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THE MULTI-RESIDUE DETERMINATION OF COUMARIN-BASED ANTICOAGULANT RODENTICIDES IN ANIMAL MATERIALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The rodenticides brodifacoum, difenacoum, coumatetralyl and warfarin are determined in animal relicta by high-performance exclusion chromatography on porous silica. The first three compounds are not separated, but are subsequently differentiated by adsorption or reversed-phase high-performance liquid chromatography of the appropriate eluate fraction collected from the exclusion column. The method is rapid, and clean-up (on Sep-Pak silica cartridges) is simple. Mean recoveries from spiked substrates were generally above 80% at levels of 0.1–1.0 mg/kg. Routine limits of determination are about 0.05–0.1 mg/kg for warfarin and about 0.02 mg/kg for the other compounds. If analysis for warfarin is not required, the latter limit can be lowered to about 1 μg/kg by a slight modification to the clean-up step.

INTRODUCTION

Methods for the diagnostic determination in animal relicta of the anticoagulant rodenticides warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin] and difenacoum [3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin] by high-performance liquid chromatography (HPLC) have been described[1,2]. A method for determining brodifacoum {3-[3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin} was needed.

Preliminary experiments showed that brodifacoum could be determined at levels of 0.01 mg/kg and above in animal materials by capillary gas chromatography, the brodifacoum being thermally degraded to a mixture of 3-(4′-bromobiphenyl-4-yl)naphthalene and the corresponding tetrahydronaphthalene, both of which were detected[3]. Subsequent results were insufficiently consistent to encourage the develop-

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ment of the method for routine use however, and methods based on HPLC were considered.

Brodifacoum has been determined in baits by reversed-phase HPLC[4] and in rat tissues by adsorption HPLC[5]. Its determination in animal tissues and blood[6] by an adaptation of the reversed-phase method[4] was described during the preparation of the present publication. Exclusion HPLC appeared to offer two advantages: simpler clean-up, because brodifacoum would be eluted before most of the co-extracted material, and more sensitive fluorimetric detection because the use of acetic acid (which quenches fluorescence) in the mobile phase would be avoided. Moreover, determination by exclusion chromatography combined with identification by adsorption or reversed-phase HPLC seemed likely to provide a convenient multi-residue method for the four coumarin anticoagulants used as rodenticides in this country: warfarin, difenacoum, brodifacoum and coumatetralyl [4-hydroxy-3-(1,2,3,4-tetrahydro-1-naphthyl)coumarin].

The present communication describes the determination by HPLC of the four compounds mentioned above in animal tissues and fluids. Extracts are cleaned-up on Sep-Pak silica cartridges and chromatographed on a porous silica exclusion column, from which the rodenticides are eluted before co-extractives. Difenacoum, brodifacoum and coumatetralyl are resolved from warfarin, but not from one another; the three compounds are differentiated by HPLC of collected eluates on an adsorption or reversed-phase column, with UV detection. Since the presence of more than one rodenticide is unlikely, exclusion chromatography normally serves for quantification and reversed-phase or adsorption chromatography for identification. If more than one of the unresolved rodenticides is present, quantification by reversed-phase chromatography is possible, but lower limits of determination can be achieved on the exclusion column.

EXPERIMENTAL

Materials and apparatus

Brodifacoum, mixed isomers of analytical standard grade, was supplied by Sorex (London), Wembley, Great Britain; other rodenticides were from previously specified sources[1]. The *cis*- and *trans*-isomers of brodifacoum and difenacoum were separated for experimental purposes by thin-layer chromatography (TLC) on precoated silica gel plates (Schleicher & Schüll G1500 LS 254, from Anderman & Co., East Molesey, Surrey, Great Britain), with ether–hexane–acetic acid (75:25:1, v/v) as developing solvent.

Methanol was of HPLC grade and chloroform was Distol grade containing about 2% of ethanol as stabilizer (both from Fisons Scientific Apparatus, Loughborough, Great Britain). Other solvents were Analar or of similar quality.

Sep-Pak silica cartridges were from Waters Assoc., Northwich, Great Britain; they were prepared for use by washing successively with methanol–chloroform (15:85, v/v) and chloroform (10 ml of each).

The HPLC columns were of stainless steel, 5 mm I.D., internally polished. Exclusion and adsorption columns were 250 mm long, slurry-packed with Magnusil 5 μm porous silica; reversed-phase columns were 100 mm long, packed with Magnusil 8H C_{22}. All columns and packings were from Magnus Scientific Instrumen-

tation (Sandbach, Great Britain). The liquid chromatograph was as previously described[7], with the addition of a Perkin-Elmer Model 2000 fluorescence spectrophotometer fitted with a 20-μl flow-cell.

