Reversed-Phase Ion-Pair High-Performance Liquid Chromatographic Quantitation of Difethialone Residues in Whole-Body Rodents with Solid-Phase Extraction Cleanup

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A reversed-phase ion-pair high-performance liquid chromatographic method was developed for the determination of difethialone residues in laboratory rats (*Rattus norvegicus*). Difethialone was extracted from rat tissue with chloroform/acetone/formic acid. The extracts were cleaned up by a solid-phase extraction (SPE) procedure using both silica and aminopropyl SPE sorbents, concentrated, and analyzed by reversed-phase ion-pair high-performance liquid chromatography. Difethialone was quantitated via ultraviolet absorbance at 262 nm. A surrogate compound, brodifacoum, was used to correct for method performance. The mean surrogate-corrected recoveries for whole ground rodent fortified at 0.2, 1.0, and 20 μg/g difethialone were 92.3 ± 7.7, 84.8 ± 6.6, and 90.2 ± 3.1%, respectively. The method limit of detection was 0.054 μg/g.

Keywords: *Difethialone; secondary hazard; rodenticide; anticoagulant; solid-phase extraction (SPE)*

INTRODUCTION

Difethialone is a second-generation anti-vitamin K anticoagulant rodenticide of the chemical class hydroxy-4-benzothiopyranones (Lechevin and Poché, 1988; Marshall, 1992). Difethialone has LD_{50} values between 0.29 and 0.51 mg/kg in three wild warfarin-resistant and nonresistant rodent species (Lechevin and Poché, 1988). Baits containing 0.0025% difethialone are registered with the U.S. Environmental Protection Agency for the control of rodents in and around buildings in urban and nonurban settings. A registration for broadcast application of difethialone-treated baits for the control of field rodents in agricultural settings is being pursued. One requirement of this registration (EPA Pesticide Assessment Guidelines Subdivision E: Wildlife and Aquatic Organisms) is a secondary hazard study to determine the risk to nontarget species consuming the carcasses of target rodent species.

The National Wildlife Research Center conducted a secondary hazard study under contract with LiphaTech, Inc. (Milwaukee, WI). A vital portion of the secondary hazard study was the quantitation of difethialone residues in the carcasses of the target rodent species. Rats were fed a diet of difethialone-treated baits and their carcasses analyzed for difethialone residue levels. The half-lives of ^{14}C-labeled difethialone in livers and serum of laboratory rats were 108 and 2.3 days, respectively (Lechevin and Poché, 1988). Even though anticoagulants accumulate in the liver of the target species, it is possible that difethialone residues in other portions of the carcass pose a secondary hazard to wildlife. This required that the entire body of the target animal be analyzed for difethialone residues. The residue levels observed will be used to determine bait fortification levels for feeding to scavenger species in later stages of the secondary hazard study.

MATERIALS AND METHODS

Apparatus. An SPE manifold (Jones Chromatography, Lakewood, CO) was used for the SPE cleanup steps. A horizontal shaker (Eberbach, model 6550, Ann Arbor, MI) was used to agitate the samples. A centrifuge (Fisher Scientific, Denver, CO) was used to separate the liquid extracts from the samples. A water bath (N-Evap, model 115, South Berlin, MA) was used to evaporate samples.

Reagents. Acetone, chloroform, ethyl acetate, ethyl ether, hexane, 2-propanol, and methanol were of liquid chromatography grade. Formic acid and glacial acetic acid were of high purity grade. Concentrated phosphoric acid was used to make the 4 N phosphoric acid in water solution. Reagent grade ascorbic acid was used to make the aqueous solution of 1.0 M ascorbic acid. A solution of 50% ammonium hydroxide in water (Fisher Scientific) was used to make the 5% ammonium hydroxide in methanol solution. Deionized water was purified using a Milli-Q water purification system (Millipore, Bedford, MA).

