A method for the separation of residues of nine compounds in cattle liver related to treatment with oxfendazole

Martin D. Rose

CSL Food Science Laboratory, Norwich Research Park, Colney, Norwich, UK NR4 7UQ.
E-mail: m.rose@csl.gov.uk

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A method for the determination of nine compounds closely related to oxfendazole has been developed for the monitoring of residues in food. The method is based on a multi-residue procedure for basic drug residues and used strong cation exchange solid phase extraction for sample clean-up. These nine compounds include fenbendazole, which is itself a licensed veterinary product. The pro-drug febantel converts quickly to fenbendazole or oxfendazole soon after administration. The method is therefore suitable for monitoring residues following the use of any of these compounds. Some of these analytes have been shown to be present as residues following the treatment of farm animals with oxfendazole. Average recoveries for the nine compounds from tissue fortified with 100 μg kg^{-1} were between 34% and 96% with relative standard deviations between 3% and 22%.

Anthelmintic agents, including benzimidazoles such as oxfendazole, are used in animal husbandry for the prevention and control of internal worm parasites. A number of these compounds have been shown to cause teratogenic and embryotoxic effects in some species.[1] Their use with farm animals raises the possibility that residues may be found in food produced for human consumption. Maximum residue limits (MRLs) for these compounds are set in European legislation[2] and range from 10 to 1000 μg kg^{-1} depending upon the compound and food type. Details of these limits are summarised in Table 1.

Several methods exist in the literature for the analysis of one or more benzimidazole compounds as residues in a variety of food types. For example, a method for the determination of fenbendazole, oxfendazole, thiabendazole and 5-hydroxythiabendazole in milk was described[3] with a limit of detection at 5 μg kg^{-1}. This procedure was based on partition between organic and aqueous phases with pH adjustment followed by solid phase extraction (SPE) clean-up on silica. Butylated hydroxytoluene (BHT) was added during the extraction to prevent further oxidation of residues. A method for the determination of six benzimidazoles in sheep or chicken liver was described using sequential SPE clean-up, first loading an acidic alumina cartridge with a hexane–chloroform (25 + 75) extract, eluting with methanol, adding water and applying to a C_{18} SPE cartridge. The final eluant was acetonitrile.[4] Matrix

Table 1 MRLs for benzimidazole anthelmintics[2]

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs/ μg kg^{-1}	Target tissues
Febante	Sum of extractable residues which may be oxidised to oxfendazole sulfone	Bovine, ovine, porcine, Equidae	500 50 10	Liver Muscle, kidney, fat Milk
Fenbendazole and Oxfendazole				
Thiabendazole	Sum of extractable residues that may be oxidised to ketotriclabendazole	Bovine, ovine	100	Muscle, liver, kidney
Thiabendazole	Sum of thiabendazole and 5-hydroxythiabendazole	Bovine	100	Muscle, liver, kidney, fat, milk
Flubendazole	Sum of flubendazole and (2-amino-1*H*-benzimidazol-5-yl)(4 fluorophenyl) methanone	Poultry and game birds, porcine	400 50 300	Liver Muscle, skin, fat Kidney
	Flubendazole	Chicken	400	Eggs
Netobimin	Sum of netobimin and albendazole and metabolites of albendazole measured as 2-aminobenzimidazole sufone	Bovine, ovine, caprine	1000 500 100 100	Liver Kidney Muscle, fat Milk
Oxibendazole	Oxibendazole	Porcine	100 500 200	Muscle, kidney Skin, fat Liver
Albendazole sulfoxide	Sum of albendazole, albendazole sulfoxide, albendazole sulfone, and albendazole 2-amino sulfone, expressed as albendazole	Bovine ovine, pheasant Bovine, ovine	1000 500 100 100	Liver Kidney Muscle, fat, milk Milk

solid phase dispersion has also been used as part of the analysis procedure for these compounds in cattle liver[5] and milk.[6]

