Journal of Chromatography, 270 (1983) 267–276
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,101

DETERMINATION OF COUMARIN ANTICOAGULANT RODENTICIDE RESIDUES IN ANIMAL TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. FLUORESCENCE DETECTION USING POST-COLUMN TECHNIQUES

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(Received June 26th, 1983)

SUMMARY

A multi-residue method was developed for the determination of the rodenticides warfarin, coumatetralyl, bromadiolone, difenacoum and brodifacoum in animal tissues by high-performance liquid chromatography with fluorescence detection. Extracts were cleaned-up by gel permeation chromatography on Bio-Beads SX-3 and residues determined by normal and reversed-phase high-performance liquid chromatography using post-column pH-switching, with chloroform–*sec.*-butylamine and borate buffer (pH 10.4) respectively, to maximise the native fluorimetric responses. Confirmation of identification was possible by re-chromatographing extracts in the absence of the post-column reagent. Chloroform–acetone (1:1) was significantly better than chloroform for the extraction of residues of these rodenticides from liver tissues. Recoveries from spiked liver tissue were generally greater than 90% at levels of 0.05–1 mg kg^{-1}. Detection limits in animal tissues of 0.002 mg kg^{-1} for coumatetratyl, difenacoum and brodifacoum, 0.01 mg kg^{-1} for bromadiolone and 0.02 mg kg^{-1} for warfarin and could be routinely achieved.

INTRODUCTION

Suspected poisoning of animals by pesticides is investigated routinely in this laboratory and a multi-residue method of analysis for anticoagulant rodenticides in animal tissues was required. The coumarin anticoagulant rodenticides warfarin [3-(3-oxo-1-phenylbutyl)-4-hydroxycoumarin] and coumatetralyl [3-(1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin] have been used for many years in the U.K., and more recently difenacoum [3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin], brodifacoum (3-[3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin) and bromadiolone (3-[3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl]-4-hydroxycoumarin) have been brought into use. The latter three compounds have a much higher acute oral toxicity to mammals and birds than

0021-9673/83/$03.00

the earlier rodenticides and hence poisonings of non-target species could arise from their usage along with a greater risk of secondary poisoning. A number of analytical methods have been described for the first four compounds but a method that included the determination of bromadiolone residues in animal tissues was needed.

Although warfarin residues in animal tissues have been determined by gas chromatography (GC[1,2]), reliable GC methods for difenacoum and brodifacoum have not been reported. Warfarin and coumatetralyl residues in biological material have been analysed by high-performance liquid chromatography (HPLC)[3,4]. Both difenacoum and brodifacoum have been determined in baits[5,6] and in animal tissues[4,7–9] by HPLC.

Corn and Berberich[10] described a fluorimetric assay for warfarin residues in plasma and demonstrated that the native fluorescence of warfarin was quenched in acidic solutions. Similar fluorimetric characteristics are shown by the other coumarin anticoagulants. The use of fluorescence detection in HPLC assays of these rodenticides has been restricted because either carboxylic acids or acidic buffers in the mobile phases have been employed for HPLC separation. Kieboom and Rammell[8] used fluorescence detection for the HPLC analysis of brodifacoum but were limited to the quenched fluorescence because of an acidified mobile phase. The full detection potential of the native fluorescence of these compounds has been exploited by Mundy and Machin[4], who used exclusion HPLC with a porous silica column and methanol as the mobile phase. However difenacoum, brodifacoum and coumatetralyl were not resolved and additional HPLC separations of fractions from the exclusion column were required for identification by ultraviolet (UV) detection.

Fluorescence detection usually increases sensitivity and selectivity over UV detection, so a method which fully exploited the native fluorescence of coumarin anticoagulants in an HPLC assay would be advantageous. For this reason the use of post-column pH-switching techniques that allow sensitive fluorescence detection was examined. Experiments were restricted to reversed-phase separations until the fluorescence detection of warfarin and its metabolites by post-column manipulation after adsorption chromatography was reported by Lee *et al.*[11]. Subsequently the use of post-column pH-switching in normal-phase HPLC was investigated. The extraction of the five rodenticides from animal tissue and the clean-up of crude extracts before HPLC determination were also examined.

EXPERIMENTAL

Materials and apparatus

Solvents of HPLC grade were obtained from Rathburn, Walkerburn, U.K. All other chemicals were supplied by BDH, Poole, U.K., and were of AnalaR grade where available. Bio-Beads SX-3 (200–400 mesh) were purchased from Bio-Rad Labs., Watford, U.K. Analytical quality standards of warfarin and coumatetralyl were obtained from the Laboratory of the Government Chemist, London, U.K. Analytical standards of difenacoum and brodifacoum were supplied by Sorex (London), Wembley, U.K.; and bromadiolone by Rentokil, Kirkby, U.K.

