Quantitative Analysis of 10 Anticoagulants in Human Blood by UPLC Triple Quadrupole Mass Spectrometry (LCMSMS)

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INTRODUCTION

Anticoagulant drugs within the warfarin and superwarfarin class are commonly used as the active ingredient in commercial rodenticides. Currently, these drugs are unregulated and commercially available for purchase. As such, these anticoagulants drugs have been implicated in different forensic casework, including suicides, homicides and accidental poisonings. In recent years, these anticoagulant drugs have emerged as toxic adulterants in illicit drugs in United States. Two major anticoagulant outbreaks in Chicago (2018) and Tampa (2021) have been reported, where synthetic cannabinoids were laced with brodifacoum. Consumers of these laced synthetic cannabinoids products suffered severe adverse effects including spontaneous internal and external bleeding, and some cases resulted in death. The detection and quantification of these compounds in forensic casework is challenging due to the low concentrations of the drugs. In addition, these drugs are typically not detected using routine screening workflows. The objective of this research was to develop and validate a quantitative, targeted method to isolate ten anticoagulants from human blood with subsequent analysis by liquid chromatography tandem mass spectrometry.

METHODS

Analysis by Waters ACQUITY UPLC Xevo TQ-S

Column: ACQUITY UPLC BEH C18 (2.1 x 100 mm; 1.7µm)
Mobile Phase A: 0.02% NH_4OH in Water
Mobile Phase B: 0.02% NH_4OH in Methanol
Flow Rate: 0.4 mL/min
Injection Volume: 10 µL

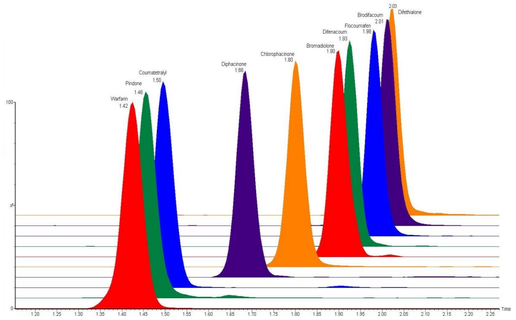


Table 1: LC Gradient

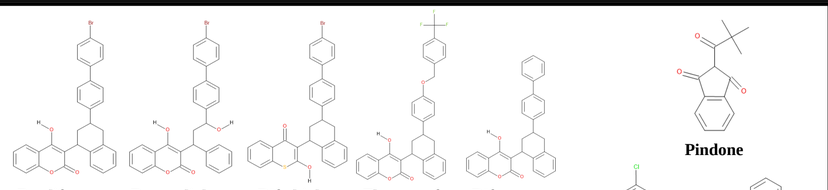
Time (min)	%MPB
0.00	5
1.60	95
3.50	95
3.60	5
4.00	5

Figure 1: Chromatographic separation achieved using LC parameters (50 ng/mL).

Table 2: MRM Transitions and MS Parameters

Drug	Precursor Ion to Quantification Ion (m/z) Precursor Ion to Qualifier Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)	Retention Time (min)
Brodifacoum	521.03 → 135.02 521.03 → 93.08	6 6	36 62	2.01
Bromadiolone	527.03 → 250.00 525.03 → 250.01	66 18	34 34	1.90
Chlorophacinone	373.10 → 201.10 373.10 → 145.20	4 4	20 20	1.80
Coumatetralyl	290.97 → 141.09 290.97 → 106.05	4 4	24 24	1.50
Difenacoum	443.30 → 135.20 443.30 → 293.20	4 4	35 35	1.93
Difethialone	537.03 → 79.01 537.03 → 151.04	90 90	42 38	2.02
Diphacinone	339.10 → 167.20 339.10 → 172.10	4 4	25 30	1.68
Flocoumafen	541.10 → 381.92 541.10 → 161.01	86 86	22 38	1.98
Pindone	229.40 → 116.10 229.40 → 144.00	4 4	35 25	1.46
Warfarin	306.97 → 161.00 306.97 → 249.93	56 56	16 20	1.42

ANALYTES OF INTEREST



EXTRACTION PROTOCOL

Protein Crash
- Aliquot 0.5 mL of blood
- Add 25 µL internal standard
- Add 0.5 mL ammonium formate buffer (pH 3)
- Add 2 mL acetone
- Cap and rotate for 5 minutes
- Centrifuge at 3000 rpm for 5 minutes
- Transfer top layer

