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HPLC Determination of Anticoagulant Rodenticide Residues in Animal Livers

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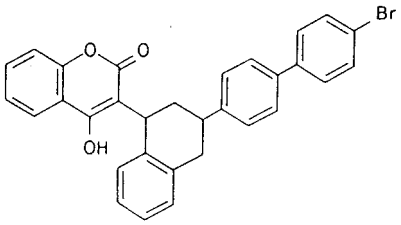
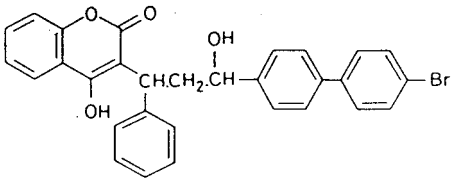
Anticoagulant rodenticides based on a 4-hydroxycoumarin ring structure have been used for many years to control a variety of rodents. In the United Kingdom, six coumarin anticoagulants are currently approved for use (Figure 1). This laboratory investigates suspected cases of poisoning of non-target animals by rodenticides and other pesticides through the Ministry of Agriculture, Fisheries and Food's Wildlife Incident Investigation Scheme. Coumarin anticoagulants are slow acting poisons. Residues are usually absent from the stomach contents of poisoned animals. Of the other internal tissues, liver generally contains the highest residues of coumarin anticoagulants making it the most diagnostically useful tissue. Liver concentrations in poisoned animals are usually below 1 mg/kg and sometimes below 0.1 mg/kg. Sensitive and selective analysis methods for determining coumarin-based anticoagulants are therefore required to diagnose poisoning and monitor exposure.

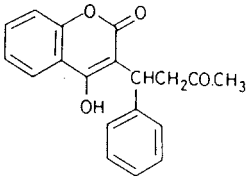
The low volatility of these compounds makes high-performance liquid chromatography (HPLC) the method of choice for their determination. A number of HPLC methods have been described for the determination of individual coumarin-based anticoagulants in biological materials including methods for warfarin (Mundy et al. 1976, Lee et al. 1981, de Vries et al. 1993), difenacoum (Mundy et al. 1977, Kelly et al. 1993) and brodifacoum (Koubek et al. 1979, Kieboom et al. 1981, Hogenboom et al. 1983). Multi-residue methods have also been described: Mundy and Machin (1982) used HPLC to determine four coumarin anticoagulants, but difenacoum, brodifacoum and coumatetralyl were not resolved and had to be identified by further HPLC analysis of the fractions from the column. Reynolds (1980) determined six coumarin anticoagulants in baits and stomach contents using HPLC, but the method was not sufficiently sensitive for sub-ppm determination. Hunter (1983a, 1983b, 1985) described a number of HPLC methods for the sensitive determination of coumarin anticoagulants. In this laboratory the reversed-phase HPLC method of Hunter (1983a) has proven useful for routine screening of liver samples for coumarin anticoagulants. In that procedure, samples are extracted with chloroform:acetone (50:50), the



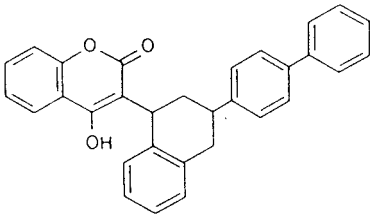
Brodifacoum



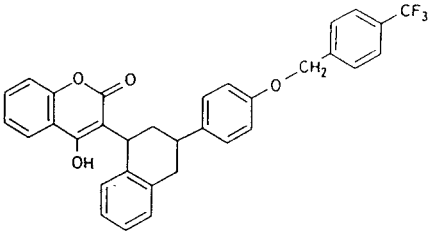
Bromadiolone



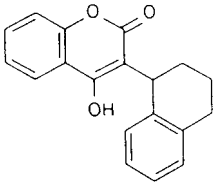
Coumatetralyl



Difenacoum



Flocoumafen



Warfarin

Figure 1. Structures of the six coumarin anticoagulants investigated in this study