Tissue samples were homogenised with an Ultra-Turrax 18N tissue disperser. Gel permeation chromatography was done using an Altex glass column (500 × 25 mm I.D.) slurry packed with Bio-Beads SX-3 in hexane–chloroform–acetone (75:20:5)

to a bed height of 375 mm. The gel was then compressed to a height of 340 mm using an adjustable plunger. The mobile phase was supplied by a Gilson Minipuls 2 peristaltic pump with an isoversinic flowtube. All other tubing was PTFE. Samples were loaded on to the column via a rotary injection valve (Altex) with a 5-ml sample loop, and the column eluate was collected by a FRAC-300 fraction collector (Sephadex).

The HPLC system consisted of a Spectra-Physics SP 8700 solvent delivery unit and a Rheodyne valve injector (20 μl), together with a Varian post-column reagent pump and reaction coil cassette, and a Perkin-Elmer LS-4 fluorescence detector. The reaction coil was PTFE tubing (2 m × 0.3 mm I.D.). Spherisorb silica (5 μm) and Spherisorb ODS (5 μm) columns (250 × 4.6 mm I.D.) were slurry packed using a Haskel pneumatic amplifier pump with isopropanol as the slurry medium and methanol as the packing medium.

Extraction

Tissue samples (10 g) were chopped, dried by admixture with anhydrous sodium sulphate and homogenised in chloroform–acetone (1:1). The extract was filtered, the residual material re-extracted and the second extract added to the filter together with washings from the homogeniser. The combined filtrate was carefully evaporated to dryness at 35°C on a rotary evaporator and made up to 10 ml in hexane–chloroform–acetone (75:20:5).

Clean-up

An aliquot (5 ml) of the crude extract was applied to the gel permeation column and eluted with hexane–chloroform–acetone (75:20:5) at 5 ml min^{-1}. The first 150 ml of eluate were discarded, the next 250 ml were collected and finally the column was washed with a further 50 ml of mobile phase. Prior to experimental use the gel permeation column was calibrated with each rodenticide, and the elution volumes for this column were: warfarin, 160–260 ml; coumatetralyl, 160–250 ml; difenacoum, 220–370 ml; brodifacoum, 260–400 ml; and bromadiolone, 240–400 ml. The elution volumes vary from column to column so that each new column must be calibrated.

HPLC determination

Normal-phase chromatography. The cleaned-up extract was carefully evaporated to dryness at 35°C and the residue redissolved in dichloromethane (1 ml). Aliquots were analysed by adsorption chromatography on Spherisorb silica using mobile phases consisting of various proportions of dichloromethane containing 2% acetic acid and hexane at a flow-rate of 1.5 ml min^{-1}. The post-column reagent, chloroform–*sec.*-butylamine (5:1) was supplied at a flow-rate such that an effluent of apparent pH $\geqslant$ 8 was obtained. *sec.*-Butylamine was used as a pH modifier because it was to hand, but it is probable that other basic organic amines would be equally satisfactory. The fluorescence of the rodenticides was monitored at excitation and emission wavelengths of 310 nm and 390 nm respectively. The slits for both monochromators were set at 10 nm.

Reversed-phase chromatography. For reversed-phase separations the extracts were dried and redissolved in methanol (1 ml) and aliquots chromatographed on Spherisorb ODS using gradient elution with mixtures of methanol and water, both containing 0.25% acetic acid, at a flow-rate of 1.5 ml min^{-1}. In this case the post-

column reagent was 0.3 *M* borate buffer (pH 10.4) and again the flow-rate was adjusted until an effluent of pH $\geqslant$ 8.0 was obtained.