LLE
- Add 1.5 mL n-butyl chloride
- Cap and rotate for 5 minutes
- Centrifuge at 3000 rpm for 5 minutes
- Transfer top layer

Recon
- Evaporate to dryness at 35 ºC for 25 minutes
- Reconstitute with 200 µL of 95:5 MPA:MPB
- Vortex and transfer solution to a Costar® Spin-X® centrifuge filter tube
- Centrifuge filter tube at 2000 rpm for 10 minutes
- Transfer filtered solution to auto-sampler vial

METHOD VALIDATION

The method was validated in accordance with ASB Standard 036. Parameters evaluated included calibration model, bias/precision, limit of detection (LOD), limit of quantification (LOQ), carryover, interferences, and ionization suppression/enhancement. Additional parameters evaluated included recovery, auto-sampler stability, and matrix matching.

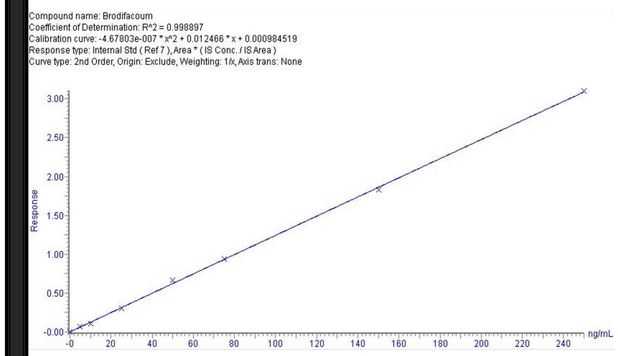


Figure 2: Brodifacoum **c**alibration curve (5-250 ng/mL).

Table 3:Validation Results

Calibration range	5-250 ng/mL
Bias	4.5% at LOQ
LOD	1 ng/mL
Carryover	> 250 ng/mL
Interferences	None
Matrix matching	Serum, urine, and oral fluid
Recovery	73-137%

AUTHENTIC SPECIMEN TESTING

Authentic deidentified cases (n=79) from the recent outbreak in Florida were provided by NMS Labs for research purposes. In 74 samples, brodifacoum was detected and quantitated across three different matrices. In six serum samples, difenacoum was detected along with brodifacoum. All urine samples (n=8) were negative for any anticoagulant drug within the scope of the method. Quantitative results are shown below in Table 4. According to news reports, a total of 42 individuals were hospitalized and two individuals died during this outbreak.

Table 4: Results from Authentic Specimen Testing

Drug	Matrix	Mean Concentration (ng/mL)	Median Concentration (ng/mL)	Range (ng/mL)
Brodifacoum	Blood (n=13) Serum (n=45) Plasma (n=16)	159 (±131) 575 (±432) 134 (±133)	110 476 53	48 – 429 86 – 1995 11 – 365

Samples were additionally screened for synthetic cannabinoids. Four urine samples were positive for 4F-MDMB-BICA 3,3-dimethylbutanoic acid, a metabolite of 4F-MDMB-BICA. Because of the long detection window of urine, it is unknown if this drug was present from this incident or previous consumption. One plasma sample was positive for MDMB-4en-PINACA, and one serum sample was positive for 4F-MDMB-BICA.

DISCUSSION AND CONCLUSIONS

We developed a unique and highly efficient liquid-liquid extraction for the isolation of anticoagulant drugs from various biological matrices, which are highly lipophilic. The analytical method proved to be very sensitive, selective and suitable for the detection and quantitation of ten anticoagulant drugs in human blood or other matrices. Following method validation, authentic samples were acquired and analyzed. Brodifacoum was identified in several cases in three different matrices. Additionally, some samples were found to have another anticoagulant present, difenacoum, which was detected in the serum with the presence of brodifacoum. From a clinical perspective, the findings from this research demonstrate the utility of detection anticoagulant drugs in serum using the described extraction technique and method. The difenacoum findings suggests that laboratories should consider including additional anticoagulant drugs into their panels or pursue additional testing in cases that appear to be related to anticoagulants, but test negative. Additional research will need to be done to understand additional risks and potential for adverse outcomes when multiple anticoagulant drugs are co-ingested. As the number of incidents involving anticoagulant drugs adulterating seized material increases, laboratories should be aware of the challenges associated with the detection of anticoagulant drugs and consider incorporating these drugs into their panels or pursue additional testing in cases that appear to be related to anticoagulants.

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