Extraction

Liver and stomach contents (10 g) were homogenised with anhydrous sodium sulphate (20 g) and chloroform (30 ml). The extract was filtered through sintered glass, the residue was homogenised with a further 15 ml of chloroform and filtered, and the filtrates were combined. Serum or urine (10 ml) was acidified with hydrochloric acid (5 *N*, 2 ml) and extracted successively with 15 and 10 ml of chloroform. The combined extracts were dried with anydrous sodium sulphate and filtered. Filtrates were concentrated under a stream of nitrogen at 35°C.

Clean-up

The chloroform extract was concentrated to 10.0 ml, a 2-ml aliquot was injected on to a Sep-Pak cartridge, and the rodenticide(s) were eluted with 4 ml of methanol–chloroform (15:85, v/v). The eluate was taken to dryness at 35°C under nitrogen, and the residue was dissolved in methanol (0.1–2.0 ml, according to the expected rodenticide content). If analysis for warfarin was not required, the chloroform extract was concentrated to about 1 ml and transferred to the cartridge, which was eluted with chloroform (4 ml) instead of the mixed eluent; any warfarin present was quantitatively retained by the cartridge.

Determination

Duplicate aliquots (20 μl) of extracts and of standard solutions in methanol were chromatographed on the exclusion column with methanol as eluent at a flow-rate of 1 ml/min. The fluorescence detector was operated at excitation and emission wavelengths of 315 and 410 nm, respectively.

The rodenticides were quantified by reference to standard solutions containing mixtures of warfarin with one of the other three compounds; this procedure was justified because the detector responded equally to equal mass/volume concentrations of brodifacoum, difenacoum and coumatetralyl. If a peak was detected at the retention time of the three excluded rodenticides, the eluate fraction producing it was collected for identification by adsorption or reversed-phase chromatography. It was usually necessary to combine the corresponding fractions from several injections of the same extract.

Identification

The eluate fraction containing brodifacoum, difenacoum or coumatetralyl was taken to dryness under nitrogen, and the residue was dissolved in 50 μl of cyclohexane–dichloromethane–acetic acid (75:25:0.6, v/v) for adsorption chromatography or methanol–water–acetic acid (80:20:0.8, v/v) for reversed-phase chromatography. In either case the solvent was used as the mobile phase for HPLC, at a flow-rate of 1 ml/min. Detection was by UV absorption at 260 nm. If more than one of the three rodenticides was identified, quantification was by reversed-phase chromatography with reference to suitable standards.

RESULTS AND DISCUSSION

Previous work[2] had shown that difenacoum could be separated from both warfarin and most co-extractives in animal materials by exclusion chromatography on porous glass with a mean pore diameter of 200 Å. Coumatetralyl was eluted together with difenacoum and, as was subsequently found, with brodifacoum. Warfarin was eluted later, within the retention volume of the main co-extractive fraction. More recently, the substitution of porous silica (mean pore diameter 60 Å) for porous glass has given better resolution and, because peaks were narrower, higher sensitivity. Difenacoum, brodifacoum and coumatetralyl were still eluted together; warfarin was eluted after these, but well before co-extractives. Exclusion chromatography on porous silica therefore provided a basis for a multi-residue method, provided that brodifacoum, difenacoum and coumatetralyl could be differentiated before or after the determinative step.

Calibration

Chromatography of standard solutions gave a linear calibration curve for warfarin over the range 1.5 ng to 2.5 μg and for the other rodenticides over the range 300 pg to 2.5 μg.

Extraction

Extraction with chloroform was essentially by the procedures previously used for the separate determinations of warfarin[1] and difenacoum[2] in animal fluids and liver. Von Meyer *et al.*[8] have also used chloroform to extract warfarin and coumatetralyl (as well as other anticoagulants) from biological fluids. Koubek *et al.*[5] have shown that incurred brodifacoum is well extracted from rat tissues by methanol–chloroform (1:9, v/v) and it can be assumed that this mixture would be equally effective for difenacoum. In the present work, comparison of chloroform with methanol–chloroform showed that the former yielded cleaner extracts from spiked liver, and was therefore to be preferred if it was no less efficient. Since any advantages of incorporating methanol in the extractant would presumably be at least as marked with the more polar rodenticide coumatetralyl as with brodifacoum, the extraction of this compound was examined. Samples of pheasant liver containing incurred coumatetralyl were analysed after extraction with chloroform and with methanol–chloroform (1:9, v/v). The coumatetralyl levels found were 0.96 and 0.89 mg/kg, respectively; it was therefore concluded that chloroform was a suitable extractant for the substrates analysed in the present work. It should be noted that the Distol chloroform used contained about 2% of ethanol: the effectiveness of pure chloroform was not examined.

