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DETERMINATION OF THE RODENTICIDE DIFENACOUM IN BIOLOGICAL MATERIALS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH CONFIRMATION OF IDENTITY BY MASS SPECTROMETRY

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SUMMARY

A method for determining difenacoum in liver, plasma, urine and feedingstuffs by high-pressure liquid chromatography is described. Samples are cleaned up by molecular exclusion chromatography on porous glass. In some cases this also serves for determination; if not, the separated difenacoum is determined on an adsorption column. Identity is confirmed by chemical ionisation mass spectrometry.

Recoveries at levels of 0.025–5 ppm from plasma were 101–113% by exclusion chromatography alone and 93–101% after adsorption chromatography. Recoveries from liver after both chromatographic steps were 62–86%. Reasons for the lower recoveries from liver are suggested.

INTRODUCTION

Difenacoum is the common name** for 3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin. The usual product is a mixture of isomers thought to be *cis* and *trans*[1]: this is supported by the nuclear magnetic resonance spectra of the separate isomers, where the 1-proton of the tetrahydronaphthalene ring resonates at δ 4.90 or 4.75. It is an anticoagulant rodenticide which is effective against warfarin-resistant rats[2,3]. A method of identifying and determining it was required as an aid to diagnosis in cases of suspected poisoning of farm animals and wildlife. Recently developed methods of analysis for other coumarin anticoagulants, including warfarin and its metabolites, at residue levels have been based on gas chromatography[4–7] or high-pressure liquid chromatography (HPLC)[8–12]. Difenacoum is too involatile for convenient determination by gas chromatography but has been separated from warfarin and some other anticoagulants by HPLC[8]. The clean-up of biological samples by the pyrophosphate procedure used for warfarin residues[8,13] would not be effective

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** Recommended by the British Standards Institution; a draft common name of the International Organisation for Standardization (ISO).

however, because difenacoum is insufficiently polar. Preliminary attempts to analyse tissue extracts by adsorption HPLC without prior clean-up gave good results with new columns, but contamination by co-extractives was so rapid as to make the procedure impracticable for regular use. Clean-up by molecular exclusion chromatography, which has been used successfully for other pesticides (see Masud *et al.*[14], Pflugmacher and Ebing[15] and Johnson *et al.*[16], where other references are given), seemed likely to be successful, particularly as difenacoum would probably be eluted before rather than after its co-extractives.

This communication describes a method for the determination of difenacoum at residue levels in animals tissues, body fluids and feedingstuffs by HPLC. An extract of the sample is cleaned up by molecular exclusion chromatography on a column of porous glass. In some cases this suffices for determination: otherwise the difenacoum fraction is collected and analysed by HPLC on an adsorption column. Identity is confirmed by chemical ionisation mass spectrometry (CI-MS).

EXPERIMENTAL

Materials and apparatus

The sources of anticoagulant rodenticides were as previously specified[8]. Anhydrous sodium sulphate, chloroform, methanol, isopropyl alcohol and iso-octane (2,2,4-trimethylpentane) were of analytical reagent grade. In preliminary work, chloroform used as a minor component of eluent solutions was washed, dried and distilled before use: this procedure did not affect the chromatographic properties of the eluent and was subsequently omitted.

The liquid chromatograph with UV detector, mass spectrometer with CI source and homogeniser were previously described[8].

Extraction

Solid samples (10 g) were macerated with anhydrous sodium sulphate (20 g) and chloroform (30 ml). The extract was filtered through sintered glass, the residue re-extracted with chloroform (15 ml) and again filtered. Plasma and urine (10 ml) were extracted with 15 and 10 ml of chloroform; the extract was dried with sodium sulphate and filtered. The chloroform filtrates were evaporated to dryness under a stream of nitrogen and re-dissolved in methanol (0.5 ml).

Exclusion chromatography

The column was stainless steel, 2 m × 4 mm I.D., packed with Bio-Glas 200, 200–325 mesh (Bio-Rad Labs., Bromley, Great Britain), a porous glass with average pore diameter 200 Å. The eluent was methanol at a flow-rate of 2.5 ml/min and UV detection was at 260 nm. As a pneumatic amplifier solvent pump was used, a restrictor of high resistance in the solvent delivery tube was necessary to avoid an excessive flow-rate.

Replicate aliquots of the methanolic extract (100 μl, the largest volume that could be used without danger of overloading the column with co-extractives) were injected. The difenacoum content was estimated by comparing the mean peak heights with those produced by standard solutions. Normally four aliquots of each extract were injected and the eluate fractions containing difenacoum (eluted before the main

bulk of the UV-absorbing material) were combined for adsorption chromatography or mass spectrometry.