During a study on the effect of cooking on residues of oxfendazole in food, conducted in this laboratory,[7] several metabolites and breakdown products were identified in the tissue. These arose from the treatment of farm animals with oxfendazole, and there was some evidence of the presence of other metabolites which were not characterised. There was also evidence of an 'unstable equilibrium' between oxfendazole, oxfendazole sulfone and fenbendazole in incurred tissue: an overall instability of these compounds in tissue during frozen storage, an uneven distribution of residues within the tissue and the possibility of an effect of protein binding on extractability of residues from tissue. None of the available methods in the literature was suitable for studying the interaction of the various metabolites and breakdown products found in this study. The structures and relationship of 11 compounds known to be part of the metabolic pathway of oxfendazole are shown in Fig. 1.

The aim of the work described here was to develop a method capable of the determination of all 11 of these compounds, which could potentially be present as residues in cattle treated with oxfendazole, fenbendazole or febantel.

Febantel is a pro-drug, *i.e.*, a product known to convert into an active compound soon after administration.[8] It is converted either directly to fenbendazole or to oxfendazole, which is arrived at *via* febantel sulfoxide as an intermediate. During preliminary investigations febantel and its sulfoxide were both found to be unstable as they converted to fenbendazole during the developed extraction and clean-up procedure. Because of their mode of action, residues of these compounds are unlikely to be found in animal tissue. Methodology which excluded these compounds was therefore deemed acceptable.

A method for the remaining nine compounds in raw and cooked tissue at normal residue levels was developed and validated. The approach was based on the multi-residue procedure for basic drugs developed in this laboratory.[9]

Experimental

Standards

Analytical standards for all compounds identified as potential metabolites and breakdown products were either purchased, given by manufacturers or synthesised at the University of East Anglia (UEA). The origins of the range of standards are shown in Table 2. Standard purity checks were performed on each compound with satisfactory results. These included melting point, ^{1}H NMR (270 MHz), ^{13}C NMR (270 MHz), IR, TLC, MS and elemental analysis.

Principle

Tissue was extracted with acetonitrile. The extract was dried with sodium sulfate and acidified with acetic acid before loading onto a conditioned strong cation exchange (SCX) solid phase extraction (SPE) cartridge. The cartridge was washed successively with acetone, methanol and finally acetonitrile

