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

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

This procedure allows for the screening and confirmation of six anticoagulant rodenticides (brodifacoum, bromadiolone, coumachlor, coumatetralyl, difenacoum, and warfarin) in whole blood. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

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 Specimens

This procedure uses two 1 mL portions of blood. It can also be adapted for analysis of rat bait pellets and other commercial products. When analyzing commercial samples, a 20-fold dilution or 20x homogenate in deionized water will typically be used. Smaller volumes may be used, with appropriate dilution, if available specimen is limited.

5 Equipment/Materials/Reagents

a. Screw-top test tubes with caps

b. Culture tubes with caps

c. Volumetric flasks (10, 50, and 250 mL)

d. Pipetters with disposable tips

e. Vortex mixer

f. Centrifuge

g. Evaporator with nitrogen

h. SPE manifold (vacuum or positive pressure)

i. Bond Elut Certify II solid-phase extraction cartridges

j. Liquid chromatography-high resolution (30000 resolving power) mass spectrometry system equipped with a 15 cm x 2.1 mm x 5 µm d_p Grace Altima C18 (or equivalent) column

k. Routine laboratory supplies, including: Pasteur pipets, pH paper, graduated cylinders, etc.

l. Purchased chemicals and reagents

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

m. 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.

n. 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.

o. 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.

p. Rodenticides 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.

q. Rodenticides 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.

r. 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.

s. Rodenticides 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.

t. Rodenticides 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.

6 Standards and Controls

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. 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.

d. 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.

e. 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.

f. 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.

g. 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.

h. 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.

i. 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.

j. 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.

k. 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.

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the authorized individual performing the procedure.

a. Into properly-labeled test tubes, add 1 mL of control or case samples and enough DI water to bring the volume to 1.2 mL. Prepare in duplicate if specimen volume allows in order to prepare a Positive Control Blood sample as directed in Section 6 above.

b. Add 4 mL of acetonitrile drop wise while vortexing sample. Vortex thoroughly for a minimum of 3 minutes.

c. Centrifuge samples for 15 minutes at 3000 rpm.

d. Transfer the supernatant to a clean test tube and concentrate to about 2 mL under a slow stream of nitrogen at approximately 50°C.

e. Add 6 mL of 0.1 M sodium acetate buffer (pH 7).

f. Prepare the Bond Elut Certify II columns by sequentially passing 2 mL methanol and 2 mL 0.1 M sodium acetate buffer (pH 7) with 5% methanol. Do not allow sorbent bed to dry.

g. Pour the sample into the appropriately labeled column reservoir. Draw the sample through the column at a flow rate of approximately 1 - 2 mL/minute.

h. Rinse column with 1 mL of 0.1 M sodium acetate buffer (pH 7).

i. Dry column under full vacuum for 1 minute.

j. Sequentially rinse column with 2 mL of Rodenticide Wash Solvent followed by 5 mL of methanol:water (1:1).

k. Dry column under full vacuum for 1 minute.

l. Elute into 12x75 mm culture tubes with 2 mL of the Rodenticide Elution Solvent at about 1 mL/minute. Evaporate under nitrogen at approximately 50°C.

m. Reconstitute dried extract in 100 µL of Rodenticide Mobile Phase #2.

n. Analyze 10 µL of the LC-HR-MS/MS Performance Mix to determine that the LC-HR-MS/MS is in proper working condition.

o. Analyze 10 µL of each extract by LC-HR-MS/MS.

9 Instrumental Conditions

Following are the instrumental parameters used in this procedure. Appendix 2 contains an abbreviated version of these parameters that may be used by the authorized individual performing the procedure.

9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
1: Water with 0.06% Acetic Acid	flow rate		0.3 mL/min	type	C-18
	time (min)	%1	%2	length	15 cm
2: Methanol with 0.06% Acetic Acid	0.0	22	78	internal diameter	2.1 mm
	3.0	22	78	particle size	5 µm
	8.0	5	95	temperature	40°C
	20	5	95		
	21	22	78		
	28	22	78		