Technical grade difethialone (100.45%, LiphaTech) and brodifacoum (98%, ICI Agrochemicals, Kent, England) were dried for 4 h at 110 °C. The purity of the difethialone was certified by comparing the response factors produced from three separate lots. A concentrated stock solution of difethialone was prepared by dissolving 2.5 mg in 25 mL of methanol. A concentrated stock solution of brodifacoum was prepared by dissolving 10 mg in 10 mL of acetone. Working standards that ranged from 0.1 to 30 μg/mL were prepared by adding 500 μL of brodifacoum stock solution to a 10-mL volumetric flask and evaporating the acetone under a gentle stream of nitrogen. An appropriate aliquot of difethialone concentrated stock solution was then added to the flask and diluted to volume with mobile phase. All standard solutions were stored at 5 °C.

An extraction solvent was prepared by mixing 500 mL of chloroform with 500 mL of acetone and 5 mL of concentrated formic acid in a 1000-mL graduated cylinder. The solution was mixed by mechanical stirring and stored in a glass bottle until used.

Tetrabutylammonium dihydrogen phosphate (97%, Aldrich, Milwaukee, WI) was used to prepare a 5 mM solution in

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methanol. An aqueous solution was also prepared that contained a 0.10 M potassium dihydrogen phosphate buffer.

Procedures. *Fortification of Controls.* The head, feet, tail, and pelt were removed from five euthanized control white laboratory rats (*Rattus norvegicus*). The carcasses were ground with a variable-speed batch sample processor (Robot Coup U.S.A., model RSI 6V, Jackson, MS), and the resulting ground tissue was stored at −5 °C in plastic freezer bags. Portions (2.00−2.10 g) of tissue were fortified with 40 or 200 μL of a 10 μg/mL standard solution or 400 μL of a 100 μg/mL standard solution in acetone to produce the 0.2, 1.0, and 20 μg/g difethialone fortification levels.

Extraction Procedure. Ground tissue was accurately weighed in 2.0-g portions into 50-mL screw-cap glass tubes. A 100-μL aliquot of 100 μg/mL brodifacoum in acetone solution was added to each sample tube. The acetone was evaporated at room temperature under a gentle stream of nitrogen for 10 min. Each sample was acidified by the addition of 100 μL of 1.0 M ascorbic acid solution. The sample tubes were vortex mixed for 30 s and then allowed to stand for 5 min. Between 10.0 and 10.5 g of sodium sulfate (Fisher Scientific) was added to each sample tube and vortex mixed for 30 s, and then 15.0 mL of extraction solvent was added immediately to each sample tube. The mixture was vortex mixed for 30 s and placed on a horizontal shaker (Eberbach, model 6550) at high speed for 20 min. The sample tubes were then centrifuged (Fisher, Centrific centrifuge) at ≈2500 rpm for 5 min. The extracts were decanted into a 30-mL plastic syringe fitted with a 0.45-μm Teflon syringe filter (Scientific Resources Inc., Eatontown, NJ). The syringes were positioned over clean 50-mL screw-cap glass tubes and allowed to filter by gravity. The extraction procedure was repeated with two additional 10-mL aliquots of extraction solvent. The extracts were combined in the syringes, and the remaining extract solution was forced through the filter by applying pressure to the plunger. The filtered extracts were placed in a 65 °C water bath (N-Evap, model 115) and the solvent was evaporated under a gentle stream of nitrogen. The samples were redissolved in 10.0 mL of hexane and vortex mixed for 30 s. The sample tubes were then placed in a sonicating bath (Sonicor, model SC-100, Copiague, NY) for 15 min. Finally, the samples were mixed using the vortex mixer for 30 s.

SPE Cleanup (Phase I). A 2-g IST SiO_2 (silica) SPE column with a 6-mL reservoir (Jones Chromatography) was conditioned for each sample by eluting with 5 mL of hexane. Without allowing the packing material to go dry, the reconstituted extracts were loaded onto the conditioned columns via gravity. Each column was rinsed with 10 mL of hexane followed by 10 mL of 20% ethyl ether in hexane solution. The wash eluate was discarded. The analytes were eluted from each column with 22.5 mL of 90% ethyl ether in hexane, in 2.5-mL increments. After the final aliquots passed through the columns, gentle vacuum was applied to remove all of the eluting solvent.

Each sample tube was placed under a gentle stream of nitrogen at room temperature until approximately half of the solvent had evaporated. Tubes were then immersed in a water bath at 65 °C and evaporated to dryness under a gentle stream of nitrogen. The samples were redissolved in 5.0 mL of hexane and vortex mixed for 30 s. After the sample tubes were placed in a sonicating bath for 15 min, they were vortex mixed for 30 s.

