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Determination of Diphacinone Residues in Hawaiian Invertebrates

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Abstract

A reversed-phase ion-pair liquid chromatographic analysis combined with a solid-phase extraction clean-up method is used to assess the quantity of diphacinone residue found in invertebrates. Three invertebrate species are exposed to commercially available diphacinone-fortified bait used for rat control. The invertebrate samples are collected, frozen, and shipped to the laboratory. The samples are homogenized after cryogenic freezing. A portion of the homogenized samples are extracted with acidified chloroform–acetone, followed by cleanup with a silica solid-phase extraction column. Diphacinone is detected by UV absorption at 325 nm after separation by the chromatographic system. The method limit of detection (MLOD) for snail and slug samples averaged 0.055 and 0.066 mg/kg, respectively. Diphacinone residues in snail tissue ranges from 0.83 to 2.5 mg/kg for Oxychilus spp. The mean recoveries from snails at 0.20 and 2.0 are 97 ± 21% and 84 ± 6%, respectively. Diphacinone residues in slug tissue ranges from 1.3 to 4.0 mg/kg for Deroceras laeve and < MLOD to 1.8 mg/kg for Limax maximus, respectively. The mean recoveries from slugs at 0.20 and 2.0 mg/kg are 91% ± 15% and 86% ± 5%.

Introduction

Diphacinone [2-(Diphenylacety1)-1H-indene-1,3(2H)-dione] is a registered late-first generation or early-second generation anticoagulant rodenticide commonly used to control populations of rats and mice in urban areas. This anticoagulant is also effective in the control of other rodents such as pocket gophers (Thomomys bottae), Beldings’ ground squirrels (Spermophilus beldingi), and California ground squirrels (Spermophilus beecheyi) in rangeland rodents. The acute oral toxicity of diphacinone for rats is approximately 2 mg/kg as compared with the acute oral toxicity for other anticoagulants such as warfarin and pindone, which are approximately 59 mg/kg.

Rats (Rattus spp.) on the Hawaiian Islands are a nonindigenous species that have impacted the native ecosystems. On the Hawaiian Islands, as well as other islands, rats have contributed to the extinction of indigenous flora and fauna (1). Control methods being studied for rats in remote areas include the broadcast application of acute toxicants, including anticoagulants such as diphacinone (2). In remote areas, broadcast application of rodenticide baits has been shown to effectively control rat populations (3). In humid environments such as Hawaii, the use of all weather rodenticide baits usually consist of grain-fortified (at 0.005% w/w) diphacinone encapsulated in wax or pressed with oil.

Invertebrates (snails and slugs) have been observed to consume portions of rodenticide baits in bait stations and baits that were located on forest floors in treated areas (2). When considering the use of pesticides to control problem species, the risk of secondary toxicity to nontarget species such as birds must be evaluated. Therefore, the objectives of this study were to determine if snails and slugs consume enough bait to accumulate measurable quantities of diphacinone and, if so, to determine if the detected residues of diphacinone are significantly high enough to pose a risk to avian species that consume these invertebrates.

An analytical method to assess the levels of diphacinone residues in snails and slugs exposed to 0.005% diphacinone-fortified baits was developed. Typically, liver and serum are analyzed for residues of anticoagulants, as they accumulate and are metabolized in the liver. Obviously, this is not possible for snails and slugs because of the small body mass of individual animals. For all three species of invertebrates, whole body diphacinone residues were determined, and multiple numbers of each species were ground together into composite samples.