Adsorption chromatography

The column was stainless steel, 0.5 m × 1.5 mm I.D., packed with Corasil II (Waters Assoc., Stockport, Great Britain). The eluent was isopropanol–chloroform–isooctane (1:2:397) and the flow-rate 1 ml/min.

The combined difenacoum fractions from the exclusion column were evaporated to dryness under a stream of nitrogen and the residue re-dissolved in the eluent for the adsorption column (100 μl). Duplicate aliquots (10 μl) were injected. The isomers of difenacoum were eluted as two well-separated peaks. For quantitative measurement, the height of the earlier peak was compared with that produced by appropriate standards. To confirm identity the two fractions believed to contain the difenacoum isomers were collected, the remaining cleaned-up difenacoum extract was chromatographed and the combined difenacoum eluates were examined by MS.

Mass spectrometry

The solvent was evaporated from the difenacoum fractions and the residue transferred in acetone to a capillary tube. The acetone was evaporated and the capillary heated in the solid probe of the mass spectrometer with methane, at a pressure of about 1 torr, as reagent gas. The spectrum of difenacoum appeared at a temperature of 225°, reached after about 2½ min.

RESULTS AND DISCUSSION

In preliminary experiments the apparent recovery of difenacoum added to extracts of livers immediately before exclusion chromatography, at a level equivalent to 1 ppm in the liver, ranged from 104 to 114%. In later recovery experiments with liver and plasma, difenacoum was added to freshly thawed material, which had been stored at −20°, before extraction. Liver and plasma were chosen for detailed examination because they are usually the most useful materials for diagnosis and liver is particularly difficult to analyse.

Exclusion chromatography

Fig. 1 shows chromatograms of extracts of pig liver and plasma, with and without added difenacoum. Difenacoum is eluted as a single peak well before the bulk of the UV-absorbing co-extractives, although the chromatogram of unfortified liver extract shows a peak in the same position as difenacoum. When extracts of livers from 2 cows, a dog, fox and horse were chromatographed, all except that from the fox showed the interfering peak, but it was virtually absent from extracts of plasma from several other species, urine, oats and milk powder. It is clear that the extracts are effectively cleaned up by the porous glass column, at least for the purpose of subsequent adsorption chromatography with UV detection.

When the other commonly used anticoagulant rodenticides warfarin, coumatetralyl and chlorophacinone, and the main mammalian metabolites of warfarin, were chromatographed, all except coumatetralyl were eluted in the main co-extractive fraction but coumatetralyl was eluted at the same retention time as difenacoum. This

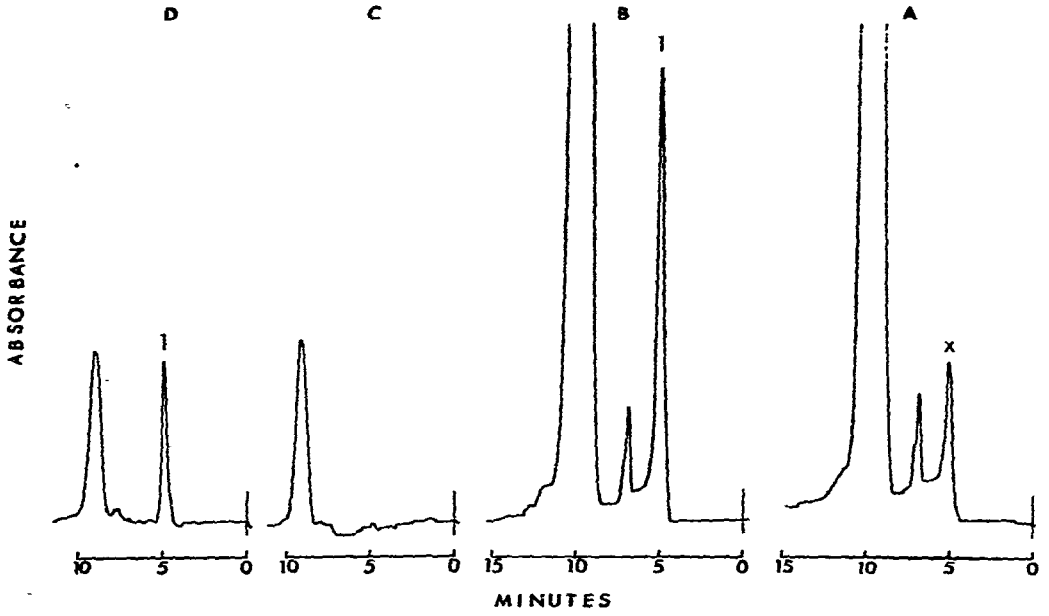

Fig. 1. Molecular exclusion chromatography of extracts from (A) 2 g unfortified liver, (B) 2 g liver + 1 ppm difenacoum, (C) 2 ml unfortified plasma, and (D) 2 ml plasma + 0.1 ppm difenacoum. 1 = Difenacoum; × = co-extractive at difenacoum retention time. A and B at 0.2, C and D at 0.05 absorbance units per mV.

was surprising, since the behaviour of coumatetralyl on an exclusion column might be expected to resemble that of warfarin rather than difenacoum. Clearly, however, difenacoum cannot be differentiated from coumatetralyl by this procedure.

