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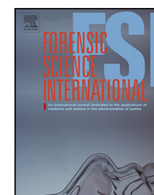
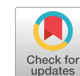


Sensitive determination of nine anticoagulant rodenticides in blood by high resolution mass spectrometry with supported liquid extraction pretreatment



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ABSTRACT

Anticoagulant rodenticides (ARs) have been widely used for controlling rodents in agriculture and households. It often occurs that non-target animals are poisoned by ARs. Also, the abuse of ARs has been often encountered in poisoning and suicide cases. Herein we report the determination of nine commonly used ARs by high resolution mass spectrometry (HRMS) with supported liquid extraction (SLE) pretreatment. The factors affecting SLE (elution solvents and pH values) were systematically tested andoptimized. The application of parallel reaction monitoring (PRM) mode led to the highest sensitivity obtained for these compounds, with LODs ranging for 0.006–0.02 ng/mL. Reasonable extraction recoveries for all the analytes were obtained ranging in 73.9%–110.7%. Good precision was achieved for the spiked blood samples, with intra-day RSD ranging in 5.0%–9.2% and inter-day RSD ranging in 6.3%–10.5%. The values of ME ranged in 82.9%–103.2% for QC sample, which are reasonable. The application of HRMS in PRM mode also resulted in high selectivity. The method was applied to the detection and quantification of ARs in blood samples from real forensic cases. This methodology possesses high potential for determination of rodenticides in clinical and forensic cases.

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1. Introduction

Anticoagulant rodenticides (ARs) are a group of pesticides which are widely used in agriculture and households for the purpose of controlling rodents [1–3]. Commonly used ARs include brodifacoum, bromadiolone, warfarin, coumachlor, coumatetralyl, difenacoum, pindone, difethialone and flocoumafen. These rodenticides inhibit vitamin K epoxide reductase, leading to the lack of vitamin K and the deactivation of blood clotting factors (II, VII, IX and X) [1,4]. Although they are meant to control rodents many poisoning incidents of non-target animals involving ARs have been reported [3,5–7]. Furthermore, ARs have been abused in human poisoning and suicide cases [8–10]. Therefore, the development of a sensitive and selective methodology for analysis of ARs is important for agricultural, clinical and forensic applications.

Mass spectrometry is one of the most important tool for the analysis of poisons from various matrices. Several studies concerning the determination of ARs by liquid chromatography–tandem mass spectrometry (LC–MS/MS) have been reported [11–14]. In forensic and clinical practices, it occurs often that the patients or victims are examined after a time span since the ingestion of poisons, because the development of symptoms can be slow. At the time of analysis, the concentrations of the original poisons in biological specimens can be very low due to metabolism, and a sensitive method is needed. High-resolution mass spectrometry (HRMS) possesses multiple advantages, including reduced optimization procedure for acquisition parameter, higher specificity, stronger qualitative capability and the ability to provide more detailed structural information [15–17]. So far, the application of HRMS has been focused on the sequencing and determination of peptides [18–20], while the reports on determination of small molecules with this technique are relatively fewer [21].

Herein, we report a new methodology for the sensitive and selective determination of ARs in blood with supported liquid

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extraction (SLE) pretreatment and HRMS determination. Parallel reaction monitoring (PRM) is a high resolution mass spectrometry mode based on Q-Orbitrap. The conventional selected reaction monitoring (SRM) mode performs one transition at a time, whereas the PRM mode performs a full scan for all transitions of a precursor ion, which means parallel monitoring of all fragments from the precursor ion. With the application of the PRM mode of HRMS for data acquisition, the lowest LODs were achieved for these target analytes among all reported methods along with superior selectivity. The methodology is of significant value for clinical and forensic analysis of rodenticide poisons.

2. Materials and methods

2.1. Chemicals and reagents

Standard samples of brodifacoum, bromadiolone, warfarin, coumachlor, coumatetralyl, difenacoum, flocoumafen were obtained from Sigma-Aldrich (Shanghai, China). Standards of pindone, difethialone and stable isotope-labeled brodifacoum-D4 were purchased from J&K Scientific (China). Sodium chloride was provided by Merck (Darmstadt, Germany). HPLC-grade solvents ethyl acetate, toluene, dichloromethane and methyl tert-butyl ether (MTBE) were obtained from Anhui Tedia (Anhui, China). The buffer solutions pH 2.0 (citric acid/sodium hydroxide/hydrogen chloride), pH 4.0 (citric acid/sodium hydroxide/hydrogen chloride), pH 6.0 (citric acid/sodium hydroxide) and pH 8.0 (boric acid/sodium hydroxide/hydrogen chloride) were obtained from Sigma-Aldrich (Shanghai, China). A 2 mL Isolute SLE+ cartridge was provided by Biotage (Biotage Trading Co., Ltd, Shanghai, China). LC–MS grade methanol and formic acid were obtained from Merck (Darmstadt, Germany). Deionized water was generated with an arium® comfort II water purification instrument (Sartorius, Göttingen, Germany) and used throughout the experiments. Stock standard solutions of anticoagulant rodenticides were generated in methanol (10.0 mL) at the concentration of 10 mg/L for all analytes. Methanol containing internal standard (Brodifacoum-d4) at the concentration of 1 mg/L was stored in a freezer at −20 °C. Working solutions were generated by appropriate dilution of the stock standard solutions. Six blank blood samples were provided by volunteers in our lab in good health condition, who had not ingested any drugs for at least 3 months prior to the experiments. The whole blood (unmodified collected blood) samples were provided by Chongqing Institute of Forensic Science (Chongqing, China) from real forensic cases, and were preserved at −20 °C prior to experiments.