Several methods have been developed for analysis of indanediones (Figure 1) in baits, formulations, and tissues. A gas chromatographic method with derivatization (4) is sensitive and selective but suffers from low recoveries and is time consuming. Spectrophotometric methods (5,6) have been utilized for baits and formulations, but they are not selective when assaying multi-residue samples. Thin-layer chromatography (7–9) methods are not suited for determining low levels of residues in complex matrices such as plant and animal tissues. Reversed-phase (RP) high-performance liquid chromatography (HPLC) methods (10–14) provide sufficient sensitivity but often produce poor chromatographic resolution for the indandiones. Ion-pair RP-HPLC (15–20) for diphacinone (Figure 1) using tetrabutylammonium ion pairing reagent is sensitive and selective, but column lifetime is often short because of adsorption of the ion-pairing reagent onto the stationary phase of the column packing material. For this study, ion-pair RP-HPLC was used because good chromatographic...
resolution can be achieved, and column lifetime can be extended with regular column washing. Sample extraction utilized a normal-phase solid-phase extraction (SPE) sample cleanup with a silica column with an automated SPE workstation.

Experimental

Sample collection

Whole snails (*Oxychilus spp*) and slugs (*Deroceras laeve* and *Limax maximus*) were collected and placed in individual plastic bags, sealed, labeled, and frozen in a freezer at -12°C. The samples were stored in a freezer until shipped to our laboratory where they were stored in freezers at -12°C until homogenized and assayed. Method validation and analyses were completed under U.S. Environmental Protection Agency Good Laboratory Practice guidelines (40 CFR 160) (21).

Reagents

Chloroform, hexane, ethyl acetate, and methanol were liquid chromatography grade reagents (Fischer Scientific, Denver, CO). Deionized water was purified using a reverse-osmosis water purification system (U.S. Filter Corp., Schaumburg, IL). Concentrated phosphoric acid (Fischer Scientific) was used to prepare the aqueous 1.33M phosphoric acid solution. The extraction solution of 1.7% (v/v) formic acid in acetone–chloroform (1:1) was prepared by mixing 20 mL of concentrated (88%) formic acid (Fischer Scientific) with 500 mL of acetone and 500 mL of chloroform. Anhydrous sodium sulfate (Fischer Scientific) was mixed with tissue samples to remove water.

Diphacinone (98.9%) was obtained from Hacco (Madison, WI). Concentrated stock standards of diphacinone were prepared by first drying the technical-grade compound for 4 h at 110°C, then dissolving 10,000 mg in 10.0 mL of ethyl acetate. Working standards, ranging in concentration from 0.050 to 2.3 mg/mL, were prepared by dilution of stock solutions with mobile phase. All standard solutions were stored in a refrigerator at 5°C.

Tetrabutylammonium dihydrogen phosphate (97%) was purchased from Aldrich (Milwaukee, WI) and used to prepare a 5mM solution in methanol. An aqueous solution of 5mM tetrabutylammonium dihydrogen phosphate with 50mM potassium dihydrogen phosphate buffer (Alltech, Inc., Deerfield, IL) was prepared.

Sample preparation

Sample homogenization and extraction

Approximately 2 to 10 g were frozen and homogenized with a Spex Centiprep 6850 freezer mill (Metuchen, NJ) (22). It required approximately 30 to 50 snails and 2 to 4 slugs to attain a 10-g sample. The samples were transferred to a polycarbonate cylindrical vessel that holds a metal rod. Each end of the cylindrical container was capped with a metal lid. The sample and container were placed in the freezer mill sample holder with liquid nitrogen and frozen over a 5-min period. The sample was homogenized by forcing the metal rod back and forth magnetically at 10 cycles/s between the metal end caps. This is typically done for approximately 1 to 2 min for 2 to 3 periods. The powdered, frozen snail or slug sample was transferred to a 20-mL glass amber sample jar. For snail samples, the soft tissue was not removed from the shell of the animal. Samples were stored at -12°C until assayed.

Homogenized tissue samples were weighed (0.500 to 0.550 g) into a 25-mL glass tube, and 5 g of anhydrous sodium sulfate was added. All samples were analyzed in duplicate if sufficient sample mass was available. The tissue and sodium sulfate were vortex mixed together for 10 s. A 15-mL aliquot of the extraction solution was added to each sample and vortex mixed for 5 s. The samples were shaken horizontally on a mechanical shaker (Eberbach, Ann Arbor, MI) at high speed (56 displacements/min) for 10 min. The samples were placed in an ultrasonic bath (power level of 150 W) for 10 min (a beaker partially filled with water was used to hold the tubes). Sample tubes were centrifuged at approximately 1000 × g for 5 min.