crude extracts cleaned-up by gel permeation chromatography (GPC),and the analytes determined by HPLC on a C_{18} column at a pH of 3.3. In order to use, for detection, the native fluorescence of these compounds at high pH, a basic solution is added post-column. Although sensitive and selective, the clean-up procedure is laborious and time-consuming and uses large volumes of solvents. Additionally the HPLC procedure does not separate difenacoum and flocoumafen, the latter compound having come into use since the method was described. A simple and reliable method was required to determine the six compounds in Figure 1, at sub-ppm level, in liver tissue.

This paper presents an HPLC method that enables the determination of six coumarin anticoagulants in liver tissue. A simple extraction and clean-up by alumina cartridge is employed which gives rise to considerable reductions in solvent use compared to the previously used GPC clean-up of Hunter (1983a). The analytes are separated by gradient elution HPLC on an Cl8 column with fluorescence detection.

MATERIALS AND METHODS

Anticoagulant standards were obtained from Greyhound Chemical Co., Birkenhead, UK. HPLC-grade water, methanol and dichloromethane and glass-distilled-grade acetone were from Rathburn Chemical Co. Ltd., Walkerburn, Scotland. Acetic acid (glacial) and 'ammonia 880' (0.88 g/mL aqueous solution), both of analytical grade, were from BDH Ltd., Poole, UK. Sodium sulphate (analytical grade), also from BDH Ltd, was heated to 400°C for 12 hr before use. 'Sep-Pak' Alumina N cartridges (1850 mg) from Waters Ltd., Watford, UK were used for clean-up.

The HPLC system was comprised of two model 510 pumps, WISP 712 autosampler, Baseline 810 work station (Waters, Watford, UK), Shimadzu RF-535 fluorescence detector with excitation wavelength of 310 nm, emission wavelength of 390 nm (Dyson Instruments, Houghton-le-Wear, UK) and a Spherisorb ODS column, 250 x 4.6 mm with Spherisorb ODS guard column, 10 x 4.6 mm (HPLC Technology, Macclesfield, UK). The HPLC conditions were as follows: Solvent A, 0.25%(v/v) acetic acid in water; solvent B, 0.25%(v/v) acetic acid in methanol. Solvent B was linearly increased from 25 to 80% over the first 1.5 min, then to 100% over the next 7.5 min, maintained at 100% for 8 min and then decreased to 25 % over the next 1 min. This was then maintained for 5 min. The flow rate was kept constant at 0.8 mL/min.

An additional pump (Waters 510) was used to deliver the post-column solution of 12% (v/v) aqueous 'ammonia 880' at 0.3 mL/min. This was mixed with the column effluent via a stainless steel T-fitting and a 0.5 m x 0.25 mm id length of stainless steel tubing connected to the detector inlet.

Liver tissue (up to 1 g) was cut into small pieces and ground with 10 x the sample weight of sodium sulphate using a pestle and mortar. The mixture was transferred to a 100-mL screw-top conical flask and shaken with 15 ml extraction solvent (30%v/v acetone in dichloromethane) on the extraction apparatus (IKA model HS250, Janke and Kunkel, Germany) at 300 oscillations/min for 1 hour. The extract was then decanted into a centrifuge tube, spun at 1000g (Minifuge 2, Hereaus, Brentwood, UK) for 10 min, and the supernatant taken. A further 10 mL of extraction solvent was added to the conical flask which was shaken for a further 30 min. This extract was again

centrifuged at 1000g for 10 min and the supernatants were combined in a 25-mL volumetric flask which was made to volume with extraction solvent.