Clean-up

Previous work[5,7] had shown the convenience of Sep-Pak cartridges for the rapid clean-up of extracts before HPLC. Such a clean-up step was not strictly necessary for the analyses described here, but it increased the effective life of analytical columns and shortened analysis time by removing co-extractives which were strongly adsorbed by them.

All four rodenticides were eluted with methanol–chloroform (15:85, v/v). When only the three less polar compounds were of interest however, a cleaner extract

was obtained by eluting with chloroform. Fig. 1A and 1B show chromatograms from extracts of unspiked liver and liver spiked with a mixture of all four rodenticides, after elution from the Sep-Pak cartridge with methanol–chloroform; the aliquots injected represent 20 mg of liver. Fig. 1C and 1D are from liver, unspiked and spiked with brodifacoum, after elution from the cartridge with chloroform; the aliquots injected represent 200 mg of liver. The absence of the large peak for co-extractives (numbered 4 in Fig. 1A and 1B) from chromatograms C and D, despite the higher concentrations of the extracts producing them, is notable and there is clearly scope for further concentration without interference. The sharpness of the rodenticide peaks in Fig. 1B and 1D is also striking, particularly in view of the rather large flow-cell volume of 20 μl.

In routine application of the method, clean-up was found to be satisfactory for serum, liver, kidney, brain, muscle and stomach or rumen contents from several species.

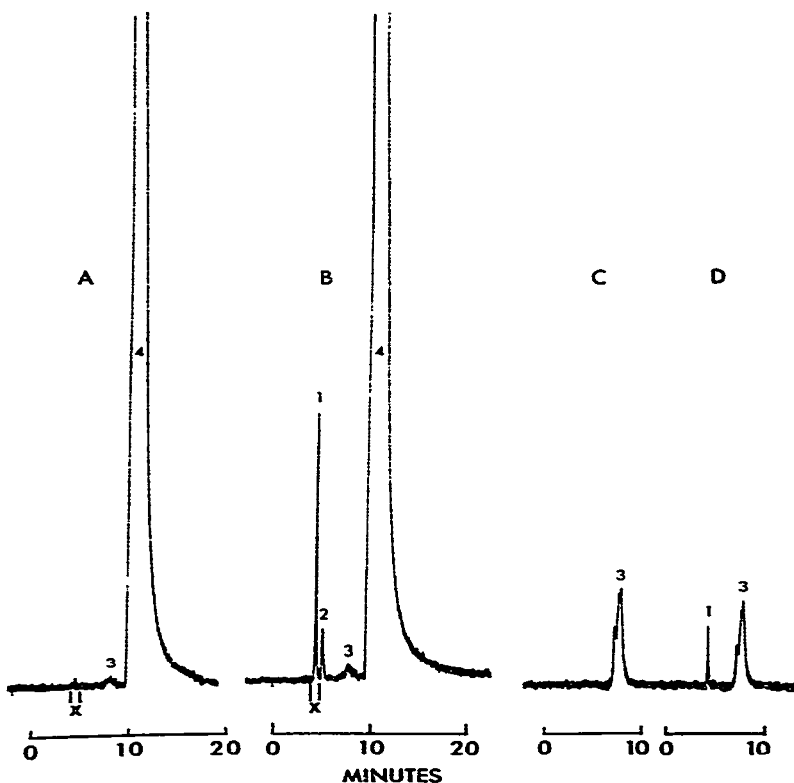


Fig. 1. Exclusion chromatography of liver extracts on porous silica. A, extract equivalent to 20 mg unfortified liver, after clean-up on Sep-Pak silica cartridge and elution therefrom with methanol–chloroform (15:85, v/v); B, as A, but liver spiked with brodifacoum, difenacoum and coumatetralyl (0.1 mg/kg of each) and warfarin (0.5 mg/kg); C, extract equivalent to 200 mg of unfortified liver, after elution from Sep-Pak cartridge with chloroform; D, as C, but liver spiked with brodifacoum (5 μg/kg). Peaks: 1 = brodifacoum, difenacoum and coumatetralyl in B, brodifacoum in D; 2 = warfarin; 3 and 4 = co-extractives. X is the fraction collected for identification by reversed-phase HPLC (see Fig. 3).