SPE Cleanup (Phase II). A 1-g IST NH_2 (aminopropyl) SPE column with a 6-mL reservoir (Jones Chromatography) was conditioned by the addition of 2.5 mL of hexane. The reconstituted extracts were loaded onto the conditioned SPE columns via gravity. Each column was sequentially washed with 5 mL of hexane, 10 mL each of 2:1 chloroform/2-propanol, chloroform, and ethyl acetate, and 6 mL of 2% acetic acid in ethyl ether. The washings were discarded. The analytes were eluted from the columns by addition of four 2-mL aliquots of 5% ammonium hydroxide solution in methanol. After the final aliquot had passed through the columns, gentle vacuum was applied to remove any remaining solvent.

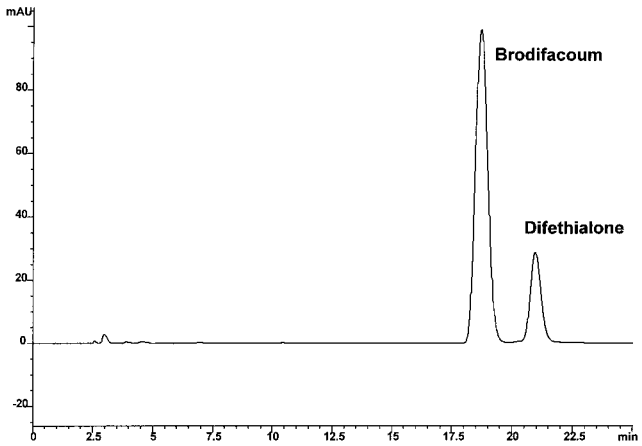

Figure 1. Chromatogram of a standard solution containing 5 μg/mL brodifacoum and 1 μg/mL difethialone.

The eluate from each SPE column was placed in a 65 °C water bath and evaporated to dryness under a gentle stream of nitrogen. The sample residues were reconstituted with 2.00 mL of mobile phase and sonicated for 15 min. The reconstituted samples were filtered through a 0.45-μm Teflon filter prior to injection into the HPLC.

High-Performance Liquid Chromatography (HPLC). The HPLC system consisted of a Hewlett-Packard 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a column oven and a diode array detector. Injections of 100 μL were made automatically by the pneumatically controlled injector valve. Analyte separation was achieved on a 250 mm × 4.6 mm i.d. stainless steel analytical column packed with 5-μm Keystone ODS/H (Keystone, Bellefonte, PA). The column temperature was 40 °C, and the diode array detector was monitored at 262 nm. The analytical column was fitted with a guard column of the same packing material. The system was operated at a flow rate of 1.0 mL/min. The mobile phase consisted of 77% 5 mM tetrabutylammonium dihydrogen phosphate in methanol and 23% 5 mM tetrabutylammonium dihydrogen phosphate with 0.1 M dihydrogen phosphate buffer in water. The mobile phase was premixed, and the pH was adjusted to 8.5 by the addition of 4 N phosphoric acid. It was filtered through a 0.45-μm nylon membrane filter (Alltech Associates, Inc., Deerfield, IL) and degassed by sparging with helium prior to use. Following each set of sample analyses the HPLC system was flushed with a mixture of 1:1 methanol/water for 40 min at a flow rate of 1.0 mL/min.

Brodifacoum and difethialone were quantitated by monitoring the UV absorbance at 262 nm and comparing the ratio of difethialone to brodifacoum response to the ratio of responses observed in a calibration standard. The retention times of brodifacoum and difethialone were approximately 18 and 20 min, respectively, for the conditions listed above. A chromatogram of a standard solution can be seen in Figure 1.

