Multiresidue Analysis of Nine Anticoagulant Rodenticides in Serum

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A rapid procedure was developed for the determination of nine anticoagulant rodenticides in animal serum by high-performance liquid chromatography (HPLC) with a fluorescence and photodiode array detector (DAD). The anticoagulants coumafuryl, pindone, warfarin, coumachlor, diphacinone, chlorphacinone, bromadialone, brodifacoum, and difethialone were extracted and quantitated simultaneously with this method. Anticoagulants were extracted at pH 5.5 with 5% (v/v) ethanol in ethyl acetate and 1% (w/v) trichloroacetic acid. Sample extracts were subjected to a cleanup on a Florisil solid-phase extraction (SPE) column. Separation was performed with a reversed phase amine-deactivated C_{18} column with a gradient of phosphate buffer, acetonitrile, and methanol. The eluent was monitored with the fluorescence detection at an excitation wavelength of 310 nm and an emission wavelength of 390 nm, and the photodiode array detection was set to 325 nm. Recoveries from spiked serum samples at ranges of 0.004−1 ppm were between 73 and 105%. The precision (CV) data range was between 1.6 and 15.6%. Detection limits were between 0.005 and 0.002 ppm for the fluorescence detection and between 0.020 and 0.050 ppm for the photodiode array detection at 325 nm.

Keywords: *Anticoagulants; rodenticides; HPLC; DAD; analysis; fluorescence detector*

INTRODUCTION

Anticoagulant rodenticides are commonly used to control mice and rat populations. However, poisoning of nontarget animals such as dogs, cats, and wildlife also occurs. The poisoning of these animals arises from ingesting either the baits or the poisoned animals themselves (Mount, 1988). The residues of anticoagulant rodenticides in the serum of poisoned animals are very low because animals may not show any signs of poisoning until a hemorrhage occurs, which will occur only after the clotting factors are depleted (Mount, 1988). The modes of action for all anticoagulant compounds are similar as they block vitamin K epoxide reductase, which is needed to reduce to vitamin K epoxide. Reduced vitamin K epoxide is essential in the biosynthesis of clotting factors II, VII, IX, and X (Mount, 1988). Diagnosis of such poisoning cases has led to a requirement for a suitable analytical method for the diagnostic laboratory. A comprehensive multiresidue method with high sensitivity and specificity will have advantages in aiding the diagnosis and treatment of anticoagulant rodenticide poisoning.

Anticoagulant rodenticides are classified as 4-hydroxycoumarins and indandiones. The differences in chemical structures present a difficulty in high-performance liquid chromatography (HPLC) determination of anticoagulants, as indandiones do not fluorescence. A variety of techniques have been used for the determination of anticoagulants (Berny et al., 1995; Bullard et al., 1975; Daenens et al., 1971; Rengel et al., 1993; De Vries et al., 1991); nevertheless, their chemistry still renders HPLC the most suitable technique.

Several HPLC methods have been reported for the analysis of anticoagulant rodenticides in biological samples, but most of them have focused on single-compound analysis (Addison, 1982; Hoogeinboom et al., 1983; Hunter, 1984; Kieboom et al., 1981; Mura et al., 1992; Murphy et al., 1989). Multicomponent procedures have been described for the analysis of 4-hydroxycoumarin compounds by fluorescence detection only (Felice et al., 1991; Hunter, 1983; Mundy et al., 1982; Rengel et al., 1993). These methods do not integrate both classes of anticoagulants, and they are not suitable for screening purposes in the diagnostic laboratory.

A sensitive multicomponent method for liver and serum has been reported by Chalmerchikit et al. (1993). With this method, simultaneous extraction of eight anticoagulants was possible, but the analysis was performed by two different HPLC systems. Hunter (1985) has studied four different HPLC systems for the multiresidue determination and confirmation of anticoagulants in animal tissue. In his report, as one of the strategies, the two classes of anticoagulant rodenticides were analyzed simultaneously by ion-pair chromatography. Even though a single chromatographic condition was established by ion-pair chromatography, screening and confirmation were still achieved by four different HPLC systems. Kujipers et al. (1995), on the other hand, reported an analysis of five superwarfarin anticoagulants in human serum by establishing a single chromatographic condition by utilizing fluorescence and fixed-wavelength UV detection.