Recoveries

Recoveries from spiked liver, serum and stomach contents were examined, as these are normally the most useful materials for diagnostic analysis. Attention was concentrated on liver because it is the more difficult of the two well-defined substrates. In the recovery experiments, five separate samples of each substrate were spiked at each fortification level, either individually with brodifacoum, difenacoum or coumatetralyl or with a mixture of brodifacoum and warfarin.

Recoveries of the four rodenticides added to pig liver at levels of 0.05–1.0 mg/kg, and of brodifacoum added at 5 µg/kg, are given in Table I. Recoveries of brodifacoum and warfarin from serum and stomach contents spiked at 0.05–1.0 and 0.1–1.0 mg/kg, respectively, are shown in Table II. In both Tables, results are presented as the mean percentage recovery at each level of each compound in each

TABLE I

DETERMINATION OF RODENTICIDES BY EXCLUSION HPLC: RECOVERIES FROM SPIKED LIVER

Column, 250 mm × 5 mm I.D., of Magnusil porous silica (5 µm); mobile phase, methanol; flow-rate 1 ml/min. Extraction and clean-up as described in text. Five separate samples at each level analysed. Spiked before homogenization

Fortification level, mg/kg	*Percentage recovery (mean ± 95% confidence interaval)*							
	Brodifacoum		*Difenacoum*		*Coumatetralyl*		*Warfarin*	
1.0	86	± 4.5	96	± 4.2	90	± 7.6	80	± 5.9
0.5	81		100		91		84	
0.1	87		100		87		76	
0.05	88		87		93		65	
0.005	78							

TABLE II

DETERMINATION OF RODENTICIDES BY EXCLUSION HPLC: RECOVERIES FROM SPIKED SERUM AND STOMACH CONTENTS

Conditions as Table I.

Substrate and fortification level, mg/kg	*Percentage recovery (mean ± 95% confidence interval)*			
	Brodifacoum		*Warfarin*	
Serum				
1.0	100	± 6.5	98	± 9.0
0.5	91		96	
0.1	98		96	
0.05	86		90	
Stomach contents				
1.0	92	± 10.4	80	± 5.7
0.1	87		74	

substrate, with the 95% confidence interval for each combination of compound and substrate. (Statistical analysis justified the application of a single 95% confidence interval to all fortification levels of each compound–substrate combination.)

Recoveries of brodifacoum, difenacoum and coumatetralyl at levels of 0.05–1.0 mg/kg were all satisfactory, with mean values of 81–100% and only 6 of 120 individual results below 80%. The recovery of brodifacoum from liver at 0.005 mg/kg (range 71–85%, mean 78%) was also adequate, although lower than recoveries at the higher levels. Recoveries of difenacoum from liver were higher than these obtained previously by adsorption HPLC[2], perhaps because the putative causes of the lower recoveries (transfer of the initial chloroform extractives to methanol and delay between spiking and extraction) were avoided.

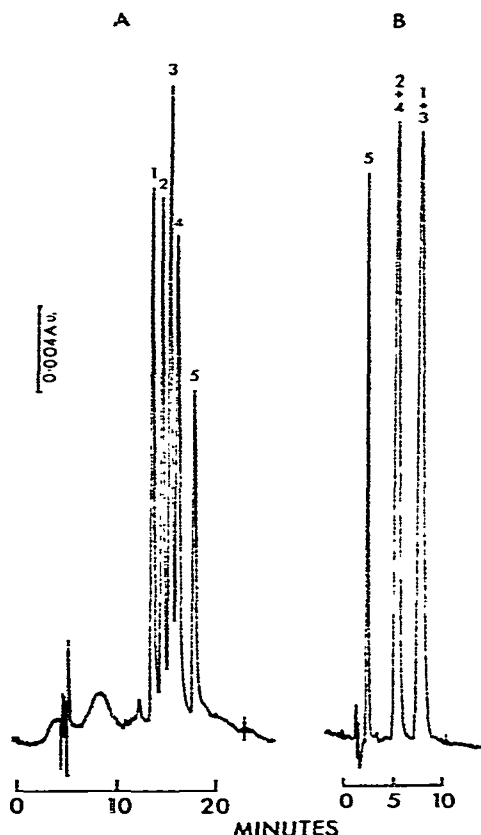
Warfarin was well recovered from serum at all levels: only one of 20 individual results was below 80%. Recoveries from liver and stomach contents were lower (and lower than those obtained from liver by the method previously used at this laboratory[1]), but sufficient for diagnostic purposes. Mean recoveries at fortification levels of 0.1–1.0 mg/kg ranged from 74 to 84%, and all individual values were 70% or higher. The mean recovery from liver spiked at 0.05 mg/kg was 65%, with a range of 62–72%.

