NOV 15, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.eq2ly752mlx9/v1

Protocol Citation: kyle.francis, megan.romano, Rupam Sarma 2023. Quantitation of Anticoagulant Rodenticides in Serum. **protocols.io** https://dx.doi.org/10.17504/protocols.io.eq2ly752mlx9/v1

MANUSCRIPT CITATION: Comprehensive Evaluation of HPLC-MS/MS Method for Quantitation of Seven Anticoagulant Rodenticides and Dicoumarol in Animal Serum. J Anal Toxicol.2023 Mar 3;bkad017. doi: 10.1093/jat/bkad017. Kyle A Francis 1, Andriy Tkachenko 2, Joseph T Johnson 1, Lori L Smith 1, Robyn T Noonan 3, Michael S Filigenzi 3, L Cynthia Gaskill 1, Megan C Romano. https://pubmed.ncbi.nlm.nih.gov/36869712/

Quantitation of Anticoagulant Rodenticides in Serum

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DISCLAIMER

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

ABSTRACT

This SOP describes the extraction and sample clean-up method for the quantitative determination of eight anticoagulant rodenticides in animal serum. Analytes were extracted with 10% (v/v) acetone in methanol and analyzed by reverse phase high-performance liquid chromatography–tandem mass spectrometry using electrospray ionization (negative mode) combined with multiple reaction monitoring. Limits of quantitation at 2.5 ng/mL for all analytes.

Method validation/evaluation/verification:

In-house method validation data and evaluation by an independent laboratory (Vet-LIRN) in collaborative multi-laboratory studies are published: https://pubmed.ncbi.nlm.nih.gov/36869712/

ATTACHMENTS

Target-SOP-Serum-ACRs-uploaded-to-Protocols.io_2023-11-10.pdf

MATERIALS

I.Materials / Equipment

A.Supplies

Protocol status: Working
We use this protocol and it's working

Created: Mar 22, 2023

Last Modified: Nov 15, 2023

PROTOCOL integer ID: 79271

Keywords: Anticoagulant, dicoumarol, rodenticides, chlorophacinone, coumachlor, bromadiolone, brodifacoum, difethialone, diphacinone, warfarin, LC-MS/MS, animal, serum, pesticides, diagnostics

Funders Acknowledgement:
U.S. FDA's Vet-LIRN
Grant ID: 1U18FD005015

Micropipettors and corresponding pipette tips (0.5 uL to 1000 uL)
Disposable 5-mL syringes with Luer-lok tips (BD Syringe, #309646)
Disposable syringe filters, 0.45 µm, PVDF membrane (MicroSolv Filters, #58045-V04-C)
Disposable micro-centrifuge tubes, 1.5 mL, polypropylene (VWR, #89000-028)
Silanized autosampler vials (2-mL; silanized; amber; Fisher Scientific #03-377F)
Vial closures and septa (Fisher Scientific #03-379-113)
Glass Pasteur pipettes

B.Chemicals (Higher grade chemicals may be substituted)

Distilled, deionized (DDI) water
Methanol, Acetonitrile, Acetone, Chloroform (HPLC or LC-MS grade; Fisher Scientific)
Ammonium acetate, HPLC grade (Fisher Scientific #A639-500)
Ammonium hydroxide, ACS grade (ACS grade, #BDH3014-500MLP, VWR International)
Standard Reference Materials:

a .Coumachlor, 98% (Sigma-Aldrich #189219-1G)
b. Dicoumarol, 98.0% (Sigma-Aldrich #M1390-5G)
c. Brodifacoum, 98.1% (US EPA National Pesticide Standard Repository)
d. Bromadiolone, 98.9% (US EPA National Pesticide Standard Repository)
e. Chlorophacinone, 99.8% (US EPA National Pesticide Standard Repository)
f. Difethialone, 99.7% (US EPA National Pesticide Standard Repository)
g. Diphacinone, 99.3% (US EPA National Pesticide Standard Repository)
h. Warfarin, 99.5% (US EPA National Pesticide Standard Repository)

C.Equipment / Instrumentation (Equivalent may be substituted)

Analytical Balance (Model PB303-S, Mettler Toledo)
Vortex (Vortex Genie 2, Fisher Scientific)
Centrifuge w/ cooling capability suitable for 1.5 mL microcentrifuge tubes (Micromax RF, Thermo Electron)
Ultrasonic Cleaner (FS30, Fisher Scientific) & micro-centrifuge floatation rack (VWR)
Thermo Scientific / Dionex UltiMate 3000 Rapid Separation Liquid Chromatography (UPLC) system with autosampler, binary pump and thermostatted column compartment
Thermo Scientific Quantum Access Max triple quadrupole mass analyzer with heated electrospray ionization
Analytical Chromatography Column (Zorbax XDB-C18 Eclipse, 2.1 x150 mm, 5µm; Agilent, #993700-902)
Guard Column Cartridge System (Syncronis C18 cartridges, 2.1 x 10 mm, 5 µm, Thermo Scientific, #97105-012101)

Prepared Reagents

1 Primary Stock Solutions – 1000 ug/mL: For each anticoagulant rodenticide, dissolve 5.0 ± 0.1 mg standard reference material in 5 mL of the appropriate solvent (as per Table 1), using 5-mL volumetric flasks. These eight solutions should be stored at -20°C for up to one year.