A rapid and cost-effective multiresidue screen that includes both classes of anticoagulant rodenticides is needed for the diagnostic laboratory. Single HPLC analysis with photodiode array and fluorescence detection ensures a fast turnaround time and avoids false positive results. This paper presents a rapid and selective analysis of two different classes of anticoagulants in animal serum using a single extraction, cleanup, and analysis. For the first time nine anticoagulant

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Table 1. Precision Data for the Retention Times of Anticoagulant Rodenticides (Within-Run Data) and Concentration of Each Anticoagulant Rodenticide in the Standard Mixture

anticoagulant	retention time[c] (min)	%CV	concn (μg/mL) in standard mixture
coumafuryl[a]	4.59	0.61	1
pindone[b]	6.98	0.70	5
warfarin[a]	7.33	0.97	1
coumachlor[a]	8.42	1.00	1
diphacinone[b]	9.09	0.78	5
chlorpacinone[b]	10.04	0.71	5
bromadialone[a]	11.10	0.70	1
brodifacoum[a]	12.20	0.47	0.2
difethialone[b]	12.44	0.34	5

[a] Fluorescence detection. [b] Photodiode array detection. [c] Typical average retention times ($n = 21$).

rodenticides are separated by a gradient using phosphate buffer, with one chromatographic system followed by a photodiode array and fluorescence detection.

EXPERIMENTAL PROCEDURES

Reagents and Solvents. Phosphoric acid 85% and glacial acetic acid were from Fisher Scientific (Pittsburgh, PA), ACS certified. Acetonitrile and methanol were of Optima grade and water was of HPLC grade from Fisher Scientific. Potassium dihydrogenphosphate and sodium phosphate heptahydrate, ACS certified, were from Fisher Scientific. HPLC solvents were filtered through 0.45 μm nylon filters (Gelman Sciences, Ann Arbor, MI).

Phosphate buffer, pH 6.3, was prepared by dissolving 0.68 g of potassium dihydrogenphosphate and 0.26 g of sodium phosphate heptahydrate in 1 L of HPLC grade water.

Phosphate buffer, pH 5.5, was prepared by adjusting the pH 6.3 phosphate buffer with concentrated phosphoric acid.

Mobile phase consisted of 65% pH 6.3 phosphate buffer, 25% methanol, and 10% acetonitrile.

A Sep-Pak Florisil SPE column, Baker analyzed, was purchased from J. T. Baker (Phillipsburg, NJ).

Standard Solutions. Coumafuryl and chlorpacinone were from Axact Standards Inc. (Commack, NY). Bromadialone, warfarin, pindone, and diphacinone were from Chem Service (West Chester, PA). Brodifacoum was from Zeneca (Richmond, CA), and difethialone was obtained courtesy of Dr. Bill Birdsall, Pennsylvania Animal Drug and Diagnostic Laboratories (Kenett Square, PA). Stock solutions of 1000 μg/mL were prepared in acetonitrile for each anticoagulant except for difethialone and brodifacoum, which were dissolved in chloroform and methanol, respectively. Solutions of standard mixtures were prepared in acetonitrile at concentration ranges 1−100 μg/mL by dilution of stock solution. The standard mixtures were stable for 6 months when stored at 5 °C in amber quapark bottles protected from light. Subsequent dilutions of standard mixtures were prepared in mobile phase for HPLC analysis at concentrations of 0.05−0.25 μg/mL every week, and they were stored at 5 °C. Each anticoagulant compound had a different concentration in the standard mixture. Table 1 lists the concentrations of individual compounds in the standard mixtures.

Apparatus. Rotary shaker was model Reax 2, from Hiedolph, Germany.

Analytical nitrogen evaporator N-Evap was from Organomation Associates, Inc., Berlin, MA.

Solid-phase extraction (SPE) vacuum manifold was from Supelco, Bellefonte, PA.

A Hewlett-Packard (HP) model 1100 high-performance liquid chromatograph was equipped with an HP 1100 photodiode array (Palo Alto, CA) and Waters model 474 scanning fluorescence detector. The analytical column was a Pinnacle ODS amine-deactivated (20 cm × 4.6 mm × 5 μm, Resteck Corp., Bellefonte, PA) equipped with a Pinnacle ODS amine-deactivated guard column (10 mm × 4.6 mm × 5 μm). Quantification was performed by comparison with external standard peak areas generated by the HP Chemstation version 04.A01.

Chromatographic Conditions. For HPLC analysis the mobile phase consisted of a gradient mixture of phosphate buffer, pH 6.3, acetonitrile, and methanol. The solvent gradient program was as follows: from 0 to 2 min, 65% phosphate buffer, 10% acetonitrile, and 25% methanol; from 2 to 10 min, a linear gradient to 25% phosphate buffer, 65% acetonitrile, and 10% methanol; from 10 to 11 min, hold at 25% phosphate buffer, 65% acetonitrile, and 10% methanol; and from 11 to 15 min, linear change to original conditions. The column was equilibrated for 10 min between sample injections. Oven temperature was set to 35 °C. The flow rate of the mobile phase was 1 mL/min. The injection volume was 10 or 25 μL. For fluorescence detection, the excitation wavelength was 310 nm and the emission wavelength was 390 nm; bandwidth was 18 nm for both excitation and emission wavelengths, and the fluorescence detector gain was set to 1000. For photodiode array detection the pilot wavelength was set to 325 nm with a 5 nm bandwidth. The other wavelengths acquired simultaneously were 280, 303, and 210 nm with a 5 nm bandwidth and 260 nm with a 2 nm bandwidth. Spectra were stored in the range of 200−400 nm. The spectrum threshold was set to 1 mAu.