The extract was transferred to a second 25-mL glass tube. The extraction was repeated twice with two subsequent 10-mL additions of extraction solution. The solvent in the extract was removed by placing the tubes in a warm water bath (60°C) and allowing nitrogen gas to flow over the surface of the extract until no solvent remained. The residue was reconstituted with 2.0 mL of chloroform, gently vortex mixed, and placed in an ultrasonic bath for 10 min. This was followed by the addition of 3.0 mL of hexane. This solution was vortex mixed and placed in an ultrasonic bath for 10 min. The reconstituted samples were filtered through a 0.45-μm Teflon syringe filter (30 mm, National Scientific Co., Part #P2500-3) into a 10-mL glass tube. A 1-mL portion of chloroform and a 1-mL portion of hexane was used to rinse the sample tube, filtered through the syringe filter, and added to the extract in the 10-mL glass tube.

Analyte concentration

The SPE procedure was completed using a Zymark RapidTrace automated workstation (Hopkinton, MA). Each aminoxypropyl SPE (500 mg sorbent in a 3-mL column) cartridge was conditioned with approximately 3 mL of hexane–chloroform (2:1). The packing material was not allowed to dry. The reconstituted sample extract (6.5 mL) was passed through the column at 2 mL/min. The eluate was discarded to waste. Each SPE column was rinsed by adding a 3-mL aliquot of hexane–chloroform (2:1),
Quality control samples and fortification of controls

Snails and slugs were collected by National Wildlife Research Center staff members at the Hilo Field Station in Hawaii prior to the beginning of any experiments. These control invertebrates were processed and screened for diphacinone prior to compositing of control samples. Control samples were fortified at 0.20 and 2.0 mg/kg diphacinone with aliquots of fortification standards of diphacinone in ethyl acetate. The quality control samples were then assayed with the method described previously.

Results and Discussion

Response linearity

Two sets of 6 diphacinone standard solutions were prepared, ranging from 0.050 to 2.3 mg/L. Data were collected from duplicate injections of each solution and a plot was constructed of analyte peak area (y-axis) versus diphacinone concentration (x-axis). A linear regression was performed on the data set and produced an $r^2 = 0.9988$. The plot of log (peak area) versus log (diphacinone concentration) produced a slope of 1.00608 and an $r^2 = 0.9981$. The average response factor over the range of the calibration curve produced a coefficient of variation of 4.1%. A linear and proportional relationship exists between chromatographic peak area and diphacinone concentration. Single-point calibration to calculate the concentration of diphacinone in the sample extracts was considered valid.

Method limit of detection

The method limit of detection (MLOD) was calculated as the concentration of diphacinone required in the sample to generate a signal equal to three times the baseline noise (peak-to-peak) observed in the chromatogram of the control extract. The MLOD was estimated from the chromatographic response in height of a signal equal to three times the baseline noise. The MLOD for snail and slug tissue samples was 0.055 and 0.066 mg/kg, respectively. For the chromatographic parameters chosen, the retention time of diphacinone was approximately 23.5 min, as shown in Figure 2B. No significant chromatographic response was noted at the retention time of diphacinone was approximately 23.5 min, as shown in Figure 2B.
tion time of diphacinone in the chromatogram of the control tissue extract (Figure 2A). Chromatograms of slug and snail extracts were virtually identical.

