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Determination of Brodifacoum in Animal Tissues by HPLC

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Brodifacoum (3-[3-(4'-bromophenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin) is a recently developed (HADLER and SHADBOLT 1975) anticoagulant with a high toxicity for the rat. The compound is being considered for vertebrate pest control in New Zealand, mainly against the rabbit, opossum, and wallaby (RAMMELL and FLEMING 1979). Before brodifacoum can be recommended for use in the field, environmental hazards need to be assessed. This assessment necessitates the analysis of brodifacoum residues in dosed and exposed animals.

YUEN (1978) described a high pressure liquid chromatography (HPLC) method for brodifacoum in baits but this is unsuitable for animal tissues. A method was therefore developed for determining brodifacoum in a variety of animal tissues and fluids.

MATERIAL AND METHODS

Brodifacoum material. Technical and pure brodifacoum were supplied by Imperial Chemical Industries (Yalding, Great Britain). The technical brodifacoum was labelled as 94% active ingredient and the pure as 98.6%.

Brodifacoum calibration. Pure brodifacoum (100 mg) was dissolved in 40 ml of dichloromethane and then diluted to 100 ml with methanol. One ml of this stock solution was diluted to 100 ml with HPLC eluant, methanol/water/acetic acid (85/15/1, v/v), for analysis. Spectral characteristics were recorded on a Perkin Elmer 402 spectrophotometer and a Baird Atomic SF-100 spectrofluorimeter, respectively.

Tissue samples. Samples were obtained from necropsied animals that had been orally dosed with sub-lethal and lethal doses of technical brodifacoum.

Extraction of samples. Minced tissue (5 g) was shaken for 30 minutes with 25 ml of extraction solvent, dichloromethane/cyclohexane (50/50, v/v). Sodium sulphate (5 g) was added to the extract before

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filtering through 5 g of sodium sulphate. The residue was extracted with a further 25 ml of solvent, shaking for 5 minutes. The combined extract was passed through a 10 mm diameter column of 5 g of aluminium oxide (AOAC 1975). The column was then washed successively with 25 ml of extraction solvent, 15 ml of chloroform, and 25 ml of diethyl ether before eluting the brodifacoum with 15 ml of methanol/water (95/5, v/v). The eluate was evaporated to dryness and the residue dissolved in 5 ml of HPLC eluant.

Blood (5 ml) was extracted by vigorous shaking for 1 minute with two 10 ml aliquots of extraction solvent. The combined extract was treated as above.

HPLC analysis. Extracts were chromatographed on a Whatman Partisil PXS 10/25 ODS column using a flow rate of 2 ml eluant/minute. The HPLC system comprised a Tracor 995 pump, Rheodyne 7120 injector with 100 µl loop, Tracor 970 absorbance detector set at 275 nm, and Varian fluorichrom fluorescence detector fitted with a deuterium lamp, 280 interference filter (Corion 10 nm ½ band width, 15% transmission) for excitation and a 410 nm band filter (Varian) for emission. Detector responses were monitored on a Perkin Elmer Sigma 10 data station.

RESULTS AND DISCUSSION

Spectral characteristics. The absorption maximum of brodifacoum in HPLC eluant was at 267 nm but a setting of 275 nm on the HPLC absorbance detector gave the best signal/noise ratio. Maximum fluorescence was at 305 nm excitation and 396 nm emission.

HPLC response. The response to 100 µl injections of brodifacoum standards (Figure 1) was linear up to 4 µg of brodifacoum for absorbance and up to 5 µg for fluorescence.

Sample analyses. Initial attempts to extract brodifacoum from tissues were based on methods used for the related hydroxycoumarin, warfarin, in the hope that these two anticoagulants could be differentiated and determined in the one diagnostic analysis. However, although the chloroform commonly used for warfarin extraction from tissues (MUNDY et al 1976) also extracted brodifacoum, the extract proved difficult to clean-up for injection on the HPLC, and recoveries were low. Extraction with dichloromethane/cyclohexane followed by clean-up on aluminium oxide overcame these difficulties and gave recoveries of 85-95%. Chromatograms of extracts from animals orally dosed with brodifacoum were relatively free of extraneous peaks, absorbance generally giving a cleaner picture than fluorescence (Figure 2).