Procedure. *(a) Extraction of Anticoagulants from Serum.* Serum sample (1.0 g) was weighed into a 125 × 20 mm screw-top disposable test tube (Fischer Scientific). Phosphate buffer (0.5 mL, pH 5.5), 5% (v/v) ethanol in ethyl acetate (5 mL), and 1% (w/v) trichloroacetic acid (0.5 mL) were added to the test tube, which was gently rotated on a rotary shaker for 10 min. The layers were separated by centrifugation at 2300 rpm (1200*g*) for 5 min. The organic phase was then transferred to a 125 × 20 mm screw-top test tube. The sample was re-extracted with an additional 5 mL of 5% ethanol in ethyl acetate (v/v). The organic phases were combined. The combined extracts were evaporated under a stream of nitrogen with a nitrogen evaporator set at 40 °C. The dry extract was redissolved in 1 mL of hexane.

(b) Florisil SPE Cleanup. The Florisil SPE column placed on a vacuum manifold was conditioned sequentially with 5 mL of methanol, 5 mL of methylene chloride, and 10 mL of hexane. The extract in 1 mL of hexane was loaded onto the column. The column was washed with 9 mL of hexane followed by 10 mL of 50% (v/v) hexane in methylene chloride. The anticoagulants were eluted with 20 mL of 0.5% acetic acid in methylene chloride (v/v), followed by 2 mL of 1% acetic acid in methanol (v/v). The combined eluates were evaporated to dryness as above and redissolved in 100 μL of acetonitrile and 150 μL of mobile phase.

(c) HPLC/Fluorescence and Photodiode Array Determination of Anticoagulants. Analytical standard mixtures of anticoagulants at concentrations of 0.05−0.5 μg/mL were injected into the HPLC system. Each set of samples that was analyzed also contained a negative control and a fortified control. Percentage recoveries and residue levels were calculated by using external standards for quantitation. 4-Hydroxycoumarins were quantitated by using the fluorescence detector only; quantitation of indandiones and difethialone was performed using the photodiode array detector at the pilot wavelength (325 nm).

Method Validation. The method was validated by analyzing control bovine serum (bovine serum from Sigma lot 55H9302) fortified with anticoagulant standard mixture at levels listed in Table 2. The recoveries and the coefficient of variation (CV) of the analytical method were determined for all spike levels within the same workday.

RESULTS AND DISCUSSION

The average recoveries of the anticoagulant rodenticides from serum at different concentrations are summarized in Table 2. In general, recoveries were satisfactory and were between 73 and 105%. The precision

Table 2. Average Recoveries and Precision Data Obtained from Control Bovine Serum Spiked with Anticoagulant Rodenticides Using Photodiode Array and Fluorescence Detection

anticoagulant	fortifn level (μg/g)	av recovery (%, $n = 7$)	%CV	fortifn level (μg/g)	av recovery (%, $n = 6$)	%CV
coumafuryl	0.02	92	3.8	0.2	74	5.7
warfarin	0.02	77	3.0	0.2	85	1.6
coumachlor	0.02	84	3.9	0.2	89	4.8
bromadialone	0.02	88	4.0	0.2	93	2.1
brodifacoum	0.004	96	5.6	0.04	88	5.5
pindone	0.1	70	4.0	1	85	4.0
diphacinone	0.1	81	4.2	1	85	2.8
chlorphacinone	0.1	89	8.3	1	88	2.7
difethialone	0.1	78	5.9	1	73	15.6

(CV) data for the analytical method were calculated for each spike level, and they were between 1.6 and 8.3% for all of the anticoagulant compounds except for difethialone. It had a high %CV at the 1 ppm spike level, which could not be explained. Bromadialone recovery was calculated by adding the area units from two isomers (Koppel, 1990).

Serum Extraction. In this study, a commercially available bovine serum was used to avoid batch-to-batch variations. To achieve the extraction efficiencies shown in Table 2, acetonitrile, methanol, and ethyl acetate and 5% (v/v) ethanol in ethyl acetate were tried as extraction solvents, and the latter was found to be the most suitable extraction solvent. This solvent allowed cleaner extracts and a higher extraction yield. Trichloroacetic acid (1% w/v) was used to precipitate the proteins from the serum extracts, allowing a distinct separation of the aqueous and the organic phases. Furthermore, the addition of the phosphate buffer to the serum favored the recovery of coumafuryl and bromadialone. The buffer pH was adjusted to optimize the recovery of these two compounds (Kuijpers et al., 1995). It was essential to add trichloroacetic acid following the extraction solvent because when this order was reversed, serum fortification gave no recovery for coumafuryl, bromadialone, and brodifacoum.