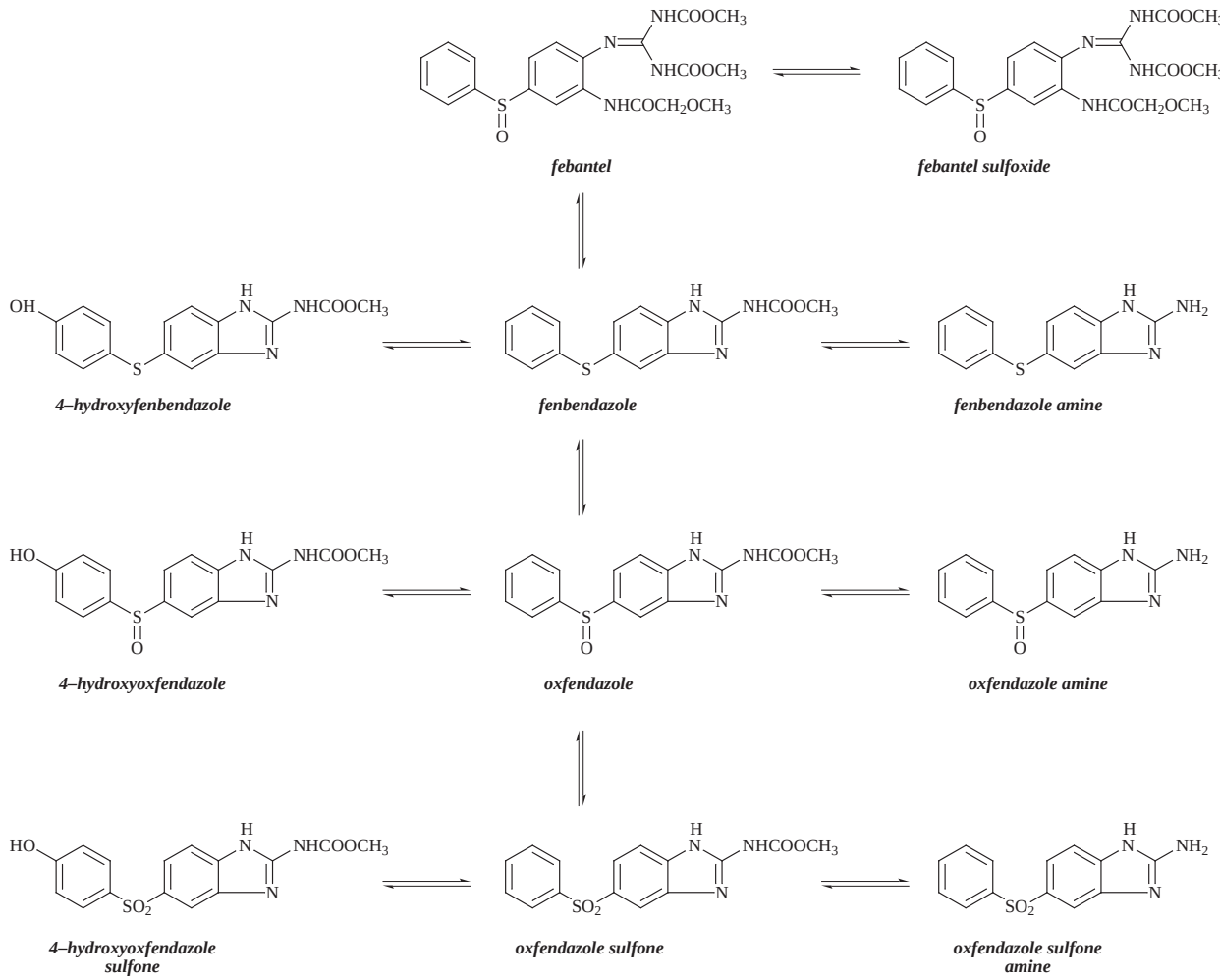

Fig. 1

before elution with acetonitrile–35% aqueous ammonia (95 + 5).

Reagents

All chemicals were of analytical grade. Solvents were HPLC or glass-distilled grade. Water was obtained from an in-house Elga water purification system.

Extraction and clean-up

Finely sliced liver tissue (5 g) was weighed into a polypropylene centrifuge tube (100 ml) and homogenised for 30 s in acetonitrile (50 ml). Sodium sulfate (5g) was added and the tubes were centrifuged (1700*g*, 5 min). A Bond-Elut SCX cartridge (500 mg per 3 ml; Varian, Walton-on-Thames, Surrey, UK) was conditioned with acetonitrile–glacial acetic acid (95 + 5, 5 ml) and a reservoir (100 ml) fitted. The sample extract was acidified with glacial acetic acid (5 ml), transferred to the reservoir and passed through the cartridge. The cartridge was washed sequentially with acetone (2.5 ml), methanol (5 ml) and acetonitrile (5ml). The analytes were eluted with acetonitrile–35% aqueous ammmonia (95 + 5, 5 ml) into a test-tube.

HPLC conditions

A gradient HPLC system was required to separate all nine compounds in a convenient run time. The column eluate was monitored for UV absorbance at 290 nm. The flow rate was constant at 0.45 ml min^{-1}. A Chromspher $5C_8$ 25 cm × 3 mm id column was used. The gradient profile is shown in Table 3.

A flow diagram of the method is shown in Fig. 2.

