Bull. Environ. Contam. Toxicol. 31, 239–243 (1983)

Improved HPLC Method for Determining Brodifacoum in Animal Tissues

Jacobus J. L. Hoogenboom and Colin G. Rammell

Animal Health Reference Laboratory, Ministry of Agriculture and Fisheries, Wellington, New Zealand

The method previously developed here (KIEBOOM and RAMMELL 1981) for determining brodifacoum (3-[3-(4'-bromophenyl-4-yl)-1, 2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin) in animal tissues was unsuitable for analysing fat and muscle samples. As data on residues in animal fat and muscle were necessary for further evaluation of environmental hazards, the method was modified to process these materials. The modifications resulted in improved recovery and sensitivity with all tissues.

MATERIALS AND METHODS

Brodifacoum. 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%.

Dichloromethane (British Drug Houses) was redistilled before use.

Aluminium oxide 90, active, acidic, for column chromatography (Merck) was used in the column clean-up procedure.

Brodifacoum calibration. Pure brodifacoum (10 mg) was dissolved in 100 ml of methanol. One ml of this stock solution was diluted to 100 ml with methanol for working standard. This standard was diluted 5-fold with HPLC eluant, methanol/5 mM sodium dihydrogen phosphate buffer, pH 3.0 (85/15, v/v), for the HPLC standard.

Tissue samples. Samples were obtained from necropsied animals that had been orally dosed with technical brodifacoum or had been poisoned as a result of experimental field operations.

Extraction and clean-up. Minced or finely cut tissue (2 g) was pulverised with 25 g of anhydrous sodium sulphate in a mortar. The mixture was then shaken for 30 minutes with 30 ml of dichloromethane/n-hexane/formic acid (30/30/1, v/v). The supernatant was filtered through Whatman No. 41 paper and the deposit extracted 3 times with 15 ml portions of dichloromethane/n-hexane (1/1, v/v) by shaking first for 10 minutes and then twice for 1 minute. The combined filtered extracts were evaporated to incipient dryness. The residue was dissolved in 5 ml of dichloromethane/n-hexane (1/1, v/v) and passed through a 10 mm diameter column of 5 g of

aluminium oxide (AOAC 1975). The column was then washed with 50 ml of dichloromethane/n-hexane (1/1, v/v) before eluting the brodifacoum with 25 ml of dichloromethane/ethanol/water (223/25/2, v/v). The eluant was evaporated to incipient dryness and the residue dissolved with gentle warming in 5 ml of HPLC eluant.

HPLC analysis. Extracts were chromatographed on a 10 µm Brownlee Labs RP8 column using an eluant flow rate of 1.2 ml/minute. The HPLC system comprised a Tracor 995 pump, Rheodyne 7125 injector with 40 µl loop, and Varian Fluorichrom fluorescence detector fitted with a deuterium lamp, 210 nm interference filter (Corion 25 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

Fluorescence characteristics. Maximum fluorescence was previously reported at 305 nm excitation and 396 nm emission using methanol/water/acetic acid (85/15/1, v/v) as eluant (KIEBOOM and RAMMELL 1981). By omitting acetic acid from the eluant the higher excitation energy available at 210 nm could be used. This effectively increased the fluorescence response 25-fold. The response to injections containing up to 400 ng of brodifacoum was linear(Figure 1). The detection limit for brodifacoum under the conditions used was 0.1 ng/40 µl injection.

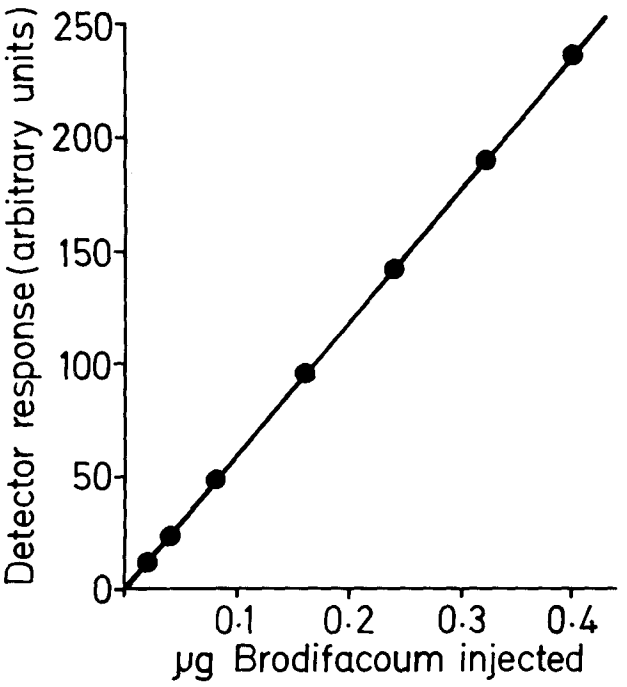

Figure 1. Fluorescence response to 40 µl injections of brodifacoum standard.