Florisil SPE column cleanup eliminated the interference at the coumafuryl and chlorpacinone retention times. It also helped to increase the life of the analytical column. Acidic methylene chloride (0.5% glacial acetic acid) eluted 100% of all anticoagulants except diphacinone and chlorphacinone. Addition of 2 mL of acidic methanol (1% glacial acetic acid) was necessary to elute diphacinone and chlorpacinone.

Figure 1 illustrates the chromatogram of the analysis of a blank serum and a serum spike using two detectors. Occasionally, a serum component with fluorescence characteristics coeluted with brodifacoum peak (Figure 1B). The peak area of this component was, however, less than the area of the detection limit standard of brodifacoum.

Chromatography. An important goal in the HPLC method was to find a chromatographic condition to separate all nine anticoagulants simultaneously. In this laboratory, ion-pair chromatography has been used to establish a single HPLC system to analyze anticoagulants simultaneously (Hunter, 1985). This approach presented problems; column performance declined after short use, affecting the reproducibility and the chromatography. The acidic enol of 4-hydroxycoumarins and the enolic structure of indandiones can cause chromatographic difficulties. The interaction of the enolic group with the residual hydroxyl groups in the HPLC column packing can cause tailing and poor peak shapes and nonlinear behavior (Vigh, 1981; Hunter, 1985; Medvodivici et al., 1997). The chromatography can be improved by ion-pairing reagents or by using a column that contains negligible amounts of hydroxyl groups. Lichrosorb NH_2 and LiChroSpher are examples of columns used to chromatograph indandiones without the ion-pairing reagents (Fauconnet, 1997; Mura et al., 1992; Addison, 1982). In this method an amine-deactivated reversed phase C18 column gave efficient chromato-

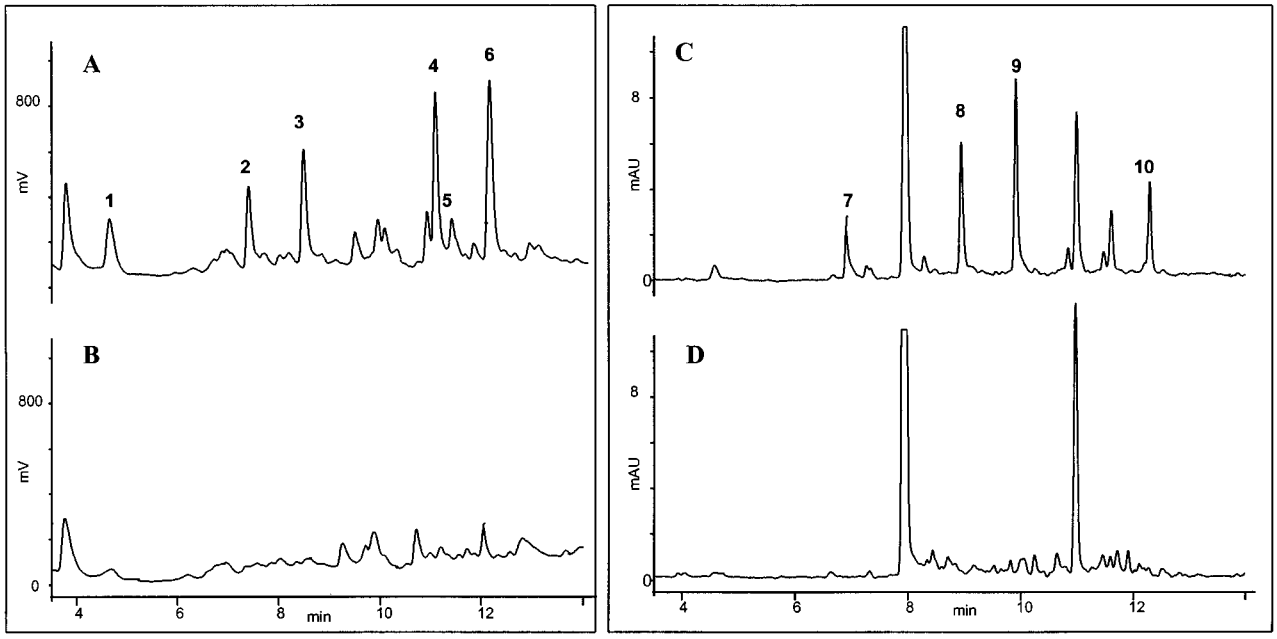

Figure 1. Typical HPLC chromatograms from a fluorescence signal: (A) bovine serum fortified with 0.020 ppm of coumafuryl (1), 0.020 ppm of warfarin (2), 0.020 ppm of coumachlor (3), 0.020 ppm of bromadialone (4), bromadialone isomer (5), and 0.004 ppm of brodifacoum (6), 100 mg injected; (B) control bovine serum, 100 mg injected. Typical HPLC chromatograms from a photodiode array signal at 325 nm: (C) bovine serum fortified with 0.1 ppm of pindone (7), diphacinone (8), chlorphacinone (9), and difethialone (10), 100 mg injected; (D) control bovine serum, 100 mg injected.