RESULTS AND DISCUSSION

Extraction

Chloroform has been widely used to extract residues of warfarin[3], coumatetralyl[12], difenacoum[9] and brodifacoum[4] from animal tissues. Kieboom and Rammell[8] found dichloromethane–cyclohexane (1:1) to be superior to chloroform for the extraction of brodifacoum whilst Koubek *et al.*[7] used chloroform–methanol (9:1) to extract the same compound. Mundy and Machin[4] compared chloroform with chloroform–methanol (9:1) as extractants for coumatetralyl in liver tissue and found slightly higher residues using chloroform. Bromadiolone has been reported to be only slightly soluble in chloroform but to be soluble in acetone[13]. This latter solvent has been used to extract warfarin from animal tissues[2]. Extraction studies on incurred bromadiolone residues in poultry liver were carried out by separately macerating liver tissue samples with the extractants; chloroform, chloroform–acetone (1:1), acetone and chloroform–methanol (4:1). The bromadiolone residues found were 0.27, 0.50, 0.51 and 0.44 mg kg^{-1} respectively. There was little difference in the amount of residues extracted by chloroform–acetone (1:1) and acetone but the former gave cleaner extracts. The efficiencies of chloroform and chloroform–acetone (1:1) in extracting incurred residues of the other coumarin rodenticides were then compared and the results are shown in Table I. In all cases higher residues were extracted by the latter solvent mixture, particularly for bromadiolone where the residue was almost doubled. Warfarin, a polar rodenticide like bromadiolone, would probably give similar high results from extraction with chloroform–acetone but unfortunately no tissues containing incurred residues were available for analysis. Overall, chloroform–acetone was found to be more suitable as an extraction solvent than chloroform.

Although co-extracted materials can make analysis of liver tissue difficult, it was chosen for recovery experiments because rodenticide residues are retained in it to a greater extent than in other tissues making it more useful diagnostically. Percentage recoveries were determined using canine liver samples spiked separately, prior

TABLE I

EXTRACTION OF INCURRED RODENTICIDE RESIDUES FROM LIVER TISSUE

Residues were extracted, cleaned-up and determined by normal-phase HPLC as described in the text. Results are expressed as mg kg^{-1} on a wet weight basis.

Rodenticide	*Species*	*Extracting solvent*	
		Chloroform	*Chloroform acetone (1:1)*
Bromadiolone	Poultry	0.27	0.50
Brodifacoum	Dog	0.43	0.57
Coumatetralyl	Cat	0.33	0.48
Difenacoum	Dog	1.23	1.51

TABLE II

RECOVERIES OF RODENTICIDES FROM SPIKED LIVER TISSUE

At each level three separate samples of liver tissue were fortified with a single rodenticide prior to extraction. Residues were extracted, cleaned-up and determined by normal-phase HPLC as described in the text.

Rodenticide	*Recovery (%) (mean ± S.D.)*		
	Fortification level (mg kg^{-1})		
	1	*0.2*	*0.05*
Warfarin	93 ± 8	95 ± 9	90 ± 5
Bromadiolone	90 ± 2	93 ± 8	82 ± 2
Brodifacoum	91 ± 6	89 ± 1	90 ± 2
Coumatetralyl	93 ± 5	93 ± 3	94 ± 2
Difenacoum	95 ± 2	97 ± 4	99 ± 3

to extraction, with one of the five rodenticides at levels of 0.05, 0.2 and 1 mg kg^{-1} and the results are shown in Table II. With the exception of bromadiolone, recoveries at all levels were consistent with mean values $\geq 90\%$, the lowest individual recovery recorded was 84%. In the case of bromadiolone initial recoveries were disappointingly low at *ca.* 66%. During recovery experiments cleaned-up extracts had been stored overnight at 4°C prior to analysis, and subsequently it was observed that very dilute solutions of bromadiolone were unstable at 4°C and degraded fairly rapidly. Overnight storage at −20°C improved the recovery of bromadiolone residues from spiked liver to a mean of 82%. The stability of dilute solutions of bromadiolone under slightly acidic and slightly basic conditions was then examined at low temperatures. Degradation of bromadiolone was increased in the presence of formic acid (0.25%) but was minimised in solutions containing isopropylamine (0.25%). Recovery experiments were repeated storing cleaned-up extracts overnight at −20°C with the addition of 0.25% isopropylamine. The volatile amine was lost when the extract was concentrated for chromatographic analysis. Percentage recoveries of bromadiolone were considerably improved at the 0.2 and 1 mg kg^{-1} levels to means of 93 and 90%, respectively, but at the 0.05 mg kg^{-1} level were lower, ranging from 81 to 84%. Additional experiments indicated that the use of isopropylamine was not suitable for samples containing warfarin, where the apparent recovery was reduced to less than 10%; however, triethylamine proved to be a satisfactory alternative giving high recoveries for both bromadiolone and warfarin. The adverse effect of isopropylamine on the recovery of warfarin may be due to reaction between this primary amine and the carbonyl group on the side-chain of the hydroxycoumarin of warfarin, whereas triethylamine as a tertiary amine would not react in this manner.