The 'Sep-Pak' cartridge was prepared by attaching to a 10-mL syringe and conditioning with 10 mL of dichloromethane. 10 mL of sample extract was added to the syringe and eluted through the column at 3-5 mL/min. The column was washed with a further 10 mL of extraction solvent and then with 2 mL of dichloromethane:acetone (25:75). The anticoagulants were eluted with 5 mL of a 5% solution of acetic acid in methanol. This was concentrated to dryness by standing in water at 70-80°C under a stream of nitrogen, and the residuum was dissolved in 0.5 mL methanol. 50 µL injections of samples and external standards were made onto the HPLC system. Quantitation was by comparison of peak height of standards and samples.

RESULTS AND DISCUSSION

In attempting to develop a satisfactory separation of the anticoagulants methanol was the preferred organic component of the mobile phase. Mobile phases containing acetonitrile have been reported to separate difenacoum, brodifacoum and flocoumafen into their separate isomers (Kelly *et al.* 1993). Whilst this is of value in detailed pharmacokinetic studies, it adds unnecessary complexity to investigations of poisoning cases. Thus the aim was to develop an acceptable separation of the components from each other without resolving any component into its individual isomers. Hunter (1983a) used C18 columns with 0.25 % solutions of acetic acid in methanol and water and a gradient program from 65-95% methanol, but this system did not resolve flocoumafen and difenacoum. Other columns were evaluated to try to improve the separation by altering selectivity whilst retaining methanol as the organic mobile phase component. Columns with bonded straight-chain alkanes gave much better separation than columns with bonded functionalities such as cyano or phenyl functional groups. Of the former, Cl8 columns gave superior separations to C8 columns. Since attempts to alter selectivity in order to separate flocoumafen and difenacoum had not proved fruitful, an improvement in the efficiency of the separation was sought instead. Starting the gradient at 25% methanol appeared to result in the analytes being concentrated in a narrower band at the head of the column, and resolution was improved. The relatively low flow-rate also contributed to narrow band widths. The gradient system employed produced satisfactory separation of the six anticoagulants (Figure 2A). An LC pump was used to deliver post column solution in this study but a cheaper alternative, such as a single piston reciprocating pump can be used at the expense of increased baseline noise. A 0.5 m length of tubing was sufficient to ensure mixing of the post column solution and column eluant; longer lengths of tubing did not result in improved responses.

