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Multi-residue determination of eleven anticoagulant rodenticides by high-performance liquid chromatography with diode array/fluorimetric detection: Investigation of suspected animal poisoning in the period 2012–2013 in north-eastern Italy

Federica Gallocchio *, Lara Basilicata, Cristiana Benetti, Roberto Angeletti, Giovanni Binato

Istituto Zooprofilattico Sperimentale delle Venezie, Chemistry Department, viale dell'Università 10, 35020 Legnaro, Padova, Italy

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ABSTRACT

Misuse or deliberate abuse of anticoagulant rodenticides (AR) may often result in incidental or malicious non-target animal poisoning. This study presents preliminary results of the analysis of 561 real suspected samples, ranging from baits to livers and stomach contents, collected at the Istituto Zooprofilattico Sperimentale delle Venezie (official referral laboratory for the regions of north-eastern Italy), in the period 2012–2013.

Samples were analyzed by a method based on a combination of liquid chromatography with diode array/fluorescence detection (HPLC-DAD/F) able to identify 11 different AR (brodifacoum, bromadiolone, chloropahacinone, coumachlor, coumafuryl, coumatetralyl, difenacoum, diphacinone, flocoumafen, pindone, warfarin).

1. Introduction

Anticoaugulant rodenticides (AR) are pesticides widely used for harmful rodent control purposes [1–4]. The most commonly employed AR are hydroxycoumarine derivatives (such as coumachlor, coumafuryl, coumatetralyl, warfarin, brodifacoum, bromadiolone, difenacoum, flocoumafen) and indandione derivatives (such as indandione, diphacinone, chlorophacinone and pindone), whose chemical structures differ (see Figs. 1 and 2).

AR can be further divided into first generation (multiple-dose form) and second generation (single-dose form) compounds [5–7]; the last show greater toxicity and are lethal for animals in a single feeding due to the greater affinity to binding sites in the liver and consequently greater accumulation and persistence [5]. In fact, first generation AR generally have shorter elimination half-lives [5] and require higher concentration (usually between 0.005 and 0.25%) [8] and consecutive intake over days in order to accumulate the lethal dose. Second generation AR, instead, are generally applied in lower concentration in bait (usually on the order of 0.001–0.005%) [8] and are lethal after a single ingestion of baits. For example, one day's feeding with brodifacoum, bromadiolone, flocoumafen and difethialone can provide an effective dose against rodents, whereas warfarin, chlorophacinone, and diphacinone generally require several doses over time [5].

AR are usually formulated and commercially available as colored soft baits or paraffin blocks, designed to attract rodents. They may contain sucrose, meat, vegetables, grains, or fruits [8]. Despite the addition of bitterants such as denatonium benzoate, baits may also attract non-target animals such as pets, livestock and wild animals, causing accidental or intentional animal poisoning [9–12].

Both hydroxycoumarin and indandione AR act as vitamin K enzyme inhibitors [7]. Vitamin K is a cofactor of primary importance in the blood coagulation process as it contributes to the activation of blood clotting factors (II, VII, IX, and X) [13]. Exposure to AR can therefore lead to a progressive decrease in blood clotting factor between 12 and 24 h after intoxication [5], resulting in massive bleeding episodes that are potentially fatal in the absence of appropriate therapy.

Other less common clinical symptoms due to AR accidental animal exposure are difficulty breathing, weakness, lethargy, coughing, vomiting, tarry blood, paleness, bleeding from the gums, seizures, bruising, shaking, abdominal distention and pain [8].

* Corresponding author. Tel.: +39 049 8084342; fax: +39 049 8080572.
E-mail address: fgallocchio@izsvenezie.it (F. Gallocchio).

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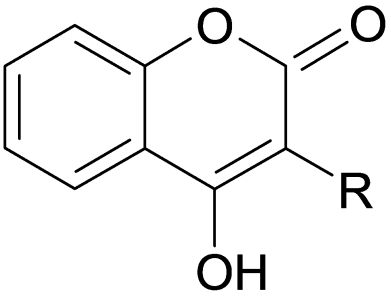

Fig. 1. Hydroxycoumarine derivatives.

Hydroxycoumarin and indandione AR act in the liver mainly where special enzymes allow vitamin K to be recycled and stored. Hence AR determination in the liver can be useful in post-mortem diagnosis and for forensic purposes.

