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Determination of rodenticides and related metabolites in rabbit liver and biological matrices by liquid chromatography coupled to Orbitrap high resolution mass spectrometry

Marina López-García, Roberto Romero-González, Antonia Garrido Frenich*

Research Group "Analytical Chemistry of Contaminants", Department of Chemistry and Physics, Research Centre for Agricultural and Food Biotechnology (BITAL), University of Almeria, Agrifood Campus of International Excellence, ceiA3, E-04120 Almeria, Spain

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ABSTRACT

An analytical method based on ultra-high performance liquid chromatography (UHPLC) coupled to Orbitrap high resolution mass spectrometry was developed for the determination of rodenticides (bromadiolone, brodifacoum, difenacoum, chlorophacinone, diphacinone, coumachlor and warfarin) in liver matrix. Different extraction conditions were tested, obtaining the best results when the "dilute and shoot" method (acidified acetonitrile as extraction solvent) and a clean-up step with primary secondary amine (PSA) were used. The optimized method was validated, obtaining recoveries ranging from 60 to 120%. Repeatability and reproducibility were evaluated obtaining values lower than 20%, except for brodifacoum at 10 μg/kg. Limits of quantification (LOQs) ranged from 0.1 to 0.5 μg/kg, except for brodifacoum, which was 100 μg/kg. Six liver samples were analyzed and diphacinone and chlorophacinone were detected in three samples at concentrations ranging from 4 μg/kg to 13 μg/kg. Moreover a retrospective screening of rodenticide metabolites in those samples and in animal forensic samples was developed based on Orbitrap capabilities. Brodifacoum was detected in three samples, and warfarin alcohol, which is a metabolite of warfarin, was also detected in one sample.

1. Introduction

Anticoagulant rodenticides (ARs) are widely used for the suppression of rodent overpopulation and that is why they have an important toxicological issue. Nowadays rodenticides are the most commonly used poisons in rodent control as well as in agriculture and household [1]. All anticoagulants contain either hydroxycoumarin or indandione nucleus, depending on their chemical structure. The most common indandione rodenticides are chlorophacinone and diphacinone, whereas two generations can be distinguished for the hydroxycoumarins. The first generation (warfarin and coumachlor) needs multiple feeding before producing their effects, while the second one, anticoagulants as brodifacoum, difenacoum and bromadiolone, are highly potent and require only a single dose to kill rats. This enhanced efficacy against rodents has resulted in increased toxicity in non-target species [2].

ARs block the vitamin K epoxide reductase required to reduce vitamin K epoxide, an essential factor in the biosynthesis of clotting factors [3]. Rodents are key dietary elements for many predators and scavengers and poisoned rodents, which are dead or dying, are easy targets. Unfortunately, predators and scavengers can also become victims if the prey or bait contains residues of any chemicals used for rodent control [4]. The main cause for accidental poisoning of domestic animals and to lesser extent wild animals, is direct consumption of anticoagulant baits. Secondary poisoning through consumption of rats and mice killed with anticoagulants may be observed in dogs and cats in urban situations but it is more likely to occur in farm situations [5].

These rodenticides can be present in numerous matrices such as blood [2,6–10], urine [2,6,11], plasma [2,5,11–16], tissues [1–5,10,17,18], human serum [2,19–24], food [6,25] and wastewater [26,27]. However the liver is the main organ in which ARs can be accumulated and that is why, this is the target tissue, where their presence can be evaluated [3].

Different extraction methods were used for the determination of rodenticides. Basically conventional solid–liquid extraction is commonly applied, using different solvents as methanol [1,3], acetone [4] or acetonitrile [2,10,25], followed by an evaporation step with a rotary evaporator [3] or under a nitrogen stream [6,8]. Moreover, in order to minimize the presence of interferents as well as

* Corresponding author.
E-mail address: agarrido@ual.es (A.G. Frenich).

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to increase the sensitivity of the method a clean-up step based on a liquid–liquid extraction [6] is needed. In the last few years, new extraction methods such as QuEChERS (quick, easy, cheap, effective, rugged and safe) [10], "dilute and shoot" approach [28,29] or semi-automated methods [30] have been applied. They are simple and fast methods, and sometimes a clean-up step with primary secondary amine (PSA) or other sorbents is necessary. Although both methods provide suitable results in different applications, the "dilute and shoot" procedure could be faster and easier than QuEChERS method and it obtains higher recovery values in some applications [31]. Due to the simplicity of this method, errors during this stage can be minimized.

In relation to the determination of rodenticides, classic detectors like fluorescence detector (FLD) [2,17,18], ultraviolet detector (UV) and photodiode-array (DAD) [2,6,10,11,13–15,18] have been used coupled to liquid chromatography (LC). In the last years mass spectrometry (MS), using single quadrupole (Q) [2,3,8,12] or triple quadrupole (QqQ) analyzers [1,2,4,5,7–9,19–21,25] has been the technique of choice, increasing the reliability of the identification process. However, these instruments have certain limitations because they require optimized acquisition parameters for each analyzed compound, the number of analyzed substances is limited, and only compounds from a target list can be detected and therefore, retrospective data analysis is not possible [32]. Nowadays, the use of liquid chromatography coupled with high resolution mass spectrometry, such as Orbitrap-MS [28–30,33] is a powerful technique that can improve the limitations of other techniques. Despite this analyzer could have some limitations as price, its use is not easy and the sensitivity could be lower than the values provided by QqQ, it is an interesting analyzer due to it operates in the full scan mode (theoretically, no limitations in number of monitored compounds) and provides accurate mass measurements (<5 ppm) [28] allowing selective detection of residues at low concentration levels in complex samples, such as baby food [29]. The main advantage of this analyzer is that enables the acquisition of unlimited number of compounds by means of accurate mass measurements combined with high resolving power [34]. As far as we know, only one previous study analyzed rodenticides in blood by LC-Orbitrap-MS/MS [35], although diphacinone and chlorophacinone were not studied.