SPE clean-up
Based on the polar nature of the analyte and the solubility of the analyte in intermediate polar solvents, an SPE clean-up was attempted by the adsorption of the analyte on aminopropyl (NH₃), 2,3-dihydroxypropyl, strong anion exchanger (SAX), florisil, and silica SPE columns. All the SPE columns were conditioned and loaded with combinations of chloroform–hexane solutions. The analyte was only partially retained on the SAX, silica, and florisil SPE sorbents during the loading steps. The only SPE sorbent to retain greater than 85% to 90% of the analyte in the presence of matrix during the loading and washing procedure were the NH₃ and silica sorbents. During method development, the NH₃ sorbent proved more reproducible and typically yielded 10% to 15% higher recoveries for diphacinone in this matrix. The analyte was only partially eluted with methanol but was completely eluted from the NH₃ sorbent with the methanolic ion-pairing reagent. The NH₃ sorbent was adopted as the SPE column of choice for the remainder of the method.

Diphacinone residues in snails and slugs
Mean recoveries of snail (n = 15) and slug (n = 16) quality control samples were 90 ± 16% and 89 ± 13% (Table II). Diphacinone residues were calculated as the average of duplicate analyses of samples, when available. When duplicate analyses differed by more than 25%, a third replicate was analyzed. The reported residue concentration was then calculated as the mean of the three replicates when this occurred. Diphacinone residues in snail and slug tissue of the three species analyzed ranged from < MLOD to 4.00 mg/kg (Table III) with a mean value of 1.68 mg/kg. In comparison, diphacinone residues (all values reported are means) determined from carcasses of species exposed to diphacinone baits have ranged from 0.52 and 1.1 mg/kg for California ground squirrels (22,23), 4.4 mg/kg for black rats (24), 2.25 mg/kg for house mice (24), and 0.40 mg/kg for pocket gophers (25).

The primary wavelength for quantitative analysis was 325 nm, though absorption at 285 nm was also recorded. The ratio of absorbance at 285 and 325 nm was used to qualitatively confirm the presence of the analyte. The molar absorptivity of diphacinone at 285 nm was typically between 1.9 and 2.1 greater than the molar absorptivity at 325 nm. Observation of the UV–vis spectra of diphacinone was also a useful tool to qualitatively confirm the presence of any indandione. As shown in Figure 3, the spectrum is unique enough to confirm the presence of diphacinone. This is the spectra of the diphacinone peak from the slug extract chromatogram in Figure 2B.

Conclusion
The methodology developed for snail and slug tissue analysis proved to be reliable, efficient, and simple. The same method was used to determine the diphacinone residues from three different invertebrate species. In estimating potential secondary hazards for proposed use of indandione rodenticides, it is critical to have analytical methods available to collect the necessary data to evaluate the risk to nontarget species.

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| Table II. Analytical Recoveries of Diphacinone in Snail and Slug Tissues |
|-----------------|-----------------|-----------------|
| **Fortification** | **Species** | **Range (%)** | **Mean (%)** | **Standard deviation (%)** | **CV (%)** |
| levels (mg/kg)   |                 |                |               |                           |            |
| 0.20 (n = 7)    | Snails          | 78–140         | 97            | 21                         | 22          |
| 2.00 (n = 8)    | Snails          | 74–92          | 84            | 5.6                        | 6.7         |
| 0.20 (n = 8)    | Slugs           | 72–120         | 91            | 15                         | 16          |
| 2.00 (n = 8)    | Slugs           | 79–95          | 86            | 4.9                        | 5.7         |

| Table III. Residues of Diphacinone in Snail and Slug Tissues |
|-----------------|-----------------|-----------------|
| **Species**     | **Range of residue concentration (mg/kg)** | **Mean residue concentration (mg/kg)** |
| Snails          | O.15–3.15 (N = 28) | 0.832–2.47 | 1.77          |
| Slugs           | L. maximus (n = 19) | < MLOD–1.80 | 0.806         |
|                | D. laeve (n = 15) | 1.30–6.00 | 2.64          |

* To calculate the mean residue for samples reported as < MLOD, the MLOD was used as the value for these samples. Residue values have not been corrected for recovery.

Figure 3. UV–vis spectra of diphacinone from the extract shown in Figure 2.
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