Table 1: Solvents for Anticoagulant rodenticides

Anticoagulant Rodenticide	Solvent
Bromadiolone, Coumachlor, Warfarin	Methanol
Brodifacoum, Chlorophacinone, Difethialone, Diphacinone	Acetone
Dicoumarol	Chloroform

2 10% (v/v) Acetone in Methanol: Transfer 25 mL acetone to a 250-mL graduated cylinder and bring to a total volume of 250 mL with methanol.

3 Secondary Stock Solution – 10 µg/mL: Transfer 50 µL of each primary stock solution to a single 5-mL volumetric flask. Complete the volume with methanol to prepare a single solution that is 10 µg/mL of each AR. This solution should be stored at -20°C for up to one month.

> **Note**
>
> *The use of a positive displacement pipette may be necessary for accurate transfer of acetone- and chloroform-containing solutions.*

4 Working Solution A – 1.25 µg/mL: Transfer 625 µL of the secondary stock solution to a single 5-mL volumetric flask. Complete the volume with methanol to prepare a single solution that is 1.25 µg/mL of each AR. This solution should be stored at -20°C for up to one month.

5 Working Solution B – 0.125 µg/mL: Transfer 62.5 µL of the secondary stock solution to a single 5-mL volumetric flask. Complete the volume with methanol to prepare a single solution that is 0.125 µg/mL of each AR. This solution should be stored at -20°C for up to one month.

6 Mobile Phase Solutions: De-gas mobile phase solutions by helium sparging

6.1 0.01M Ammonium Acetate, pH 9 – Dissolve 0.77 ± 0.01g ammonium acetate in ~750ml distilled, deionized water (DDI water) in a 1-L volumetric flask. Adjust pH to 9 by adding ammonium hydroxide dropwise. Complete volume with DDI water.

6.2 Methanol, LC-MS grade

Sample Treatment

7 **Matrix-Matched Calibrants and Quality Control Samples**

> **Note**
>
> *Prepare 7 calibrants and 3 QC samples in labelled 1.5-mL disposable micro-centrifuge tubes using control serum.*

7.1 Pipette the appropriate volumes of AR Standard Solutions followed by controlled serum into the corresponding tube, as described in Table 2, yielding a final volume of 250 µL.

7.2 Cap tubes and vortex mix for 00:00:10 to mix thoroughly. 10s

7.3 Proceed to step 8.2

Table 2: Preparation of Matrix-Matched Calibrants and Quality Control Samples

	A	B	C	D	E	F

	A	B	C	D	E	F
	Calibrant / QC Sample	Concentration (ppb; ng/g)	Volume of Secondary Stock Solution (µL)	Volume of Working Solution A (µL)	Volume of Working Solution B (µL)	Volume of Control Serum (µL)
	Cal 1	2.5	---	---	5.0	245
	Cal 2	5.0	---	---	10	240
	Cal 3	10	---	---	20	230
	Cal 4	25	---	5.0	---	245
	Cal 5	50	---	10	---	240
	Cal 6	250	6.25	---	---	243.75
	Cal 7	500	12.5	---	---	237.50
	QC Blank	0	---	---	---	250
	QC 5.0PPB	5.0	---	---	10	240
	QC 400PPB	400	10	---	---	240

8 Sample Extraction

8.1 Transfer 250 µL unknown sample serum to a labelled 1.5-mL disposable micro-centrifuge tube.

8.2 To all calibrants, QC samples, and unknown samples, add 250 µL 10% (v/v) acetone in metha 10s pre-chilled at 4°C using an accurate pipettor. Vortex mix thoroughly for 00:00:10 .

8.3 Centrifuge the samples at 16,000 g and 4°C for 00:10:00 10m

8.4 Decant the supernatant for each sample into a new, labelled 1.5-mL disposable micro-centrifuge tube.

8.5 Into the decanted tubes with precipitate, add 250 µL 10% (v/v) acetone in methanol pre-chille 10s
4°C using an accurate pipettor to all calibrants, QC samples, and unknown samples. Vortex mix thoroughly for 00:00:10 .

8.6 Place the precipitate-containing samples into the micro-centrifuge tube flotation rack and place in the sonication bath.