9.2 Mass Spectral Parameters

3 Segments		
Segment 1 – 0-5 minutes – 2 scan events		
Event #1	full scan m/z 240-400 profile at 30000 resolution	
Event #2	MS/MS at 7500 resolution	collision energy: 30 (rel) for 343.073 and 309.112; 40 (rel) for 293.117
	precursor from cyclic scan table: 2-5 min for m/z 293.117 and m/z 343.073; 1.3-4.3 min for m/z 309.112	
	isolation width: 3.0 Da	scan range: software control
Segment 2 – 5-13 minutes – 2 scan events		
Event #1	full scan m/z 390-580 profile at 30000 resolution	
Event #2	MS/MS at 7500 resolution	collision energy: 30 (rel)
	precursor from cyclic scan table: 8-11.5 min for m/z 445.180; 5.5-9 min for m/z 509.075; 9-12.5 min for m/z 523.090	
	isolation width: 3.0 Da	scan range: software control
Segment 3 – 13-28 minutes – 1 scan event		
Event #1	Full scan m/z 240-580 profile at 30000 resolution	

NOTE: The precursor ion for bromadiolone (509.08) is the protonated dehydrated pseudomolecular ion. In validation it proved impossible to produce reasonable source yield of the unfragmented pseudomolecular ion.

10 Decision Criteria

10.1 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 MSMS, 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**

10.2 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:

10.2.1 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.

10.2.1.1 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.

10.2.1.2 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.

10.2.2 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 the Guidelines for Comparison of Mass Spectra standard operating procedure (TOX104) for further guidance.

11 Calculations

Not applicable.

12 Measurement Uncertainty

Not applicable.

13 Limitations

a. Limits of Detection in Blood:

Warfarin – 10 ng/ml
Coumachlor and Bromadiolone – 5 ng/ml
Coumatetralyl, Difenacoum, and Brodifacoum – 2 ng/ml

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

14 Safety

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

15 References

Felice, L and Murphy, M.J., "The Determination of the Anticoagulant Rodenticide Brodifacoum in Blood Serum by Liquid Chromatography with Fluorescence Detection", *Journal of Analytical Toxicology* 13: 229-231 (1989).

Grobosch, T, et al., "Acute Bromadialone Intoxication", *Journal of Analytical Toxicology* 30: 281-286 (2006).

Extraction of THC and THC Metabolite from Blood Using Certify II", Publication from Varian Sample Preparation Products, Harbor City, California.

Rev.#	Issue Date	History	
1	01/19/2012	Converted to high resolution MS, and added MSMS analysis, changing: title and sections 2, 3, 5, 10, and 11. Rewrote section 1 to include information about history and pharmacological action of target analytes. Added coumachlor, coumatetraly, difenacoum, and warfarin to target analyte list, changing title and sections 2, 3, 6, 10, 11, and 14. Added additional validation data to comply with current Laboratory Division guidelines, changing: sections 11 and 14. Reduced sample volume used to 1 mL, changing: sections 4, 6, and 9. Updated procedural terminology to match current Chemistry Unit usage, changing: sections 2, 3, 5, 9, and 11. Revised "bench sheet" (appendix 1) for procedural revisions, and added an appendix 2 with instrument parameters checklist.	
2	06/15/2021	Entire document	Removed footers; updated approval lines; changed mass spec reference to "LC-HR-MS/MS" throughout
		1	Removed educational language.
		2, 3	Revised to current unit standards for organization and wording. Defined LC-HRMSMS in 3.
		4	Added possibility of specimen dilution.
		5	Removed reference to TOX103 and added prep instructions for all reagents. Combined purchased chemicals into a table and renumbered section.
		6	Combined items (a) through (e) into a single item, and renumbered subsequent items.
		7	Removed section (calibration) and renumbered subsequent sections.
		8	Changed "examiner or chemist" to "authorized individual" in preamble. Reworded items (d) and (l) for clarity. In –c, added rpm for centrifuge step.
		9	Added reference to Appendix 2
		9, 10	Changed "amu" to "Da" throughout
		10.2.1.2	Removed specific signal-to-noise algorithm requirement.
		10.2.2	Removed "reasonably degree of scientific certainty" language. Updated mass spec comparison language.
		12	Retitled as "Measurement Uncertainty"
		13	Removed all figures of merit except limits of detection.
		15	Removed references to internal FBI Laboratory documents/
		Bench Notes	Removed reagent preparation

Approval

Redact - Signatures on File

Chemistry Unit Chief: _____ Date: 06/14/2021

QA Approval

Toxicology Technical Leader: _____ Date: 06/14/2021

Appendix 1: Abbreviated version of the Rodenticide Procedure for bench use.

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Appendix 2: Abbreviated version of the Rodenticide instrumental conditions for bench use.

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