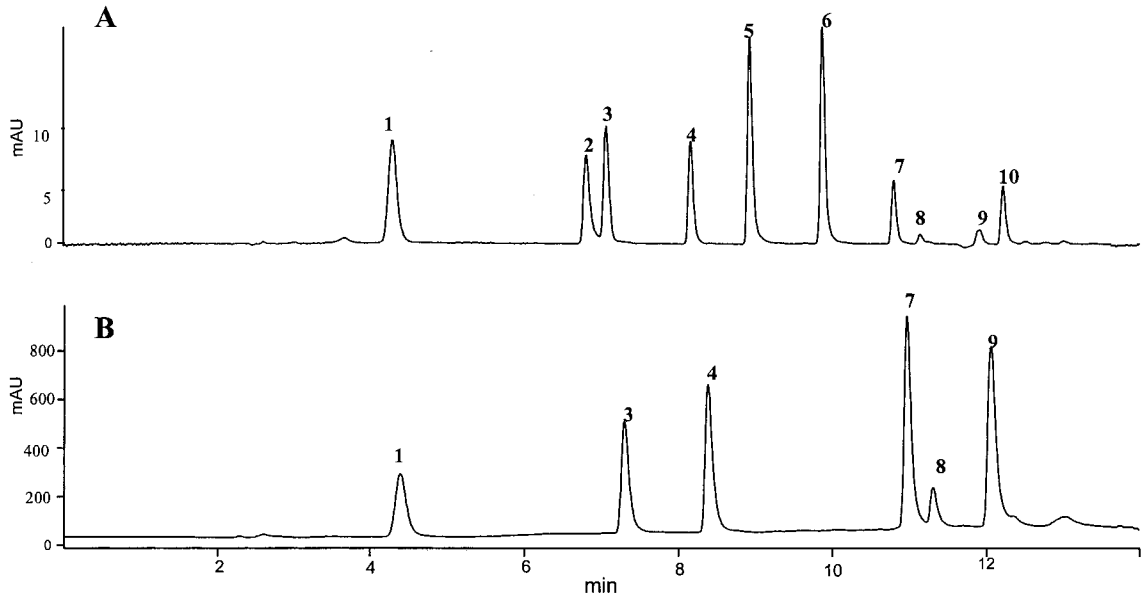

Figure 2. Typical HPLC chromatograms of a standard solution containing anticoagulant rodenticides using (A) photodiode array signal at 325 nm, 20 ng of each except brodifacoum (4 ng injected); (B) fluorescence signal, 2.5 ng of each except brodifacoum (0.5 ng injected). Peaks: coumafuryl (1); pindone (2); warfarin (3); coumachlor (4); diphacinone (5); chlorphacinone (6); bromadialone (7); bromadialone isomer (8); brodifacoum (9); difethialone (10). The run time was 15 min.