Clean-up

Clean-up of extracts from animal tissues containing certain coumarin rodenticides has been achieved using adsorption techniques[2,8] or by gel permeation chromatography[7]. The multi-residue technique described by Mundy and Machin[4] effectively used exclusion chromatography as a clean-up before final identification, although the exclusion chromatography was used in part as a determinative step. The

choice of liver as the principal tissue type and fluorescence as a sensitive and specific detection mode meant that one of the most important parts of any clean-up was the removal of lipid material from the extract, especially if reversed-phase separations were to be used. Elimination of lipid material ensured that extracts were readily soluble in small volumes of methanol. A gel permeation method similar to that employed for brodifacoum[7] and elsewhere for other pesticides[14,15] was adopted. The mobile phase differed from that of Koubek *et al.*[7] in that a more polar eluent was used to ensure solubility of warfarin and bromadiolone, and for convenience more volatile solvents were chosen. The clean-up was suitable for multi-residue analysis and has proved satisfactory in routine use for liver extracts and for extracts from kidney tissue and stomach content material.

Fluorescence spectral characteristics

Fluorescence excitation and emission spectra of solutions of each rodenticide in methanol were measured. The excitation spectra of warfarin and coumatetralyl both showed maxima at 310 nm, but those of difenacoum, brodifacoum and bromadiolone showed maxima between 250 and 260 nm and had shoulders at 310 nm. All emission spectra showed maxima at 390 nm when excited at 310 nm and when excited at lower wavelengths in the case of difenacoum, brodifacoum and bromadiolone. Excitation of the latter three rodenticides at 255 nm increased the intensity of emission at 390 nm *ca.* two-fold compared to excitation at 310 nm. The fluorimetric responses of warfarin and bromadiolone were less than those of difenacoum, brodifacoum and coumatetralyl which were very similar. The fluorimetric responses of the rodenticides were not affected by the presence of phosphate, tris or borate buffers of pH 8.0.

High-performance liquid chromatography

Adsorption chromatographic methods have been described for the HPLC determination of four of the rodenticides used[3,5–7]. In the present study experiments were done with Spherisorb silica using mobile phases consisting of hexane, dichloromethane and acetic acid. As reported elsewhere[9] warfarin was not conveniently eluted with brodifacoum, coumatetralyl and difenacoum in a single isocratic separation. Two isocratic separations were finally used; dichloromethane acidified with 2% acetic acid–hexane (35:65) was used in the analysis of brodifacoum, coumatetralyl and difenacoum whilst these solvents in the ratio 90:10 were used for warfarin and bromadiolone. The post-column reagent, chloroform–*sec*-butylamine (5:1), was used at 0.3 ml min^{-1} for the analysis of brodifacoum, coumatetralyl and difenacoum. Coumatetralyl was eluted after brodifacoum and difenacoum which each eluted as two peaks because of the resolution of *cis*- and *trans*-isomers. The *cis*-isomers of difenacoum and brodifacoum co-eluted and the *trans*-isomers showed only marginal resolution. Mundy and Machin[4] described the resolution of all four isomers of difenacoum and brodifacoum, so that the failure to resolve them in this study may be due to some band broadening, inherent in using the post-column system or as a result of a lower column efficiency or a combination of these factors. Despite this shortcoming this method has proved convenient in routine use, being essentially free from interferences. Chromatograms of brodifacoum residues in a canine liver extract are shown in Fig. 1. Additional confirmation was obtained by re-chromatographing extracts

with the post-column reagent pump switched off, when peaks resulting from coumarin rodenticides were virtually eliminated from the chromatogram (Fig. 1C).

A post-column reagent flow-rate *ca.* 0.75 ml min^{-1} was required for the isocratic analysis of warfarin and bromadiolone. Fig. 2 shows the separation of these two compounds and the identification of bromadiolone residues in a poultry liver extract. Again the analysis has proved satisfactory in routine use although in a limited number of samples some potential interference ($\leqslant 0.1$ mg kg^{-1}) at the retention time of warfarin has been observed.