Validation protocol

Tissue for use as a control was purchased locally and analysed to verify the absence of residues. Batches consisting of at least one blank control sample and at least four fortified blank samples were analysed on three separate days. The solution

Table 2 Oxfendazole related compounds and their source

		Abbreviation	Procured from
(i)	Febantel	FBT	Bayer
(ii)	Febantel sulfoxide	FBT-SO	UEA
(iii)	4-Hydroxyfenendazole	OH-FEN	UEA
(iv)	Fenbendazole	FEN	Sigma
(v)	Fenbendazole amine	FEN-A	UEA
(vi)	4-Hydroxyoxfendazole	OH-OXF	UEA
(vii)	Oxfendazole	OXF	Syntex
(viii)	Oxfendazole amine	OXF-A	UEA
(ix)	4-Hydroxyoxfendazole sulfone	OH-OXF-S	UEA
(x)	Oxfendazole sulfone	OXF-S	Syntex
(xi)	Oxfendazole sulfone amine	OXF-S-A	UEA

Table 3 Gradient elution profile

Time/min	A (%)[a]	B (%)[a]
0	75	25
18	60	40
23	0	100
30	0	100
31	75	25

[a] Mobile phase A: 0.1 M ammonium carbonate–methanol (80 + 20); mobile phase B: 0.1 M ammonium carbonate–methanol (20 + 80).