In relation to the maximum residue limit (MRL) set for these compounds in several matrices, the EU has only set MRL for warfarin in liver at 10 μg/kg [36], highlighting the need for development sensitive analytical methods for the determination of these type of compounds in biological matrices.

In the current study, a new instrumental method based on ultra-high performance liquid chromatography coupled with Orbitrap mass spectrometry (UHPLC-Orbitrap-MS) has been developed and validated for the determination of rodenticides in liver matrix, which can be considered as a target tissue where these compounds can be accumulated. In addition, a retrospective screening analysis (post-target screening) of rodenticides metabolites in 220 biological samples was developed based on a database containing 57 metabolites. Up to now few studies focused on the detection of the metabolites of rodenticides have been performed, being the warfarin metabolites the most studied due to its use in the treatment and prevention of thromboembolism [14].

2. Materials and methods

2.1. Chemicals and reagents

Diphacinone was provided from Supelco (Bellefonte, PA, USA) with purity ≥ 99.3%. Coumachlor was supplied from Fluka (Steinheim, Germany) with purity ≥ 99.9%. Bromadiolone, warfarin and brodifacoum were obtained from Riedel-de-Haën (Seelze, Germany) with purity ≥ 99.2%. Difenacoum and chlorophacinone were purchased from Dr. Ehrenstofer (Augsburg, Germany) with purity ≥ 97%. Individual stock standard solutions (208–554 mg/L) were prepared in acetone and were kept at ≤ 5 °C. Methanol was purchased by Fluka and acetonitrile and acetone by Sigma Aldrich (St. Louis, MO, USA). All solvents were LC–MS grade. A multi-compound working solution was prepared in a mixture of methanol:acetone (50:50, v/v) by a combination of each individual standard stock solution (5 mg/L). This solution was stored at ≤5 °C. Formic acid was purchased from Thermo Fisher Scientific (Geel, Belgium). Other reagents as primary secondary amine (PSA), graphitized black carbon (GBC) and Florisil cartridges were obtained from Scharlab (Barcelona, Spain), ammonium formate (purity >99%) from Fluka, C_{18} from Agilent Technologies (Santa Clara, CA, USA), and anhydrous magnesium sulfate, sodium chloride and zirconium oxide (ZrO_2) from Sigma Aldrich. LC–MS water was purchased from J.T. Baker (Barcelona, Spain). Econofilter Nylon filters (13 mm, 0.20 μm pore size, Agilent Technologies) were used for filtration of extracts. A mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrate and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative mode calibration mix) and a mixture of caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark 1600 (Proteo Mass LTQ/FT-Hybrid ESI positive mode calibration mix) from Thermo Fisher Scientific were used for accurate mass calibration of the Orbitrap analyzer.

2.2. Apparatus

The apparatus used during the extraction procedure were a rotary agitator from Heidolph (Schwabach, Germany), a vortex mixer WX from Velp Scientifica (Usmate, Italy), an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) and a Consul 21 high-volume centrifuge from Olto Alresa (Madrid, Spain).

2.3. UHPLC-Orbitrap-MS analysis

A Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) equipped with a chromatographic column from Thermo, Hypersil GOLD aQ C18 column (100 mm × 2.1 mm, 1.9 μm particle size) was used for chromatographic analysis. The organic phase consisted of methanol containing 0.1% (v/v) formic acid and ammonium formate 4 mM. The aqueous phase consisted of water containing 0.1% (v/v) formic acid and ammonium formate 4 mM. The gradient used for the chromatographic separation was carried out as follows: first, 95% of aqueous phase was set during 1.0 min, decreasing linearly to 0% in 7.0 min. After 4 min keeping 0% of aqueous phase, this percentage was increased again up to 95% in 0.5 min. Finally the initial conditions were kept constant during 1.5 min, obtaining a total analysis time of 14 min. Other chromatographic conditions as flow rate, column temperature and injection volume used were set at 0.25 mL/min, 25 °C and 10 μL respectively.

Chromatographic equipment was coupled with an Orbitrap mass spectrometric analyzer (Exactive™, Thermo Fisher Scientific, Bremen, Germany). Ionization was performed using a heated electrospray interface (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), both in positive mode (ESI+) and negative mode (ESI−). In the collision cell (based on straight multipole mounted inside a metal tube) the characteristic ions were fragmented and controlled by accurate mass measurements. The parameters were: automatic gain control (AGC) at 1×10^6, spray voltage at 4 kV, sheath gas (N_2, >95%) at 35 (adimensional), tube lens voltage at 95 V (−95 V in ESI−), skimmer voltage at 18 V (−18 V in ESI−), auxiliary gas (N_2, >95%) at 10 (adimensional), capillary voltage at 35 V (−35 V in ESI−), heater temperature at 305 °C and capillary temperature