2.2. Sample pretreatment with SLE

Samples were vortexed at high speed for several seconds. An aliquot of 0.5 mL for each sample was injected into a 2 mL test tube (Axygen, Union City, CA) that was free from contamination, and 10 μL methanol containing 10 ng working internal standard solution was added. The mixture was vortex-mixed for approximately 30 s. 0.5 mL of citric acid/sodium hydroxide buffer solution (pH = 6) was added, and the mixture was again vortexed for approximately 1 min. The sample was then loaded to a 1 mL SLE cartridge (Biotage Isolute). A positive pressure was exerted to enhance the absorption of the sample into the SLE cartridge. After the analytes equilibrated with the sorbent for 5 min, they were eluted with 2 mL of ethyl acetate as the elution solvent three times. The extraction solvent was eluted by applying a positive pressure from the top with a rubber bulb for rapid elution and evaporated to dryness under nitrogen flow at 40 °C. The residues were reconstituted with 50 μL initial mobile phase and vortexed for ∼30 s. A 2 μL aliquot of the reconstituted solution was infused into the UHPLC–HRMS system for analysis.

2.3. LC–HRMS/MS analysis

Chromatographic separation was performed with a Dionex 3000 Series UHPLC (Thermo Fisher, USA) instrument. The column temperature was set at 35 °C. The target analytes were separated with an ACQUITY UPLC BEH T3 column (1.7 μm, 2.1 × 100 mm; Waters Corp., Milford, MA, USA). The flow rate was 0.4 mL/min. The mobile phases consisted of 0.1% formic acid/water (solvent A) and 0.1% formic acid/methanol (solvent B). The gradient parameters were set as the following: initially 90% A kept for 1 min, ramped to 15% A over 0.5 min and held for additional 4 min, followed by returning to initial conditions in 0.5 min and re-equilibrating for 2 min. The overall running time was 8 min. The injection volume was 2 μL. Acquisition was performed in PRM mode with a Thermo Q-Exactive HRMS (Bremen, Germany) equipped with an electrospray ionization (ESI) source. The IonSpray voltage was set at −2.8 KV in the negative ionization mode. The capillary temperature was 350 °C. The HRMS instrument was calibrated every seven days with calibration solutions (P/N: 88323) supplied by the instrument manufacturer. Sheath gas, auxiliary gas and sweep gas were all nitrogen. The pressures were set at 40, 8, 0, respectively, in arbitrary units. In PRM mode, the mass resolution for data collection was set as 70,000 FWHM for precursor ions and 35,000 FWHM for fragment ions. The data of the precise precursor ion mass and fragment ion were collected under the corresponding normalized collision energy (NCE) for the target analytes. Automatic gain control (AGC) target was set as $1 \times e^{6}$, maximum injection time was set at 100 ms, isolation window was set as 2.0 m/z. The maximum number of precursor ions to be multiplexed in a scan event (MSX count) was set as 1. Chromatograms obtained in PRM mode for the nine ARs and one internal standard spiked at 2 ng/mL are shown in Fig. 1.

2.4. Method validation

The mass spectrometry data obtained by PRM mode were used for method validation. Blank blood samples of six individuals were fortified, and this method was validated by evaluating the corresponding linearity, sensitivity, recovery, accuracy and precision. The specificity was tested by comparing the chromatograms of spiked samples with those of blank blood (Fig. 2). Linearity was determined with blank blood spiked at different concentrations (0.02–200 ng/mL for coumatetralyl, warfarin, coumachlor; 0.07–200 ng/mL for other analytes), by calculating the area of the peaks relative to the internal standard. Typically, LOD and LOQ are determined as the analyte concentrations at which the signal-to-noise ratios are equal to 3 and 10 [22]. In the present method, with the determination by HRMS, the background noise is essentially invisible and elusive to define. Thus, the LOD was determined by the concentration at which the peak is well defined, and slightly below which the peaks disappears, using spiked samples. For the determination of LOD, the mass precision was within 5 ppm. LOQ was defined as the concentrations that is three times the LOD.

Extraction recovery and precision were assessed by analyzing blood samples spiked at the concentrations of 0.02 ng/mL, 2 ng/mL and 200 ng/mL for coumatetralyl, warfarin, coumachlor, 0.07 ng/mL, 2 ng/mL and 200 ng/mL for other analytes. Extraction recovery was calculated based on this equation: Extraction recovery (%) = (peak area of analyte in blood spiked before pretreatment/peak area of analyte in blood spiked after pretreatment) × 100. Extraction recoveries were calculated with analysis in sextuplets for each analyte. Intra-day precision was assessed by analysis of the same spiked sample for six times on one single day. Inter-day precision was assessed by spiking an extracted sample prepared daily for 6 consecutive days and analyzed in sextuplets. The intra-

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