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Quantitation of Anticoagulant Rodenticides in Serum

Purpose:

This SOP describes the extraction and sample clean-up method for the quantitative determination of eight anticoagulant rodenticides in serum.

I. Materials / Equipment

A. Supplies

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

B. Chemicals (Higher grade chemicals may be substituted)

1. Distilled, deionized (DDI) water
2. Methanol, Acetonitrile, Acetone, Chloroform (HPLC or LC-MS grade; Fisher Scientific)
3. Ammonium acetate, HPLC grade (Fisher Scientific #A639-500)
4. Ammonium hydroxide, ACS grade (ACS grade, #BDH3014-500MLP, VWR International)
5. 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)

1. Analytical Balance (Model PB303-S, Mettler Toledo)
2. Vortex (Vortex Genie 2, Fisher Scientific)
3. Centrifuge w/ cooling capability suitable for 1.5 mL microcentrifuge tubes (Micromax RF, Thermo Electron)
4. Ultrasonic Cleaner (FS30, Fisher Scientific) & micro-centrifuge floatation rack (VWR)
5. Thermo Scientific / Dionex UltiMate 3000 Rapid Separation Liquid Chromatography (UPLC) system with autosampler, binary pump and thermostatted column compartment

6. Thermo Scientific Quantum Access Max triple quadrupole mass analyzer with heated electrospray ionization
7. Analytical Chromatography Column (Zorbax XDB-C18 Eclipse, 2.1 x150 mm, 5µm; Agilent, #993700-902)
8. Guard Column Cartridge System (Syncronis C18 cartridges, 2.1 x 10 mm, 5 µm, Thermo Scientific, #97105-012101)

D. Prepared Reagents

1. 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.

2. Primary Stock Solutions – 1000 ug/mL: For each anticoagulant rodenticide, dissolve 5.0 ± 0.1 mg standard reference material in 5.00 mL of the appropriate solvent (See Table 1), using a 5-mL volumetric flask. 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

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 single 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 ug/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.
   a. 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.

   b. Methanol, HPLC grade

II. Sample Treatment:

A. 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.

1. Pipette the appropriate volume of AR Standard Solutions into the corresponding tube, as described in Table 1, yielding a final volume of 250 uL.
2. Cap tubes and vortex mix 10 s to mix thoroughly.
3. Proceed to step 2 of section B.

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

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 QC 5.0PPB	0 5.0	--- ---	--- ---	--- 10	250 240
QC 400PPB	400	10	---	---	240

B. Sample Extraction

1. Transfer 250 µL unknown sample serum to a labelled 1.5-mL disposable micro-centrifuge tube.
2. To all calibrants, QC samples, and unknown samples, add 250 µL 10% (v/v) acetone in methanol pre-chilled at 4°C using an accurate pipettor. Vortex mix thoroughly for 10 s.
3. Centrifuge the samples at 16,000 g and 4°C for 10 minutes.
4. Decant the supernatant for each sample into a new, labelled 1.5-mL disposable micro-centrifuge tube.
5. Into the decanted tubes with precipitate, add 250 µL 10% (v/v) acetone in methanol pre-chilled at 4°C using an accurate pipettor to all calibrants, QC samples, and unknown samples. Vortex mix thoroughly for 10 s.

B. Sample Extraction (continued)

6. Place the precipitate-containing samples into the micro-centrifuge tube flotation rack and place in the sonication bath.
7. Sonicate the samples for 5 minutes.
8. Centrifuge the samples at 16,000 g and 4°C for 10 minutes.
9. Transfer, via glass pipette, the supernatant for each sample and combine with the previous corresponding supernatant in step 4. Vortex mix thoroughly for 10 s.
10. Centrifuge the combined supernatant tubes at 16,000 g and 4°C for 10 minutes.
11. Filter each supernatant by syringe filtering:
    a. Remove the syringe plunger and attach a PVDF luer-lok syringe filter.
    b. Transfer the supernatant to the syringe, re-insert the plunger and filter into:
        i. a labelled glass silanized autosampler vial.

    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.

    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.

III. HPLC – MS/MS Analysis

A. HPLC Settings

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 is in Table 3.
2. Flow Rate: 0.400 mL/min
3. Column Temperature: 25°C
4. Injection Volume: 10 uL
5. Total Run Time: 34 min
6. Autosampler temperature: +24C (room temperature). Note, pesticides are usually very stable

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

B. 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 3.

1. ESI Source Conditions:
   Optimized on the basis of direct infusion of solvent-diluted reference standards
   a. Negative ion mode
   b. Spray Voltage: 4000 V
   c. Vaporizer Temperature: 380°C
   d. Sheath Gas Pressure: 50 psi
   e. Auxillary Gas Pressure: 45 psi
   f. Ion Sweep Gas Pressure: 0 psi
   g. Capillary Temperature: 300°C
   h. Skimmer Offset: (Not used)

2. Other Parameters:
   a. Collision Gas Pressure: 1.7 mTorr
   b. Collision Energy: Ion-Dependent; see MRM Transitions Table
   c. Tube Lens: Ion-Dependent; see MRM Transitions Table
   d. Q1 / Q3 Peak Width (FWHM): 0.70 u
   e. Cycle Time: 0.300 s

Table 3. MRM Monitored Transitions / Expected Retention Times

Anticoagulant Rodenticide	Retention Time (min)	Precursor Ion ($(M-H^+)^-$; u)	Fragment Ion (u)	Collision Energy (eV)	Tube Lens (V)
Warfarin	3.70	**307**	**161**[a]	22	70
		307	250	25	70
Coumachlor	7.50	**341**	**284**	26	71
		341	161	23	71
Diphacinone	10.06	**339**	**167**	28	77
		339	165	48	77
Dicoumarol	10.30	**335**	**161**	21	47
		335	117	47	47
Chlorophacinone	13.26	**373**	**201**	24	76
		373	145	25	76
Bromadiolone[b]	15.33	**525**	**250**	38	97
		525	273	40	97
Brodifacoum	17.92	**521**	**135**	40	101
		521	143	57	101
Difethialone	18.22	**537**	**151**	41	100
		537	371	35	100

[a]Transitions in bold are used for quantitation

[b]Two isomers are present for Bromadiolone; only the earliest eluting (and most abundant) isomer is used for detection and quantitation.

C. Post-Acquisition Data Analysis

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.

1. Qualitative Identification – The respective analyte is considered to be qualitatively identified in the unknown sample if the following criteria are met:
   a. The quantifying ion and the corresponding confirming ion co-elute within 0.1 min of one another, each with a signal-to-noise ratio ≥ 3.
   b. The retention times of the quantifying / 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.
   c. The quantifying ion and the corresponding confirming ion ratio is within +/- 20% of the expected ratio (typically the average of the batch standards' ion ratios).

2. Quantitative Analysis – The following parameters should be used to generate calibration curves to determine quantitative results:
   a. Perform quadratic least squares regression using peak areas for all calibrants versus concentration, ranging from 2.5 to 500 ppb
   b. Weighting: $1/x^2$
   c. Ignore Origin
   d. Correlation coefficients (R^2) are expected to be greater than or equal to 0.95
   e. The peak area of the quantifying ion is greater than the peak area of the same ion in the least concentrated calibrant.