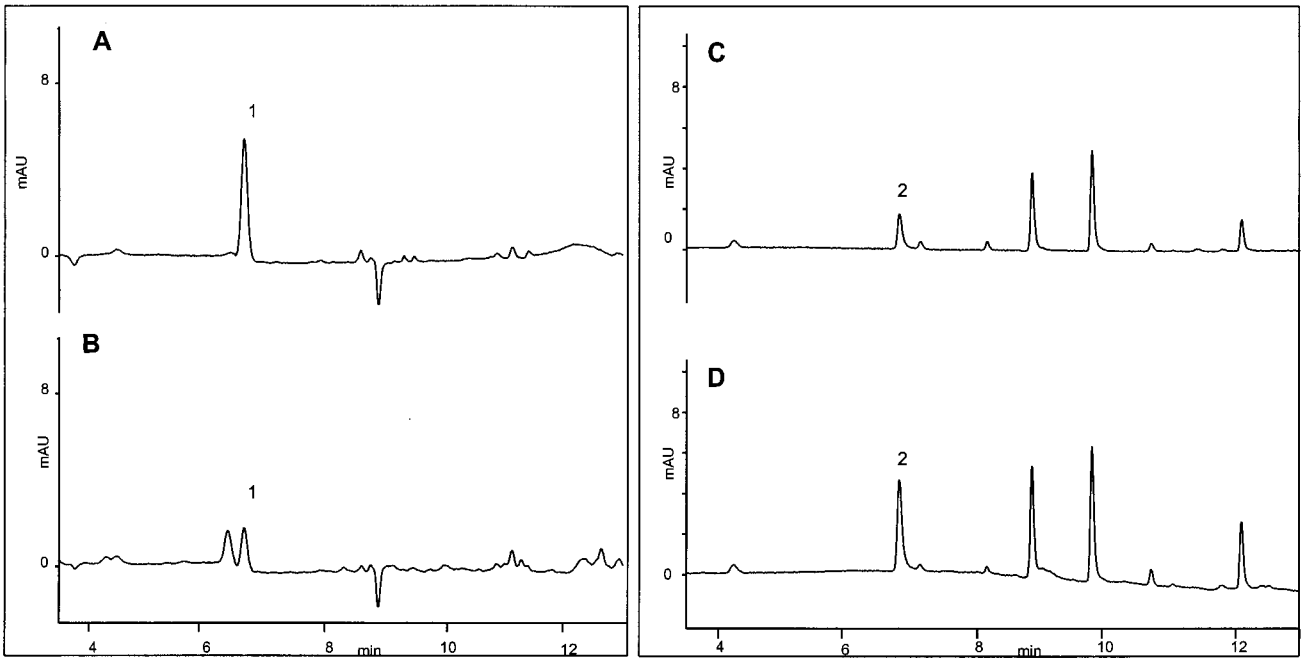

Figure 3. Chromatograms of a bobcat serum using photodiode array signal (A) at 325 nm or (B) at 280 nm. Peak 1 indicates the coeluting serum component. Chromatograms of an anticoagulant standard containing 5 ng of pindone using photodiode array signal (C) at 325 nm and (D) at 280 nm. Peak 2 indicates pindone.

graphic performance. The reproducibility of the system was excellent. After the column had been used for one year, only slight changes in the peak shapes and sensitivity were noted. However, buffer was necessary in the mobile phase to maintain stable retention times. Table 1 demonstrates that the stability of the retention times within-run is excellent and the retention times shifted only 0.2 min over a period of three months. The pH of the phosphate buffer was carefully adjusted to 6.3 to give the optimum separation (Figure 2), without quenching the fluorescence response (Hunter, 1983). Finally, to separate all nine compounds from one other and from the serum components, a gradient method was employed. Figure 2A illustrates the photodiode array chromatogram of a standard solution containing all nine anticoagulants. This demonstrates that the resulting gradient was able to resolve the anticoagulant peaks in 15 min. All anticoagulant peaks were resolved as single peaks except for bromadialone (Figure 2B). Bromadialone was resolved in its isomeric component (Rengel, 1993; Chaermchaikt et al., 1993; Felice et al., 1991; Koppen, 1990).

The excitation and emission wavelengths of the fluorescence detector were set to obtain the highest sensitivity for 4-hydroxycoumarin compounds, and the baseline drift was not significant (Hunter, 1985; Murphy et al., 1993). For the photodiode array detection, pilot wavelength was set to 325 nm, the wavelength at

