlor	5
Bromadiolone	5
Coumatetralyl	2
Difenacoum	2
Brodifacoum	2

11.2 Carryover

No significant carryover was detected during method validation.

12 LIMITATIONS

12.1 Interferences

High levels of alprazolam may lead to false negative results for warfarin. Grossly decomposed or putrefied samples may affect limits of detection.

13 SAFETY

Take standard precautions for the handling of chemicals and biological materials. See the *FBI Laboratory Safety Manual* for further guidance.

14 REVISION HISTORY

Revision	Issued	Changes
03	07/01/2022	Complete document reformat.