The minimum detectable amount of coumatetralyl was 20 pg, and of brodifacoum and difenacoum 20–40 pg, depending on the isomeric ratio. The minimum detectable amounts of bromadiolone and warfarin were 80 pg and 120 pg, respectively. The fluorimetric responses of the rodenticides were linear up to 2 μg. For tissue extracts practical detection limits of 0.002 mg kg^{-1} (wet weight) were possible for coumatetralyl, brodifacoum and difenacoum; and of 0.01 and 0.02 mg kg^{-1} for bromadiolone and warfarin. Placing a restrictor downstream of the fluorimeter to provide a back pressure of *ca.* 50 p.s.i. did not improve the baseline noise at high

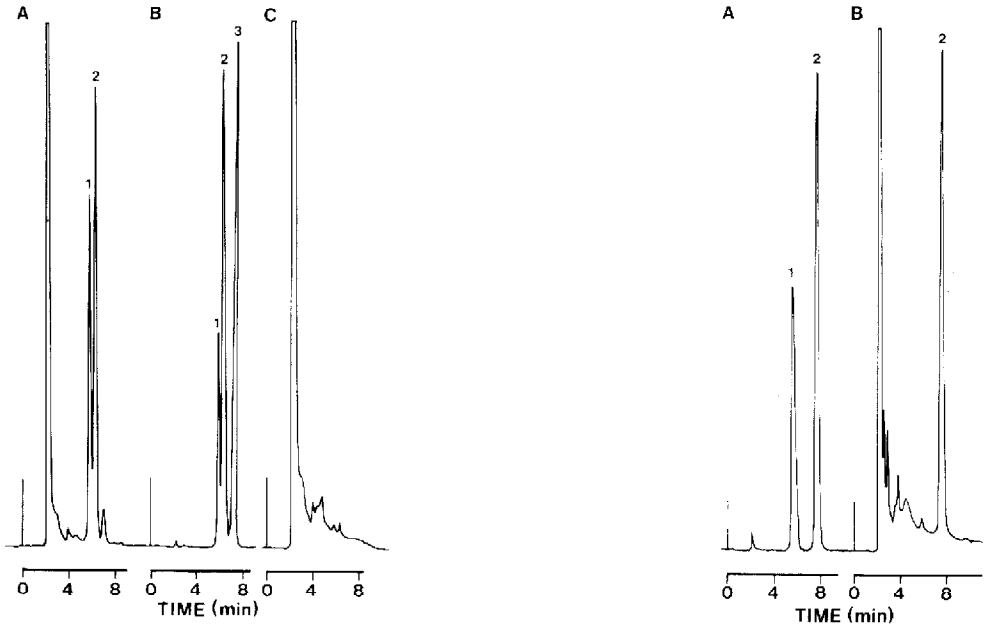

Fig. 1. Normal-phase chromatography of brodifacoum (1,2) and coumatetralyl (3) on Spherisorb silica (5 μm). Eluent, dichloromethane acidified with 2% acetic acid hexane (35:65); flow-rate, 1.5 ml min^{-1}; post-column reagent, chloroform *sec.*-butylamine (5:1); post-column reagent flow-rate, 0.3 ml min^{-1}; fluorescence detection, $\times 2$; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Extract from canine liver containing brodifacoum ($\equiv$ 0.57 mg kg^{-1}). (B) Reference standards, brodifacoum (49 ng), coumatetralyl (25 ng). (C) Canine liver extract re-chromatographed in the absence of the post-column reagent.

Fig. 2. Normal-phase chromatography of warfarin (1) and bromadiolone (2) on Spherisorb silica (5 μm). Eluent, dichloromethane acidified with 2% acetic acid hexane (90:10); flow-rate, 1.5 ml min^{-1}; post-column reagent, chloroform *sec.*-butylamine (5:1); post-column reagent flow-rate, 0.75 ml min^{-1}; fluorescence detection, $\times 3.5$; λ_{ex} = 310 nm, λ_{em} = 390 nm. (A) Reference standards, warfarin (48 ng), bromadiolone (46 ng). (B) Extract from poultry liver containing bromadiolone ($\equiv$ 0.5 mg kg^{-1}).

sensitivity settings. A longer reaction coil (7.5 m) gave only a marginal improvement in noise level. Excitation of bromadiolone, brodifacoum and difenacoum at 255 nm increased the response but did not change the signal-to-noise ratio, therefore excitation at 310 nm was adopted for routine use.