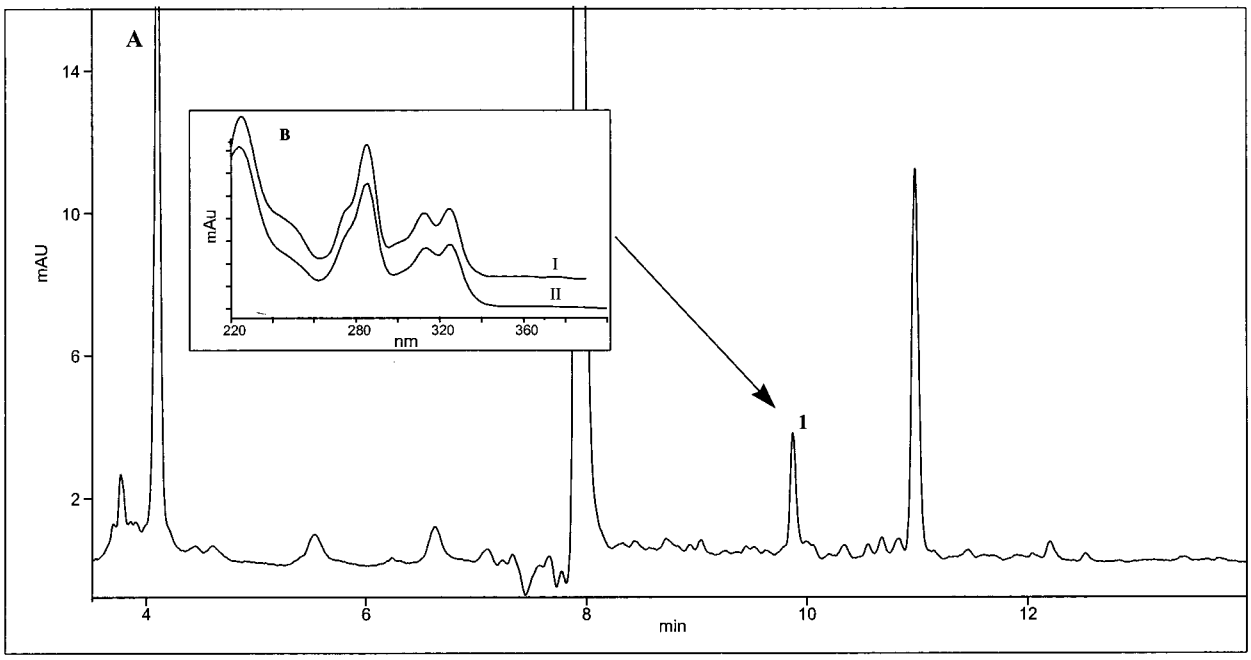

Figure 4. (A) Chromatogram of a coyote serum containing 0.040 ppm of chlorphacinone; photodiode array signal at 325 nm; peak 1 is chlorphacinone. (B) Normalized UV spectrum of chlorphacinone in coyote serum (I) compared with the UV spectrum of chlorphacinone standard (II).

which the second maximum absorbance appears for indandione and coumarin-based anticoagulants. This wavelength was not as sensitive as the 280 nm wavelength for the indandiones, but it gave a cleaner chromatogram, in most cases, free of interferences (Figure 2). Other wavelengths were also acquired for the purposes of eliminating and confirming the presence of the anticoagulant compounds. These wavelengths were employed because each one of them proved to be sensitive to different anticoagulant compounds. The wavelength at 280 nm was the most sensitive for indandiones, 260 nm was the most sensitive wavelength for brodifacoum and bromadialone, and 303 nm was used to obtain the most sensitivity for coumafuryl. The bandwidths for these wavelengths were kept narrow to increase the selectivity. 4-Hydroxycoumarin compounds were quantitated using the fluorescence signal only; however, confirmation involved matching the UV spectrum of the unknown to that of the analytical standard achieved from the photodiode array signal.

The important aspect of this analysis was the tandem combination of the photodiode array detector with the fluorescence detector, which simplified the detection of all nine anticoagulants. It should be noted that the photodiode array detector was the integral part of this method, as it had an important advantage over the widely used fixed-wavelength UV detectors. It provided the necessary spectral information for positive serum samples as coeluting serum components were easily distinguished by the aid of different wavelengths acquired during the analysis. In Figure 3 the peak at pindone retention time can easily be identified as a serum component by comparing the ratio of the area units at 280 and 325 nm to that of the pindone standard at the same wavelengths.

Linearity and Limits of Detection. Table 3 lists the linear range for anticoagulant rodenticides along with the correlation coefficients.

Table 3. Linear Concentration Ranges and Correlation Coefficient of Each Anticoagulant for the Regression Equation

anticoagulant	concn range (μg/mL)	correl coeff
coumafuryl	0.05−0.5	0.997
pindone	0.25−5	0.989
warfarin	0.05−0.5	0.999
coumachlor	0.05−0.5	0.999
diphacinone	0.25−5	0.997
chlorphacinone	0.25−5	0.989
bromadialone	0.025−0.25	0.998
brodifacoum	0.005−0.05	0.997
difethialone	0.25−5	1.000

Table 4. Detection Limits of Anticoagulant Rodenticides in Serum by HPLC with Photodiode Array and Fluorescence Detection

anticoagulant	fluorescence detection (ppm)	diode array detection (325 nm) (ppm)
coumafuryl	0.005	0.050
warfarin	0.005	0.050
coumachlor	0.005	0.050
bromadialone	0.005	0.050
brodifacoum	0.001	0.020
pindone	ND[a]	0.050
diphacinone	ND	0.025
chlorpacinone	ND	0.025
difethialone	ND	0.050

[a] ND, not detected.

Limit of detection was defined as 3 times the peak to noise ratio and calculated for each compound from both detectors (Table 4). The sensitivity of the method using fluorescence detection is approximately 10 times higher than that with photodiode array detection at 325 nm. The difference between the detection limits from both detectors indicates that the residues detected by the fluorescence detector may not be confirmed by the photodiode array detector. However, depending on each case, lower detection limits can be achieved from the photodiode array detector by employing other wavelengths.