RESULTS AND DISCUSSION

Few methods have been reported for the analysis of anticoagulant rodenticide residues in whole body rodent (Koubek et al., 1979; Odam and Townsend, 1976). Methods exist for serum (Breckenridge et al., 1985; Corn and Berberich, 1967; de Vries and Schmitz-Kummer, 1994; Felice and Murphy, 1989; Kaiser and Martin, 1974; Kieboom and Rammell, 1981; Lamiable et al., 1993; Lee et al., 1981; Midha et al., 1974; Mundy and Machin, 1977, 1982; Mundy et al., 1976; O'Bryan and Constable, 1991), liver (Hoogenboom and Rammell, 1983; Hunter, 1983a,b, 1984; 1985; Hunter and Sharp, 1988; Hunter et al., 1988; Jones, 1996; Kieboom and Rammell, 1981; Langseth and Nymoen, 1991; Mundy and Machin, 1977, 1982; Mundy et al., 1976; Odam and

Townsend, 1976; O'Bryan and Constable, 1991; Ray et al., 1989), muscle (Hoogenboom and Rammell, 1983; Hunter, 1984; Mundy and Machin, 1982), and fat (Hoogenboom and Rammell, 1983). These methods employ a variety of analytical detection methods. Gas chromatographic methods lacked the required sensitivity and precision (Kaiser and Martin, 1974; Midha et al., 1974; Odam and Townsend, 1976). Thin-layer chromatographic methods are not well suited to quantitation of residues (Caissie and Mallet, 1976; Mallet et al., 1973, Opong-Mensah and Porter, 1988). Spectrophotometric methods exist for anticoagulants but lack specificity in the presence of large quantities of matrix components (Caswell, 1959; Corn and Berberich, 1967; Kawano and Chang, 1980). Reversed-phase HPLC methods are sensitive, but chromatographic resolution tends to be poor (Breckenridge et al., 1985; de Vries and Schmitz-Kummer, 1994; Felice and Murphy, 1989; Hoogenboom and Rammell, 1983; Hunter, 1983a; Hunter et al., 1988; Jones, 1996; Kieboom and Rammell, 1981; Koubek et al., 1979; Lamiable et al., 1993; Langseth and Nymoen, 1991; Lee et al., 1981; Mundy and Machin, 1977, 1982; Mundy et al., 1976; O'Bryan and Constable, 1991; Ray et al., 1989; Yeun, 1978). Ion-pair reversed-phase HPLC has been used for a number of anticoagulant rodenticides and has proven to be adequately selective and sensitive (Hunter, 1983b, 1984, 1985; Hunter and Sharp, 1988; Hunter et al., 1988). Additionally, washing the column overcomes the shortened column lifetime associated with the use of ion-pair chromatographic methods by removing a significant portion of the ion-pair reagents from the column.

A wide variety of cleanup procedures have been cited for the extraction of anticoagulants in various matrices. Gel permeation chromatography (GPC) provides adequate cleanup of the sample but is time-consuming and requires large quantities of solvents (Hunter, 1983a,b). A method has also been reported that uses a combination of GPC and Sep-Pak SPE cartridges (C_{18}), but this method also requires large volumes of solvents (Hunter, 1984, 1985; Hunter and Sharp, 1988; Hunter et al., 1988; Koubek et al., 1979). Liquid−liquid extractions have been used for plasma and urine samples but proved to be unreliable due to the solubility of difethialone in a variety of solvents (Breckenridge et al., 1985; Caswell, 1959; Corn and Berberich, 1967; Felice and Murphy, 1989; Kaiser and Martin, 1974; Lamiable et al., 1993; Lee et al., 1981; Midha et al., 1974; Mundy et al., 1976; O'Bryan and Constable, 1991). SPE has been successfully employed for the cleanup of anticoagulant rodenticides in various matrices (de Vries and Schmitz-Kummer, 1994; Hoogenboom and Rammell, 1983; Jones, 1996; Kawano and Chang, 1980; Kieboom and Rammell, 1981; Langseth and Nymoen, 1991; Mundy and Machin, 1982; Ray et al., 1989; Yuen, 1978). We found all of these methods to provide insufficient cleanup for our purposes. In addition, the above methods focus on coumarin- and indanedione-derived anticoagulants and are not entirely applicable to the benzothiopyranone, difethialone. To overcome these limitations, we developed a two-step SPE cleanup procedure that utilizes silica and aminopropyl sorbents.

Response Linearity. Two sets of five difethialone standard solutions were prepared and injected into the HPLC in duplicate. The concentrations of the solutions ranged from 0.10 to 32 μg/mL difethialone. Each standard solution also contained 5 μg/mL brodifacoum.