In Italy, a Decree of the Ministry of Labor, Health and Social Policies (Italian Ministerial Regulation of December 18, 2008–Italian Official Journal no. 13, January 17, 2009) has been in force since January 2009, banning the improper or malicious use of poisoned baits. Whenever poisonings and intoxications are suspected or clinically diagnosed by a health official or veterinary practitioner, they must be reported to the appropriate authorities, who will take the necessary legal action. At the same time, relevant sample materials involved in the case (meat and grain baits, stomach and gut contents, liver, unknown suspicious samples) are collected and referred to official laboratories for detailed chemical analyses. The Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) is the official referral laboratory for the regions of north-eastern Italy (Veneto, Friuli Venezia Giulia, Trentino Alto Adige). Such mandatory procedures have contributed to a marked increase in the number of samples being sent for toxicological assay, prompting the need for fast, effective, easily applicable analytical methods. The fact that AR do not naturally occur in mammalian organisms or other biota warrants the development of a qualitative rather than a quantitative method which remains effective and fit for purpose. For this reason, a qualitative method to identify 11 AR (coumachlor, coumafuryl, coumatetralyl, warfarin, brodifacoum, bromadiolone, difenacoum, flocoumafen, diphacinone, chlorophacinone and pindone) by liquid chromatography with diode array/fluorescence detection (HPLC-DAD/F) has been developed for and applied to the analysis of real samples. The combined use of DAD and F-detector permitted to set up a simple and cheap method allowing to detect simultaneously different kind of ARs (fluorescent and non fluorescent compounds) with different chemical structure (Fig. 1), unlike a previous method published before where only fluorescent detector was employed [14]. Data analysis of samples from suspected cases of poisoning collected at the Istituto Zooprofilattico Sperimentale delle Venezie in the period 2012–2013 are presented and discussed here below.

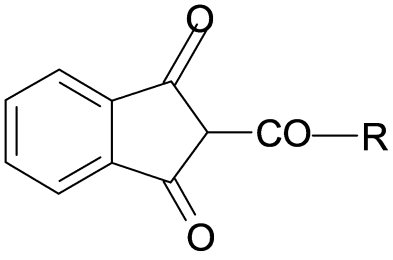

Fig. 2. Indandione derivatives.

2. Materials and methods

2.1. Reagents

Certified analytical standards of brodifacoum, bromadiolone, chloropahacinone, coumachlor, coumafuryl, coumatetralyl, difenacoum, diphacinone, flocoumafen, pindone and warfarin were purchased from Dr. Ehrenstofer GmbH (Augsburg, Germany).

HPLC-grade HiPersolv® CHROMANORM® methanol and acetonitrile and analytical grade acetone were purchased from Prolabo (VWR international, Leuven, B). Ammonium acetate (purity ≥ 98.0%) and Sodium sulphate anhydrous (purity ≥ 99.0%) were supplied by Sigma Aldrich® (St Louis, MO, USA). Distilled water was deionized by means of a Milli-Q system from Millipore™ (Bedford, MA, USA). SPE Strata Florisil FL-PR 500 mg/6 mL cartridges were supplied by Phenomenex® (Torrance, CA, USA). 589³ Blue ribbon ashless paper circles filter, 90 mm diameter, were purchased from Schleicher & Schuell GmbH (Dassel, Germany). Hydrochloric acid (HCl) 1 mol/l (0.999–1.001 N) were supplied by Carlo Erba Reagent s.r.l. (Cornaredo, Italy). Capped 15-mL polypropylene centrifuge tubes were purchased from Vakutest Kima s.r.l. (Padova, Italy).

A 1000 μg/mL stock solution in HPLC grade acetonitrile or in HPLC grade acetonitrile/methanol mixture (50/50) were prepared for each compound. Then a composite intermediate standard solution of 10 μg/mL was prepared by mixing the appropriate amounts of the individual standard solutions and diluting with ammonium acetate buffer 0.01 M and methanol (50/50).

2.2. Apparatus

Chromatographic analysis was performed on an Agilent 110 series HPLC (Agilent Technologies, Santa Clara, CA, US), equipped with a 1313A automatic injector, a G1322A degasser, a G1311A binary pump, a G1315B diode-array (DAD) and G1321A fluorescence (F) detectors.

2.3. Sample collection

In the period 2012–2013, 561 different suspect samples, ranging from baits, livers and stomach contents, were collected at IZSVe for AR analysis. Some samples were suspected to contain AR poisoning mainly due to observed clinical symptoms or to *post mortem* findings in animals; others belonged to dead animals found in areas where treatments against rodents had been conducted; others still were analyzed for AR simply because other causes of poison had been ruled out in previous analyses.

2.4. Fortified samples

For the verification of method performances twenty independent blank samples of liver, stomach content belonging to different animal species (dog, cat, fox, etc.) and twenty negative bait were fortified at 1 mg/kg chosen as Decision Point Concentration. These samples were considered as positive controls.

Table 1
Gradient conditions.

Step	Time (min)	% Phase A	% Phase B
1	1	40	60
2	20	0	100
3	25	0	100
4	26	50	50
5	30	50	50