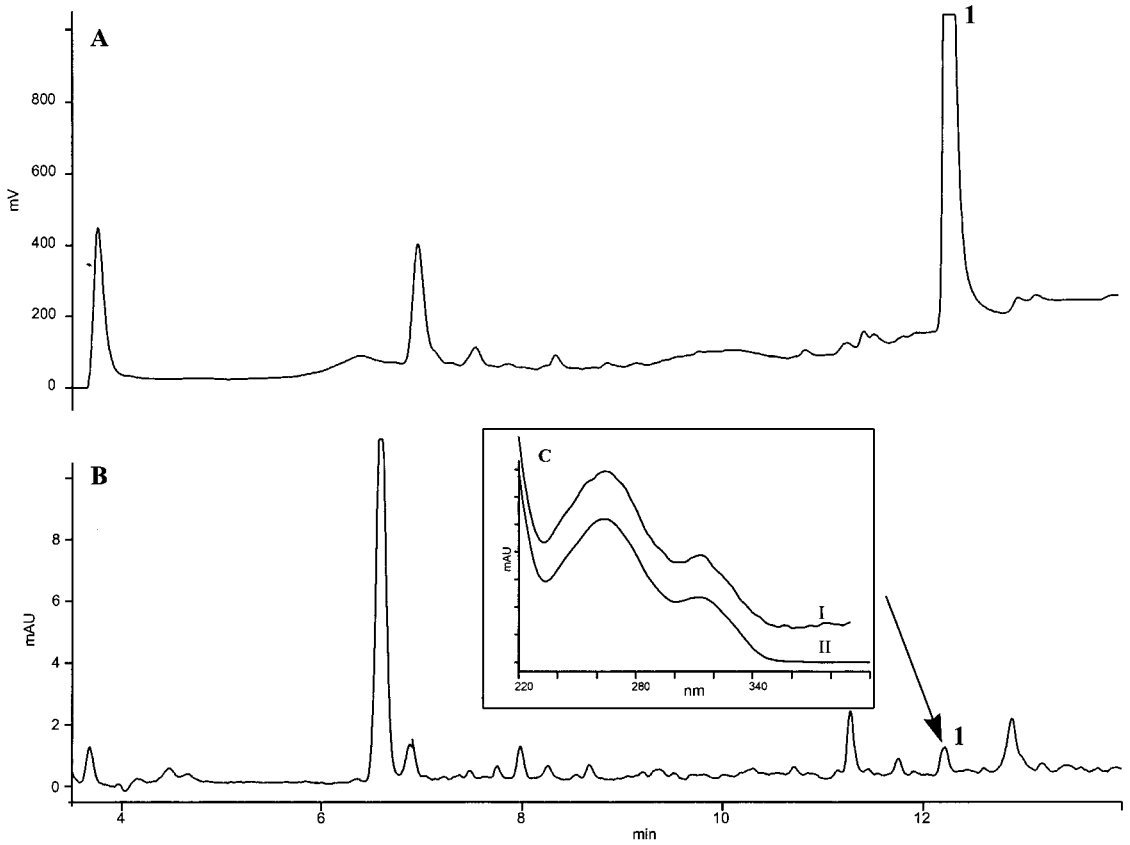

Figure 5. Chromatogram of a canine blood extract containing 0.014 ppm of brodifacoum: (A) fluorescence signal; (B) photodiode array signal at 280 nm, peak 1 is brodifacoum. (C) Normalized UV spectrum of brodifacoum in canine serum (I) compared with the UV spectrum of brodifacoum standard (II).

Application. The method described in this study was applied to a coyote serum submitted to the diagnostic laboratory. The analysis revealed the presence of chlorphacinone in coyote serum at 0.040 ppm. Figure 4 depicts the chromatogram of the coyote serum and the spectrum of chlorphacinone in the serum compared with the spectrum in the library. The presence of chlorphacinone was confirmed by retention time and spectral match (995 of 1000 using HP Chemstation software).

Yet another application involved a sample of canine blood submitted with clotting disorders. Figure 5 shows the fluorescence and photodiode array signals of the canine blood positive for brodifacoum at 0.014 ppm. The Florisil cleanup step of the experimental procedure is modified for this case; only the first fraction, 20 mL of acidic methylene chloride, from the Florisil column was collected and analyzed. Comparison of the photodiode array signal and the fluorescence detector signal along with the matching spectrum (match quality of 986 of 1000 using HP Chemstation software) of the brodifacoum constituted a substantial evidence for the presence of brodifacoum.

The application of this method was also extended to dicumarol determination in bovine serum for the diagnosis of sweet clover hay poisoning (Puschner et al., 1998).

Conclusions. The method presented in this paper provided a sensitive, selective, reliable, and precise determination of nine anticoagulants in animal serum. The use of the photodiode array detector provided the specificity for the simultaneous confirmation and identification of the anticoagulant rodenticides. The method was fast and clinically useful. It has been successfully applied to the detection of anticoagulants in serum on a wide range of animal species.

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