Table 1. Results of Linear Regression Analysis

data set	R^2	slope	Y-intercept
area responses	1.0000	254.52	2.370
ratio of area responses	1.0000	0.2298	0.004454

A plot was constructed of difethialone chromatographic peak response versus difethialone concentration. A second plot was constructed of the ratio of difethialone peak response to brodifacoum peak response versus difethialone concentration. A linear regression was performed on each data set. The regression statistics are shown in Table 1. A linear relationship was found to exist between the peak responses or the ratio of peak responses and difethialone concentration in both cases. The response was directly proportional to concentration over the range of interest.

Extraction. Solutions of acetone and chloroform have previously been used to extract anticoagulant residues from tissue samples with good results (Hunter, 1983a,b, 1984, 1985; Hunter et al., 1988; Jones, 1996); therefore, these were logical choices for difethialone and brodifacoum. Ascorbic acid was added directly to the sample prior to extraction, and the extraction solvent was acidified to keep heme in its reduced state. It has been reported that the oxidized form of heme binds anticoagulant rodenticides leading to reduced analyte recoveries (Schulert and Weiner, 1953). Preliminary experiments demonstrated that solutions lacking these acids yielded significantly reduced recoveries compared to the corresponding acidified samples.

Sodium sulfate was used to dry the samples. An excess amount was used to ensure complete absorption of water from the samples. Experiments have shown that too little sodium sulfate resulted in a cloudy hydrophilic layer that yielded reduced analyte recoveries.

SPE Cleanup. The use of two separate SPE cartridges was required for several reasons. Although the NH_2 (aminopropyl) SPE column is the primary column for sample cleanup, it could not be used without the SiO_2 (silica) column being used first. The SiO_2 column acted as a precleanup step, removing a significant portion of lipophilic material that would have interfered with the aqueous mobile phase. Since a large portion of the sample matrix was composed of lipid material, a process that would remove these components while leaving the analytes behind was essential. Samples processed without the use of the SiO_2 column yielded a biphasic end product that gave extremely poor separation and low, variable analyte recoveries.

In addition to the removal of a significant portion of the lipophilic material, the SiO_2 column also retained some of the more polar matrix components that would have interfered with the analyte's binding to the stationary phase of the NH_2 column. With the SiO_2 column removing these components, more active sites became available on the NH_2 column. This measure greatly increased the separation efficiency of the NH_2 column. The benefits of this two-step cleanup procedure are clearly observed from chromatograms of extracts obtained with (Figure 2B) and without (Figure 2A) the SPE cleanup procedure. The SPE cleanup procedure reduced the amount of matrix components introduced into the chromatographic system, thereby greatly reducing the replacement of guard columns.

Recoveries. The mean surrogate-corrected recoveries of difethialone at the 0.2, 1.0, and 20 μg/g fortification