Reversed-phase systems have been used for the analysis of coumarin anticoagulant rodenticides[4,5,8] and have the advantage that difenacoum and brodifacoum elute separately as single peaks with no resolution of *cis*- and *trans*-isomers. Reversed-phase systems have an additional advantage in that gradient elution can be used to optimise the separation for multi-residue analysis and so attention was concentrated on developing a gradient system, but the method can be used equally well with isocratic separations. Methanol and water, both containing 0.25% acetic acid, were used for gradient elution from Spherisorb ODS. The post-column reagent, 0.3 *M* borate buffer pH 10.4, was supplied at 0.3 ml min^{-1}. Fig. 3 shows the gradient separation of all five rodenticides and the use of this system to identify brodifacoum and difenacoum residues in a canine liver extract. Again additional confirmation was possible by re-chromatographing extracts in the absence of the post-column reagent. The minimum detectable amounts were somewhat lower than in normal-phase chromatography, principally because the baseline noise was considerably less in the re-

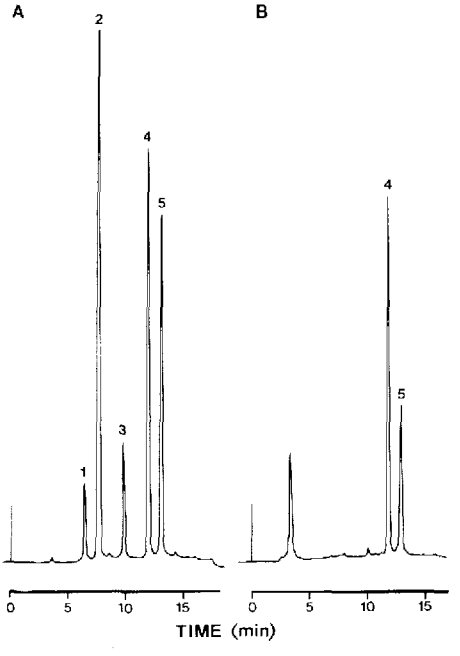

Fig. 3. Reversed-phase gradient separation of warfarin (1), coumatetralyl (2), bromadiolone (3), difenacoum (4) and brodifacoum (5). Eluent, methanol water containing 0.25% acetic acid; gradient program, 65% methanol→84%→95% in 5-min linear stages followed by a 5-min hold at the final conditions; flow-rate, 1.5 ml min^{-1}; post-column reagent; 0.3 *M* borate buffer, pH 10.4; post-column reagent flow-rate; 0.3 ml min^{-1}; fluorescence detection, × 2; λ_{ex} = 310 nm; λ_{em} = 390 nm. (A) Reference standards, warfarin (20 ng), coumatetralyl (20 ng), bromadiolone (21 ng), difenacoum (19 ng) and brodifacoum (20 ng). (B) Extract from canine liver containing difenacoum (≡ 1.47 mg kg^{-1}) and brodifacoum (≡ 0.78 mg kg^{-1}).

versed-phase system. At high sensitivity the baseline drifted upwards during gradient elution so isocratic conditions were preferable for residues at very low levels.

Although the reversed-phase system was potentially more useful in analyses of brodifacoum and difenacoum, some liver extracts exhibited interferences, particularly with brodifacoum, at high sensitivities. Koubek *et al.*[7] made use of a further clean-up with Sep-Pak cartridges after gel permeation chromatography and a similar additional clean-up could be adapted for multi-residue work to remove interferences and improve the detection limits. In routine screening of liver tissues from poisoning incidents it does not appear necessary to use any further clean-up as liver residues have invariably been greater than 0.1 mg kg^{-1}, ranging up to 2 mg kg^{-1} with a mean residue level of 0.58 mg kg^{-1} for eighteen incidents.

The order of elution in normal-phase chromatography was brodifacoum/difenacoum first followed by coumatetralyl, warfarin and finally bromadiolone. In reversed-phase chromatography warfarin was eluted first followed by coumatetralyl, bromadiolone, difenacoum and lastly brodifacoum.

In this laboratory the reversed-phase system is preferred for analysis of baits, crop contents and gizzard or stomach contents from poisoned animals. For analysis of extracts from liver tissues the normal-phase method is used with subsequent confirmation using the reversed-phase system.

CONCLUSIONS

Residues of brodifacoum, bromadiolone, coumatetralyl, difenacoum and warfarin can be detected at low levels in liver tissue using either normal-phase or reversed-phase HPLC with post-column pH-switching techniques to enhance the fluorimetric response. Quantitative analyses are possible in both chromatographic modes providing the post-column reagent is delivered at a constant flow-rate such that the eluate is slightly alkaline. The reversed-phase system has advantages in that all five rodenticides can be analysed in a single chromatographic run and brodifacoum and difenacoum are fully resolved as single peaks.

ACKNOWLEDGEMENTS

The author is grateful to Sorex (London) for gifts of difenacoum and brodifacoum and to Rentokil for bromadiolone.

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