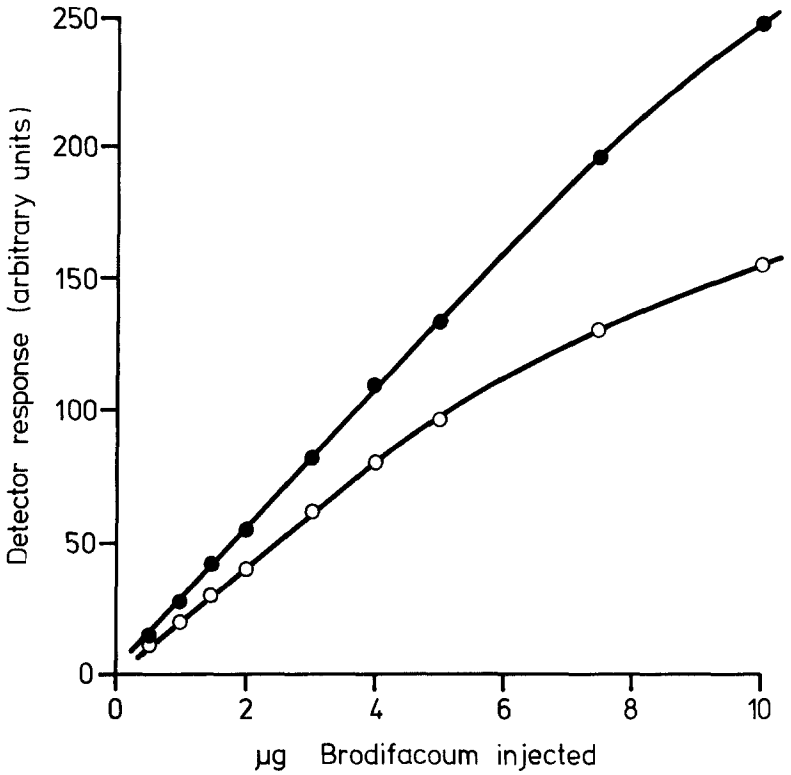

Figure 1. Absorbance (O) and fluorescence (●) responses to 100 µl injections of brodifacoum standard.

Detection limits for brodifacoum by this method were 0.05 mg/kg. Lower levels could readily be detected by adjusting the sample size and solvent volumes.

Extracts of liver and blood from sheep, cattle, and dogs that had no contact with brodifacoum showed no fluorescence peaks in the brodifacoum position. The identity of doubtful peaks as brodifacoum can be checked, if necessary, by comparing absorbance and fluorescence responses, and by varying the absorbance, excitation, and emission wavelengths. The separation of brodifacoum into its two isomers by using different HPLC conditions (YUEN 1978) may also be used for brodifacoum identification.

Fig. 2

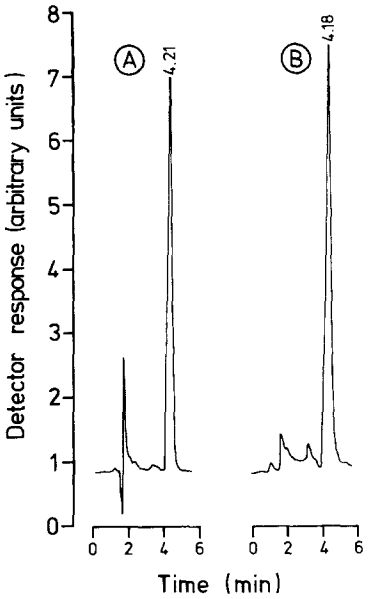

Fig. 3

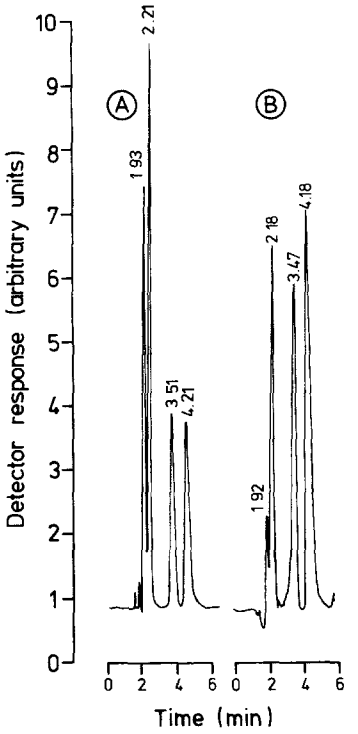

Figure 2. HPLC chromatograms of extracted liver containing brodifacoum, using absorbance (A) and fluorescence (B) detectors.

Figure 3. HPLC chromatograms of hydroxycoumarins, using absorbance (A) and fluorescence (B) detectors. Hydroxycoumarins, in order of elution, were warfarin, racumin, difenacoum, and brodifacoum.

In veterinary diagnostic work, the clinical history implicating an anticoagulant is often relatively clear, chemical analysis of tissue being mainly carried out for confirmation. As brodifacoum becomes more widely used it will therefore be necessary to distinguish it in tissue samples from other anticoagulants in use. The hydroxycoumarins warfarin, racumin, and difenacoum are all readily differentiated from brodifacoum by their relative retention times and absorbance/fluorescence response ratios (Figure 3).

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