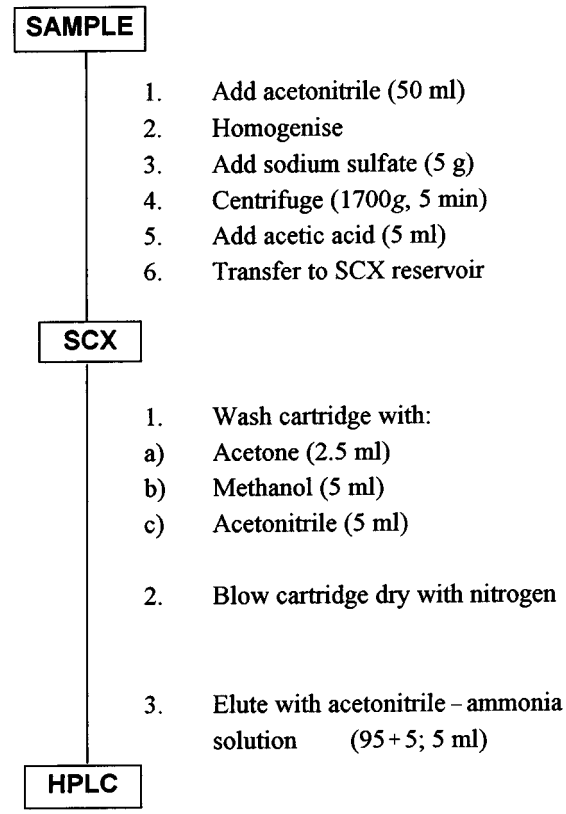

Fig. 2

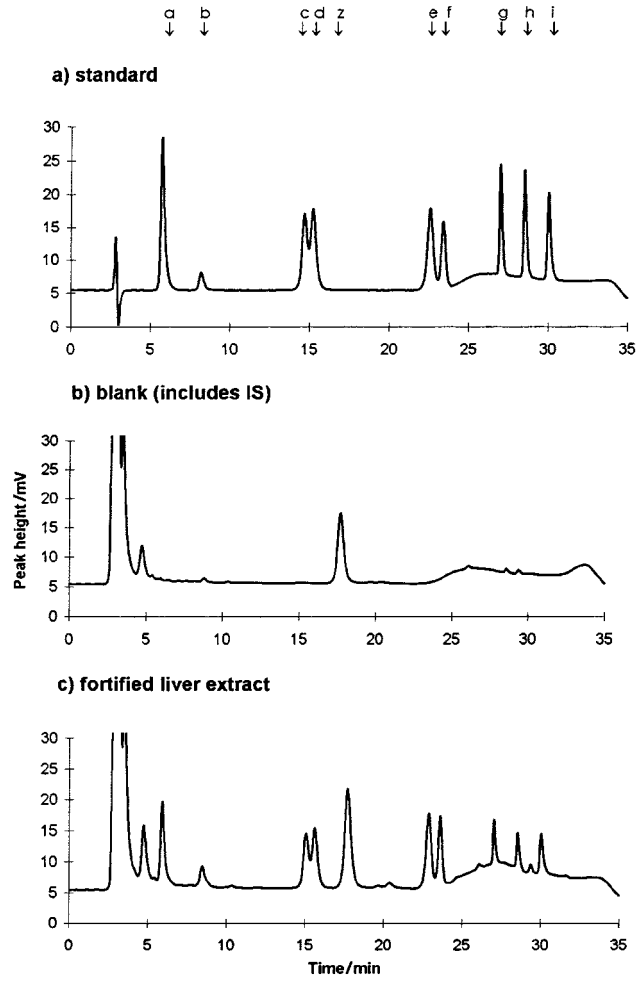

Fig. 3

Table 4 Method validation data—samples fortified at 100 μg kg^{-1}. A blank sample was analysed on each day and found to contain no trace of any analyte

	Recovery (%)								
	OH-OXF-S	OH-OXF	OXF-A	OXF-S-A	OXF	OXF-S	OH-FEN	FEN-A	FEN
Day 1 n = 6									
Mean (%)	53	74	48	51	77	66	40	28	41
RSD (%)	19	14	12	12	11	17	19	19	21
Day 2 n = 4									
Mean	59	93	77	82	84	105	48	37	49
RSD (%)	22	18	2.9	3.2	16	11	13	17	14
Day 3 n = 4									
Mean	63	105	81	85	93	117	49	36	46
RSD (%)	21	20	2.6	3.8	15	12	22	8.4	16

used for fortification was added to the tissue and allowed to equilibrate for 5–10 min prior to the addition of the acetonitrile used for extraction. Linearity of standards was checked for the range equivalent to 100–1000 μg kg^{-1}.

Results and discussion

Although there is some chemical similarity between the analytes, there is a wide range of polarity between the first and last eluting compounds. Their chemical similarity (they are all basic compounds) was exploited by applying cation exchange solid phase extraction for clean-up and variation in polarity was used to separate these compounds on a gradient HPLC system. None of the metabolites measured were found to co-elute with other benzimidazole compounds tested (thiabendazole, albendazole sulfone, cambendazole) using these HPLC conditions.

The method developed separates all nine compounds associated with oxfendazole that are likely to be found as residues in food. Chromatograms of a standard mixture, a blank sample extract and an extract from a sample fortified at 100 μg kg^{-1} are shown in Fig. 3. Thiabendazole was incorporated as an internal standard. Because of the variation in recovery between analytes, external standards were used for recovery calculations for individual compounds. Validation data for the method on three separate days for samples fortified at 100 μg kg^{-1} are shown in Table 4. Injections of standards demonstrated that the response was linear over a range 100–1000 μg kg^{-1} with correlation coefficients $\geq$0.98.

Normal quality targets in this laboratory for recovery and CV of analytes determined by a method are to give an average recovery greater than 40% with an RSD of less than 20%. These values were generally achieved with this method, although there was some compromise with some analytes, notably fenbendazole amine, where recoveries less than the target were permitted in order to allow the same method to be used for all compounds. This compromise was allowed because of the versatility of the multi-analyte method.

Conclusion

A multi-residue method for the determination of residues of compounds associated with oxfendazole treatment of farm animals has been developed. The same residues are likely to be associated with the treatment of farm animals with fenbendazole and the pro-drug febantel due to the reaction pathways and metabolism of these compounds. The method is suitable for application to surveillance programmes to detect residues arising from the use of these compounds.

Acknowledgement

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