For calibration, 100-μl aliquots of solutions containing 0.5–100 ppm of difenacoum in methanol were injected in triplicate. The response was linear over this concentration range (*i.e.*, the range 0.05–10 μg injected, corresponding to levels of 0.025–5 ppm in an original sample of 10 g or 10 ml) and was described by the equation:

$$h = (155.1 \pm 0.503)\, w + 15.9$$

where h represents the peak height (mm) at a detector sensitivity of 0.02 absorbance units for a sigmal of 1 mV (250 mm) and w the weight of difenacoum injected (μg). The coefficient of w is shown as mean $\pm$ standard error.

Difenacoum was added to pig plasma and liver over the range 0.025–5 ppm. Apparent recoveries from plasma are shown in Table I as means from five separate samples at each level with their 95% confidence limits, the result for each sample being the mean from quadruplicate injections. Results were slightly high and notably consistent over the range 0.05–5 ppm: at 0.025 ppm rather higher but still acceptable. Unfortified plasma gave a small peak in the difenacoum position, equivalent to about 0.005 ppm in the original plasma or 20% of the lowest level of difenacoum examined. For the purposes of residue analysis, contamination at this level can usually be ignored and it appears that difenacoum can be determined in plasma, at levels of 0.025 ppm and above, by exclusion chromatography of chloroform extracts.

TABLE I

RECOVERY OF DIFENACOUM FROM PLASMA BY EXCLUSION HPLC

Difenacoum was added to plasma before extraction with chloroform. Column, 2 m × 1.4 mm I.D., Bio-Glas 200; mobile phase, methanol; pressure, 45 kg/cm² (with flow restrictor); flow-rate, 2.5 ml/min.

Difenacoum added (ppm)	*Recovery (%), mean (95% confidence limits) from 5 separate samples of plasma*
0.025	113 (110–116)
0.05	104 (101–107)
0.1	106 (103–109)
1	101 (98–104)
2	105 (102–108)
5	105 (102–108)

The interfering peak from the liver used in the recovery experiments represented an apparent difenacoum content of about 0.4 ppm. Recoveries of difenacoum added over the range 0.025–1 ppm varied widely; at higher levels they were more consistent, but low. It appeared that exclusion chromatography alone would not suffice for determination in pig liver.

Adsorption chromatography

It had previously been established that warfarin, coumatetralyl, chlorophacinone and difenacoum could be separated from one another and from metabolites of warfarin by HPLC on a column of Corasil II, with 2% isopropyl alcohol in isooctane as eluent[8]. This solvent system was designed for the determination of warfarin and is too polar for determining difenacoum on the Corasil column. With the isopropanol–chloroform–isooctane eluent used in the present work, the two isomers of difenacoum were eluted as well-separated peaks, the first about three times the height of the second, and coumatetralyl was eluted slightly later than the first difenacoum isomer. Chromatography on columns of LiChrosorb SI-60 and Spherisorb ODS was also examined, but peaks from difenacoum tailed more than on the Corasil column when eluents giving convenient retention times were used. The separation of the difenacoum isomers and coumatetralyl on the Corasil column is illustrated in Fig. 2. Chlorophacinone, warfarin and its metabolites were eluted so slowly as to be undetectable at the levels likely to be encountered; they would in any case be separated from difenacoum on the exclusion column. The separation between the first difenacoum isomer and coumatetralyl is small, but sufficient for the two peak heights to be separately measured if the compounds are present at similar concentrations. If the extract contained only one of them, the presence or absence of a peak at the retention time of the second difenacoum isomer would be diagnostic.