As mentioned above, brodifacoum, difenacoum and coumatetralyl were detected with almost identical sensitivity (response relative to mass/volume concentration) under the fluorescence conditions used. In routine work, therefore, standard solutions of any one of the three can be used for the quantitation of any compound(s) eluted at their common retention time, and the compound(s) can subsequently be identified in the collected eluate fraction. The lower limit of determination for these rodenticides by the normal procedure is apparently about 10–20 μg/kg. If analysis for warfarin is not required, however, lower levels are easily determined by applying a more concentrated extract to the Sep-Pak cartridge and eluting with chloroform (see Fig. 1D), when the limit of determination appears to be below 1 μg/kg. Warfarin is much less sensitively detected by fluorescence: its limits of determination in routine use are about 0.05 mg/kg in serum and 0.1 mg/kg in liver.

Substantially lower levels of all four rodenticides can be determined by collecting eluate fractions from successive injections and combining, concentrating and reinjecting them as described elsewhere[1,7]. An internal standard might be needed: warfarin could be used as internal standard for brodifacoum, difenacoum or coumatetralyl, and any of these three would be suitable as an internal standard for warfarin. Warfarin can also be determined at very low levels by specific-ion mass spectrometry[1] in eluates from exclusion columns.

Identification

Brodifacoum, difenacoum and coumatetralyl in collected eluate fractions can be differentiated by adsorption or reversed-phase chromatography, with mobile phases similar to those used by Yuen[4] and Koubek *et al.*[5], respectively: representative chromatograms are shown in Fig. 2. The *cis*- and *trans*-isomers of both brodifacoum and difenacoum are separated on the adsorption column (Fig. 2A). Adsorption is convenient because it is carried out on the exclusion column, and is perhaps the method of choice for certainty of identification. Reversed-phase chromatography (Fig. 2B) is quicker, however, and the chromatogram is simpler. It is to be preferred



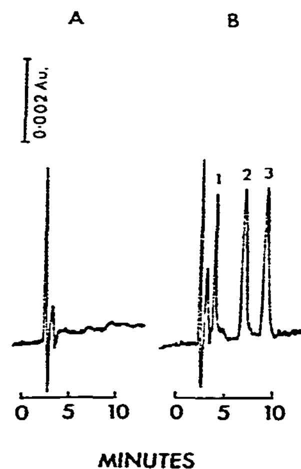


Fig. 2. Separation of brodifacoum, difenacoum and coumatetralyl (40 ng of each) by (A) adsorption chromatography on porous silica and (B) reversed-phase chromatography on a docosyl bonded phase. Peaks: 1 and 3 = isomers of brodifacoum; 2 and 4 = isomers of difenacoum; 5 = coumatetralyl.

Fig. 3. Identification of brodifacoum, difenacoum and coumatetralyl by reversed-phase chromatography. Eluate fractions X (see Fig. 1, A and B) from exclusion column chromatographed on reversed-phase column. A, unfortified liver, as Fig. 1A; B, fortified liver, as Fig. 1B. Peaks: 1 = coumatetralyl; 2 = mixed isomers of difenacoum; 3 = mixed isomers of brodifacoum.

for quantitative determinations if more than one of the three rodenticides is present. Fig. 3A and 3B show reversed-phase chromatograms from the extracts of unspiked and spiked liver whose exclusion chromatograms are shown in Figs. 1A and 1B. Ten successive aliquots of the cleaned-up liver extracts were injected on to the exclusion column, and the eluate fractions marked "X" in Fig. 1A and 1B were collected and taken to dryness, the residues being dissolved in 50 μl of the reversed-phase eluent. Fig. 3B shows the separation of coumatetralyl, difenacoum and brodifacoum in that order.

The sensitivity of detection by UV absorption is much lower than by fluorescence, but is again closely similar, when measured by peak height, for the three compounds in eluates from the reversed-phase column. The brodifacoum and difenacoum peaks are notably broader than those from coumatetralyl (Figs. 2B and 3B), however, possible owing to the incipient resolution of *cis*- and *trans*-isomers.

CONCLUSIONS

Brodifacoum, difenacoum, coumatetralyl and warfarin can be rapidly and sensitively determined in animal materials by exclusion HPLC on porous silica. The method appears to be applicable to a wide range of substrates.

If analysis is for all four rodenticides, the lower limits of determination are about 0.05–0.1 mg/kg for warfarin and about 0.02 mg/kg for brodifacoum, difenacoum and coumatetralyl. If warfarin is not included in the analysis, a more selective clean-up lowers the limit for the other three compounds to about 1 μg/kg or below. The method can be extended to determine sub-μg/kg levels of all four compounds.

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