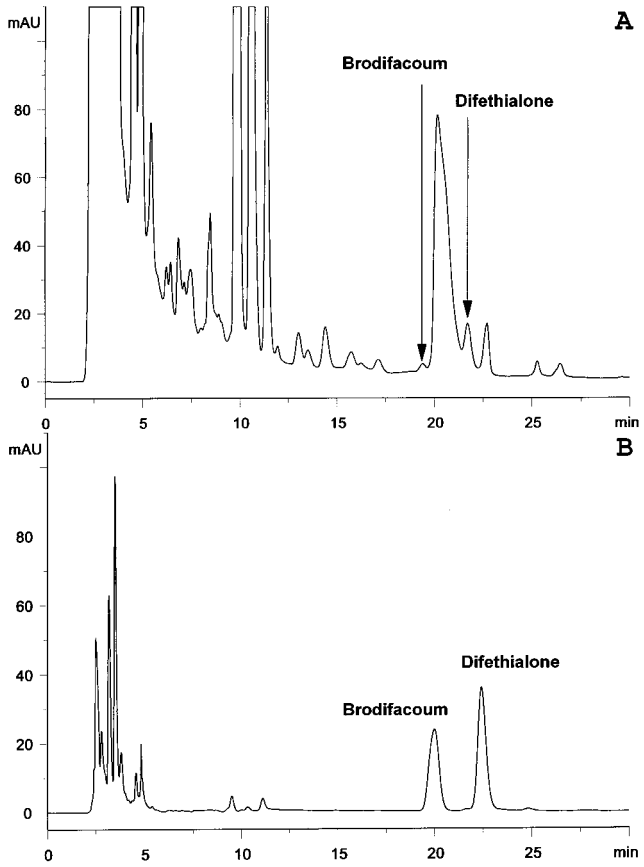

Figure 2. Chromatograms of a fortified rodent tissue that was not subjected to the cleanup procedure (A) and a corresponding fortified tissue that was subjected to the cleanup procedure (B).

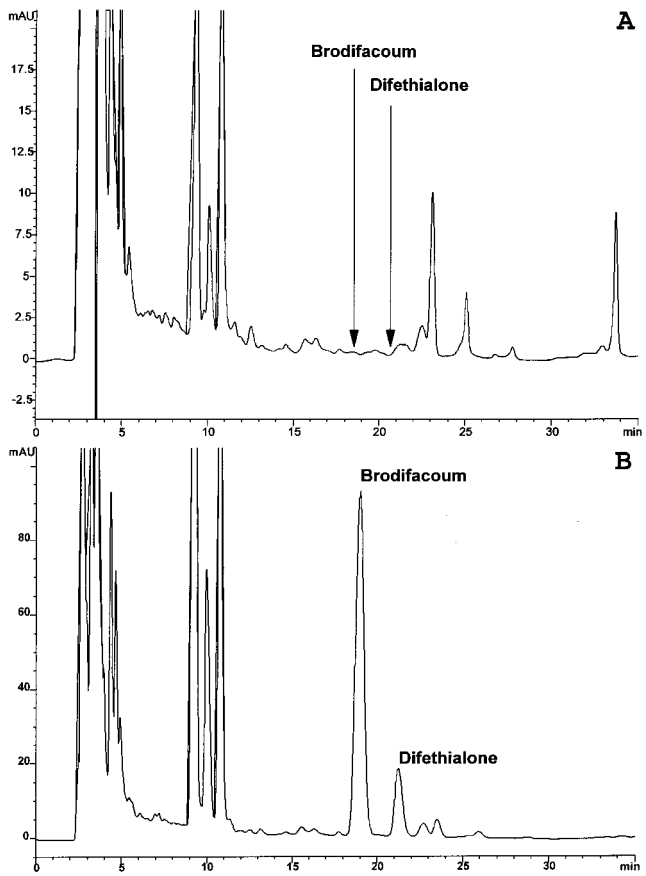

Figure 3. Chromatograms of a control rodent tissue (A) and a rodent tissue fortified at 1 μg/g (B).

levels were 92.3 ± 7.7, 84.8 ± 6.6, and 90.2 ± 3.1%, respectively. Seven replicates were analyzed at each fortification level. The analyses were performed over a 12-day period with no statistically significant difference in recoveries observed from day to day. Control tissue samples were treated according to the procedures previously outlined in this method. A representative chromatogram is shown in Figure 3A. A slight chromatographic interference was observed at the retention time of difethialone; however, this did not significantly affect method performance. A chromatogram of a tissue sample fortified at 1.03 μg/g is shown in Figure 3B.

Method Limit of Detection (MLOD). The MLOD was estimated from the chromatographic responses of five control tissue extracts and seven tissues fortified at the 0.2 μg/g level. The MLOD was calculated as the concentration of difethialone required to generate a signal equal to 3 times the baseline noise (measured peak-to-peak) plus the peak height of the chromatographic interference observed in the control tissue chromatograms. The MLOD was 0.054 μg/g.

Conclusion. The use of a two-step SPE cleanup was demonstrated to be an effective technique for the determination of difethialone residues in rat tissue. This is the first reported use of a two-step cleanup using both silica and aminopropyl sorbents. It is also the first reported use of SPE for the determination of difethiaone residues.

Most importantly, the use of this method will enable researchers to estimate the secondary hazard associated with the use of difethialone as a rodenticide and permit regulatory agencies to make an informed decision regarding the registration of this new pesticide.

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