For calibration, 10-μl aliquots of the isopropanol–chloroform–isooctane mixture containing 2.5–100 ppm (i.e., 0.025–1 μg) of difenacoum were injected in triplicate. These levels corresponded to 0.03–1.25 ppm in a 10-g sample. The response to the earlier isomer was linear according to the equation $h = (341 \pm 1.6)\, w - 1.86$,

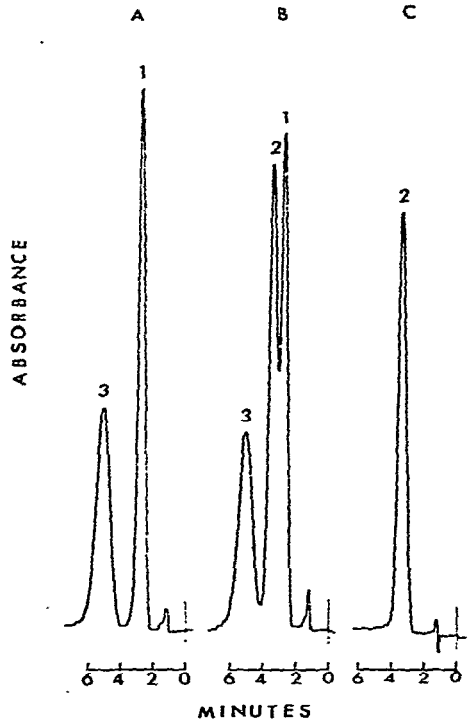

Fig. 2. Adsorption chromatography of solutions of (A) difenacoum, (B) difenacoum + coumatetralyl and (C) coumatetralyl. 1 and 3 = isomers of difenacoum; 2 = coumatetralyl.

where h and w have the same meanings as before. The sensitivity was higher than on the exclusion column because the peaks were narrower.

Recoveries of difenacoum from plasma and liver fortified at 0.025–5 ppm are given in Table II. They are based on the injections of duplicate 10-μl aliquots of collected eluates from the exclusion column and include losses incurred by both chromatographic steps. The result at each level is the mean from five separate samples with its 95% confidence limits.

Mean recoveries ranged from 93 to 101% for plasma and from 62 to 86% for liver. Since no response to unfortified plasma or liver was detected, there is scope for determining lower levels by increasing the sample size. Although recoveries from liver were low, their consistency indicated that the method was adequete for residue analysis.

Since recoveries were low when difenacoum was added directly to liver but not when it was added after extraction with chloroform and transfer to methanol, two possible sources of loss were suspected. One was a small waxy residue, insoluble in methanol, which remained after evaporation of chloroform from the initial extract and which might retain some of the difenacoum. The other was metabolic degradation. The possibility of appreciable metabolism had originally been discounted, partly because the conditions of fortification made it unlikely and partly because it had apparently not been a factor in similar experiments with the related compound warfarin[8].

TABLE II

RECOVERY OF DIFENACOUM FROM PLASMA AND LIVER BY ADSORPTION HPLC

Plasma fortified as in Table I. Liver fortified with difenacoum, then macerated with sodium sulphate and chloroform. Extracts chromatographed on exclusion column and eluate fractions therefrom on adsorption column. Exclusion chromatography as in Table I. Adsorption column, 0.5 m × 1.5 mm I.D., Corasil II; mobile phase, isopropanol–chloroform–isooctane (1:2:397); pressure, 20 kg/cm²; flow-rate, 1 ml/min.

Difenacoum added (ppm)	*Recovery (%), mean (95% confidence limits) of 5 separate samples*	
	Plasma	*Liver*
0.025	94 (90– 97)	62 (52–71)
0.05	95 (91– 98)	68 (59–78)
0.1	95 (92– 98)	73 (63–83)
1	98 (95–101)	86 (76–95)
2	100 (97–103)	72 (63–82)
5	101 (98–104)	83 (74–93)

To examine these possibilities difenacoum was added to a chloroform extract of unfortified liver, to a blank extract after transfer to methanol and to portions of liver which were then allowed to remain at ambient temperature (18°) for varying periods before extraction. Recoveries of difenacoum are shown in Table III. It appears that some loss occurs during transfer from chloroform to methanol and some as a result of metabolism. In the original recovery experiments, the interval between fortification of the liver and its maceration with chloroform varied from about 2 to 15 min. The loss on transfer to methanol was not thought sufficient to necessitate modification of the method.

TABLE III

RECOVERY OF DIFENACOUM ADDED TO LIVER OR LIVER EXTRACT AT VARIOUS STAGES

Difenacoum added to liver at various intervals before extraction, and to extracts. All additions equivalent to 2 ppm in liver. Samples chromatographed on exclusion and adsorption columns. Conditions as in Tables I and II.