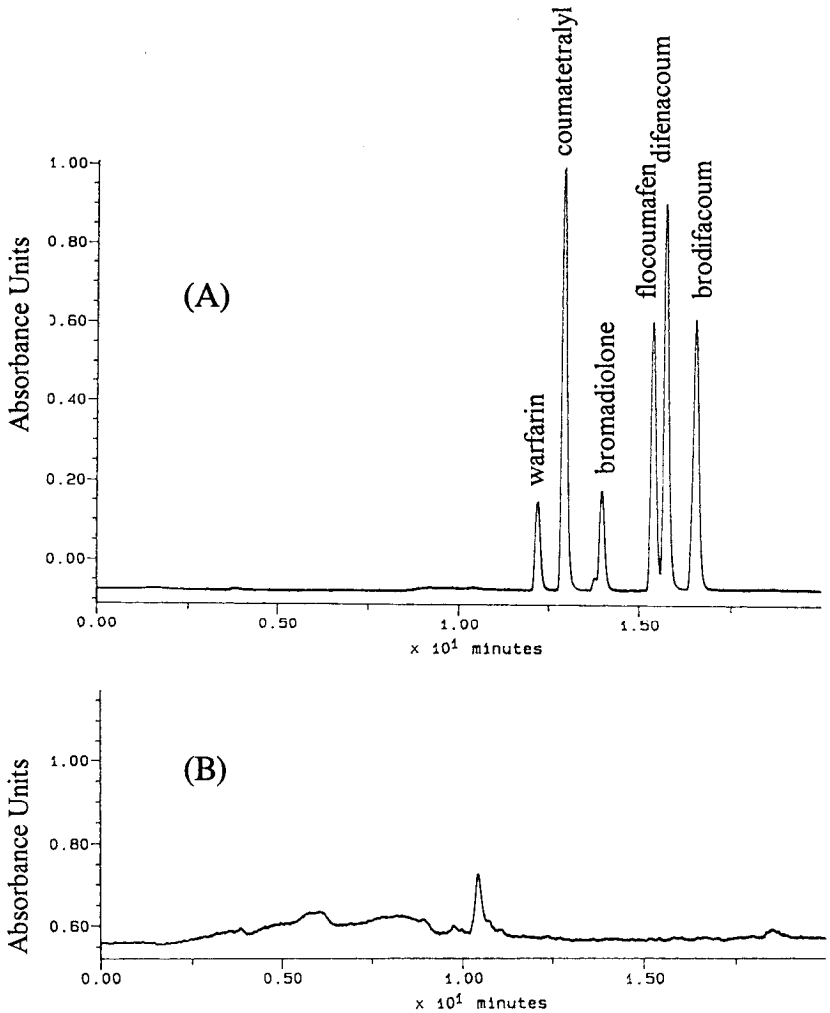


Figure 2 High Performance Liquid Chromatograms, 50 µl injections
(A) Standard mixture of six anticoagulants, 0.16 µg/mL each.
(B) Blank Cat Liver

For determination at sub-ppm levels, a clean-up is required. The clean-up procedure described here uses disposable columns packed with neutral alumina. The alumina in the columns used in this study was of Brockman activity grade 1, and all the anticoagulants studied were strongly adsorbed, allowing most co-extractives to be eluted from the column with mixtures of dichloromethane and acetone. A solution of acetic acid in methanol gave quantitative elution of the anticoagulants. The simplicity of the procedure allows several samples to be cleaned-up simultaneously. The clean-up proved capable of removing most of the lipid material from the extract and provided an adequate chromatographic

Table 1. Recoveries of anticoagulants from liver tissues (mean percentage ± standard deviation)

	Amount added (mg/kg)		
	0.04	0.20	1.00
Warfarin	96±7.6	91±6.8	96±6.0
Coumatetralyl	87±9.6	84±5.2	90±7.6
Bromadiolone	84±14.2	77±8.8	78±5.0
Flocoumafen	85±9.6	86±7.6	85±7.1
Difenacoum	91±11.4	88±9.5	90±5.4
Brodifacoum	88±9.9	88±4.9	87±6.6

background for low level determination (Figure 2B). A small amount of fat remained after the clean-up but this did not prevent the extract being re-dissolved in methanol after evaporation, and prolonged use of the method has not caused excessive column deterioration.

Accuracy and precision were evaluated by measuring recoveries. Each recovery was performed by spiking a 1-g sub-sample of liver with a mixture of the six anticoagulants. Six different liver samples were used; two cat livers and one each from a badger, fox, dog and buzzard. The results are given in Table 1. The mean overall recovery was 87 % with a standard deviation of 7.9 % . The recovery experiments were performed over six separate days and on each day three sub-samples from the same liver were spiked, one at each of the three levels used. A sub-sample of each liver was also analysed unspiked. Thus, the standard deviations given in Table 1 give an estimate of between-run variation. Overall, the results demonstrate that the method is suitable for quantitative determination in livers from a range of animals.

Limits of detection in liver tissue, (defined as the concentration that produces a signal to noise ratio of 3:1) are 0.010 mg/kg for warfarin and bromadiolone and 0.002 mg/kg for the other four anticoagulants

The method has been used in this laboratory for 18 months and has proved reliable and robust as well as producing considerable savings in time and cost. The analysis time to screen a batch of six samples is 7-8 hours, compared to 18-20 hours by the previous method (Hunter 1983a). Solvent consumption is cut by approximately 85%. Occasional interfering peaks are observed but these can usually be distinguished from anticoagulants by re-chromatographing the extract with the post-column solution pump switched off. The responses of the anticoagulants are reduced by 90-95 % under these conditions, providing an easy way of distinguishing them from chromatographic interferences.

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