Sample extraction. Although liver and blood samples were relatively easy to analyse, muscle and fatty tissue originally proved much more difficult. Brodifacoum appears to be tightly bound to muscle tissue and is soluble in fat. Attempts to facilitate extraction of muscle tissue by prior enzymatic digestion with pancreatin were unsuccessful. However the incorporation of formic acid in the extraction solvent, together with pulverisation of the tissue with anhydrous sodium sulphate to bind water and aid homogenisation, made the extraction method suitable for all tissues and improved the efficiency (Table 1).

TABLE 1

Brodifacoum results from 5 rabbit livers analysed by the improved method (A) and a previous method (B)

Method	mg Brodifacoum/kg of liver				
	1	2	3	4	5
A	9.4	7.1	9.4	6.5	8.8
B	4.7	2.6	3.6	2.5	3.7

Column clean-up. The aluminium oxide column effectively removed interfering components from the tissue extracts. A small amount of fat remained in the residue of fatty tissue after clean-up, but this did not interfere in the HPLC analysis. However, the HPLC column was routinely flushed with tetrahydrofuran after each batch of samples.

Sample analysis. Figure 2 shows typical chromatograms obtained with extracts of liver, muscle and fatty tissue from dosed sheep. Similar chromatograms have been obtained with tissue extracts from rabbits, birds and cats poisoned as a result of experimental field operations with brodifacoum. Corresponding extracts from animals that had no contact with brodifacoum showed no peaks in the brodifacoum position. Redistillation of the dichloromethane solvent was necessary to minimise extraneous peaks. Better baseline separation of peaks was achieved with a 5 µm LiChrosorb RP18 column but the 10 µm RP8 column was preferred because of shorter analysis time and lower column back-pressure.

Day-to-day analysis of blanks and tissue samples spiked with 1 µg of brodifacoum showed a mean recovery of 88 ± 7%. The coefficient of variation for six replicate analyses of sheep liver containing 5.1 mg/kg of brodifacoum was 4%.

Table 2 shows results of duplicate analyses on samples of liver, muscle and fatty tissue from two sheep orally dosed with 1 mg (A) and 3 mg (B) of brodifacoum/kg of bodyweight on each of 3 successive days. The sheep were sacrificed and sampled on the sixth day. The detected presence of residues in the muscle and fat highlights the need for care in the field use of brodifacoum near livestock.

Although this method was primarily developed for animal tissue analysis, it may also be used for the determination of brodifacoum in commercial baits. The high fat or wax content of some of these baits previously made their analysis difficult.

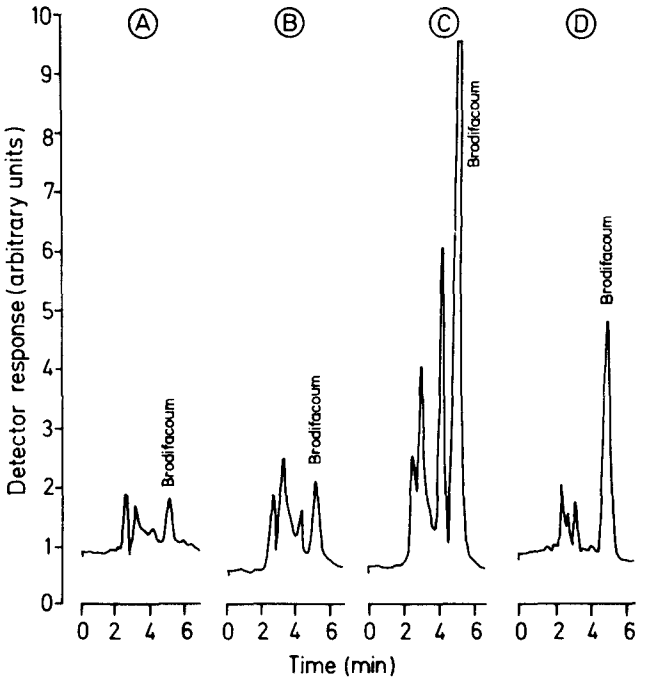

Figure 2. HPLC chromatograms of extracted muscle (A), fatty tissue (B) and liver (C) from sheep dosed with brodifacoum, and a 7 ng brodifacoum standard injection (D).

TABLE 2

Duplicate analyses of liver, muscle and fatty tissue from 2 sheep (A,B) orally dosed with brodifacoum

	mg Brodifacoum/kg of		
Sheep	Liver	Muscle	Fat
A	1.34	0.13	0.20
	1.63	0.10	0.16
B	1.51	0.12	0.21
	1.48	0.10	0.12

ACKNOWLEDGEMENTS

The authors thank ICI Tasman (NZ) Limited for samples of brodifacoum, Mr J. Bell and staff for tissues from animals poisoned during field operations with brodifacoum, and Mr A. Barkus for technical assistance.

REFERENCES

HOROWITZ, W. (Ed) : Official Methods of Analysis, 12th ed. Association of Official Analytical Chemists, Washington, D.C. (1975) 43.003 (h).

KIEBOOM, A.J. and C.G. RAMMELL : Bull. Environm. Contam. Toxicol. 26, 674 (1981).

Accepted May 19, 1983