Difenacoum added to	*Recovery (%) (mean of duplicate injections)*
Extract after transfer to methanol	98
Initial extract	88
Liver, 30 sec before extraction	83
3 min before extraction	82
10 min before extraction	78
30 min before extraction	68

Mass spectrometry

The base peak of the CI spectrum of difenacoum was at *m/e* 163 and presumably represented the protonation of 4-hydroxycoumarin or an isomeric rearrangement product. At masses above 300 a.m.u. only the characteristic ions at *m/e* 445 (M + H,

41% of the base peak), 473 ($M + C_2H_5$, 9%) and 485 ($M + C_3H_5$, 3%) showed intensities above background levels. The low mass of the base peak makes it of little value for identification, but the peaks representing the molecular ion were above the mass of most contaminants and well suited for the confirmation of identity.

Difenacoum was much less sensitively detected by CI-MS than was warfarin[8], although its initial volatilization from the heated probe was sharp. After some 20 sec, the spectrum decreased rapidly to a much lower intensity, then faded slowly to an undetectable level during periods up to 30 min. It appeared that difenacoum which had evaporated from the heated probe condensed on the ion source of the spectrometer, from which it again slowly evaporated. Because of this slow evolution, about 200 ng was needed for a satisfactory spectrum. This quantity would be present in 10 g of material containing 0.02 ppm however, so the lower limit of detection is similar to that of the chromatographic procedures. For comparison, the lower limit of visual detection after thin-layer chromatography on fluorescent plates was about 1 μg.

The extent of clean-up needed for MS was examined. In the case of plasma, the combined exclusion column eluates from four successive 100-μl injections of the concentrated uncleaned blank extract gave a spectrun with no peaks above *m/e* 350. Bulked eluate fractions from the exclusion column would therefore be suitable for MS. Eluates from the exclusion chromatography of liver extracts sometimes showed interfering peaks when the entire fraction was examined, but not when the eluate from a single 100-μl injection was used. Since this represented one fifth of the total sample and 200 ng was required for an adequate spectrum, MS of the eluate from the exclusion column should be effective if the sample contained 0.1 ppm or more of difenacoum.

CONCLUSIONS

(1) Difenacoum residues in liver and plasma can be determined by succesive exclusion and adsorption HPLC. Recoveries are satisfactory at concentrations from 0.025 to 5 ppm.

(2) Since exclusion chromatography of extracts of urine, oats and milk powder showed no peaks near the retention time of difenacoum, the method should be suitable for an extensive range of tissues and feedingstuffs.

(3) The absence of interfering peaks in adsorption chromatograms of blank substrates implies that larger samples could be taken and lower levels determined if required.

(4) Exclusion chromatography alone was adequate for the analysis of plasma, and should be successful for other substrates free from UV-absorbing co-extractives eluted in the same position as difenacoum.

(5) Identity can be confirmed by CI-MS: the quantity needed would depend upon the equipment available, and was about 200 ng in the work described. The concentrated eluate from the exclusion column representing 10 ml of plasma or 2 g of liver is sufficiently clean for CI-MS.

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REFERENCES

1 M. R. Hadler and R. S. Shadbolt, *Nature (London)*, 253 (1975) 275.
2 M. R. Hadler, R. Redfern and F. P. Rowe, *J. Hyg.*, 74 (1975) 441.
3 B. D. Rennison and M. R. Hadler, *J. Hyg.*, 74 (1975) 449.
4 H. S. Funnell and N. Platonow, *J. Forensic Sci.*, 15 (1970) 601.
5 D. G. Kaiser and R. S. Martin, *J. Pharm. Sci.*, 63 (1974) 1579.
6 K. K. Midha, I. J. McGilveray and J. K. Cooper, *J. Pharm. Sci.*, 63 (1974) 1725.
7 E. M. Odam and M. G. Townsend, *Analyst (London)*, 101 (1976) 478.
8 D. E. Mundy, M. P. Quick and A. F. Machin, *J. Chromatogr.*, 121 (1976) 335.
9 R. Vanhaelen-Fastré and M. Vanhaelen, *J. Chromatogr.*, 129 (1976) 397.
10 R. A. O'Reilly and C. H. Motley, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 35 (1976) 756.
11 M. J. Fasco, L. J. Piper and L. S. Kaminsky, *J. Chromatogr.*, 131 (1977) 365.
12 T. D. Bjornsson, T. F. Blaschke and P. J. Meffin, *J. Pharm. Sci.*, 66 (1977) 142.
13 F. B. Fishwick and A. Taylor, *Analyst (London)*, 92 (1967) 192.
14 Z. Masud, V. Bátora and J. Kovačičová, *Pestic. Sci.*, 4 (1973) 131.
15 J. Pflugmacher and W. Ebing, *J. Chromatogr.*, 93 (1974) 457.
16 L. D. Johnson, R. H. Waltz, J. P. Ussary and F. E. Kaiser, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 174.