8.7 Sonicate the samples for 00:05:00 5m

8.8 Centrifuge the samples at 16,000 g and 4°C for 00:10:00 10m

8.9 Transfer, via glass pipette, the supernatant for each sample and combine with the previous 10s
corresponding supernatant in step 8.4. Vortex mix thoroughly for 00:00:10

8.10 Centrifuge the combined supernatant tubes at 16,000 g and 4°C for 00:10:00 . 10m

8.11 Filter each supernatant by syringe filtering:
- Remove the syringe plunger and attach a PVDF luer-lok syringe filter.
- Transfer the supernatant to the syringe, re-insert the plunger and filter into:

Note

If the filtered sample's volume is below or approximately equal to the minimum volume needed for proper autosampler syringe aliquoting (i.e. syringe height) when following i., then follow ii. to ensure proper syringe aliquoting and injection onto column.

i. a labelled glass silanized autosampler vial or
ii. a 1.5-mL micro-centrifuge tube, then pipet 150 µL of the filtered sample into a labelled glass silanized autosampler vial containing a vial insert.

HPLC – MS/MS Analysis

9 HPLC Settings

9.1 Gradient Elution Profile: Profile parameters may be adjusted slightly at the discretion of the chemist to achieve baseline resolution of brodifacoum and difethialone at 500ppb (Cal 7). The recommended gradient profile when using a Zorbax XDB-C18 Eclipse, 2.1 x150 mm, 5µm analytical column along with Syncronis C18 guard column is in Table 3.

Table 3. Recommended Gradient Profile

Time (min)	0.01M Ammonium Acetate, pH 9 (%)	Methanol (%)
0	**60**	**40**
1	**60**	**40**
9	**43**	**57**
15	**23**	**77**
18	**19**	**81**
19	**10**	**90**
24	**10**	**90**
25	**60**	**40**
34	**60**	**40**

9.2 Flow Rate: 0.400 mL/min

9.3 Column Temperature: 25°C

9.4 Injection Volume: 10 µL

9.5 Total Run Time: 00:34:00 34m

9.6 Autosampler temperature: +24C (room temperature). Note, pesticides are usually very stable

10 **MS/MS Detection** These parameters are suggestions and may need to be optimized for different MS instruments. Multiple reaction monitoring transition parameters are listed in Table 4.

Table 4. MRM Transitions and Approximate Expected Retention Times

	A	B	C	D	E	F
	Anticoagulant Rodenticide	**Retention Time (min)**	Precursor Ion ((M-H+)-; u)	Fragment Ion*	**Collision Energy (eV)**	**Tube Lens (V)**
	Warfarin	3.70	**307**	161	22	70
				250	25	70
	Coumachlor	7.50	**341**	**284**	26	71
				161	23	71
	Diphacinone	10.06	**339**	**167**	28	77
				165	48	77
				161	21	47

A	B	C	D	E	F
Dicoumarol	10.30	**335**	117	47	47
Chlorophacinone	13.26	**373**	**201**	24	76
			145	25	76
Bromadiolone**	15.33	**525**	**250**	38	97
			273	40	97
Brodifacoum	17.92	**521**	**135**	40	101
			143	57	101
Difethialone	18.22	**537**	**151**	41	100
			371	35	100

*Transitions in **bold** are used for quantitation
**Two isomers are present for Bromadiolone; only the earliest eluting (and most abundant) isomer is used for detection and quantitation.

10.1 ESI Source Conditions: *Optimized on the basis of direct infusion of solvent-diluted reference standards*

- Negative ion mode
- Spray Voltage: 4000 V
- Vaporizer Temperature: 380°C
- Sheath Gas Pressure: 50 psi
- Auxiliary Gas Pressure: 45 psi
- Ion Sweep Gas Pressure: 0 psi
- Capillary Temperature: 300°C
- Skimmer Offset:(Not used)

10.2 Other Parameters:

- Collision Gas Pressure: 1.7 mTorr
- Collision Energy: Ion-Dependent; see Table 4 (MRM Transitions)
- Tube Lens: Ion-Dependent; see Table 4 (MRM Transitions)
- Q1 / Q3 Peak Width (FWHM): 0.70 u
- Cycle Time: 0.300 s

Post-Acquisition Data Analysis

11

Note

Peak area integration is performed using pre-selected software parameters (i.e. smoothing, S/N, etc.) as a starting point. The baseline setting and the peak integration start and stop points are then visually inspected in each chromatogram and manually adjusted as needed.

12 Qualitative Identification- The respective analyte is considered to be qualitatively identified in the unknown sample if the following criteria are met:

12.1 The quantifying ion and the corresponding confirming ion co-elute within 0.1 min of one another, each with a signal-to-noise ratio $\geq$ 3.

12.2 The retention times of the quantifying and confirming ions are within 2% of the mean retention time for the same analyte in all calibrants and QC samples acquired within the same batch analysis

12.3 The quantifying ion:confirming ion ratio is within +/- 20% of the expected ratio (typically the average of the batch standards' ion ratios).

13 Quantitative Analysis – The following parameters should be used to generate calibration curves to determine quantitative results

13.1 Perform quadratic least squares regression using peak areas for all calibrants versus concentration, ranging from 2.5 to 500 ppb

13.2 Weighting: $1/x^2$

13.3 Ignore Origin

13.4 Correlation coefficients (R^2) are expected to be greater than or equal to 0.95

13.5 The peak area of the quantifying ion is greater than the peak area of the same ion in the least concentrated calibrant.