Quantifying fenbendazole and its metabolites in self-medicating wild red grouse *Lagopus lagopus scoticus* using an HPLC-MS-MS approach

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Parasite control

Trichostrongylus tenuis

Abstract

On red grouse estates in the UK the nematode parasite *Trichostrongylus tenuis* is often controlled by application of grit medicated with the anthelmintic fenbendazole (FBZ). To date, assessment of the efficacy has been inhibited by the inability to quantify uptake of FBZ by the birds. We have developed a simple and sensitive HPLC-MS-MS method for detecting and quantifying FBZ and its metabolites from a 300 mg sample of red grouse liver. This method could be used to improve the efficacy of medicated grit treatment by allowing the identification of conditions and application methods that optimize the uptake of FBZ. With the necessary modifications, our method will also be applicable to other wildlife species where self-medication is used for parasite control.

Introduction

Medicated feed is frequently used for parasite control in wildlife populations, including red grouse (Newborn & Foster 2002), pheasants (Draycott et al., 2006) and bighorn sheep (Miller et al., 2000; Schmidt et al., 1979). Because these animals self-administer, drug uptake may vary among individuals and over time, for example due to differential access to feed. Assessment of drug uptake (and therefore efficacy), which is already difficult in self-administering populations, is hindered further by this variation. To aid parasite management in these situations, a reliable method to quantify drug uptake is required.

One such approach is the quantification of drug residues in the tissues of harvested animals. Here we develop a quantification method for red grouse (*Lagopus lagopus scoticus*), an economically important game bird species endemic to the British Isles. Red grouse exhibit regular population collapses owing in part to high burdens of the parasitic nematode *Trichostrongylus tenuis* (Hudson et al., 1998). Control of these intestinal parasites helps reduce the frequency and severity of population crashes (Hudson et al., 1998). The most widespread technique for controlling *T. tenuis* in red grouse has been the provision of quartz grit, which grouse consume to aid digestion, coated with the anthelmintic drug fenbendazole (FBZ). Recently, the use of grit coated with flubendazole (FLUB), has also become widespread. Medicated grit piles are distributed on red grouse habitat in December and replenished in early March (Newborn and Foster, 2002). No medicated grit is distributed after the end of March (Intervet UK Ltd, Panacur Data Sheet), resulting in a minimal withdrawal period of 19 weeks to prevent FBZ residues (i.e. FBZ and its metabolites) from entering food products when grouse are harvested for human consumption from 12 August to 16 December. Treatment with FBZ-medicated grit has been shown to reduce average worm burdens by 34% (Newborn and Foster, 2002).

FBZ is a member of the benzimidazole (BZ) group of anthelmintics which provides broad-spectrum efficacy against endoparasites and is widely used in domestic animals and humans (McKellar and

Scott, 1990). Methods have been developed for the quantification of FBZ residues in liver, muscle and plasma from a range of domestic animals, including cattle, sheep and fish, using High Performance Liquid Chromatography (HPLC) or Liquid Chromatography–Mass Spectrometry (LC–MS) (Danaher et al., 2007). Here we present a method to quantify FBZ residues in red grouse livers using HPLC coupled to an ion trap tandem mass spectrometer (HPLC-MS-MS). We measured simultaneously FBZ and two of its metabolites, fenbendazole sulphone ($FBZSO_2$) and oxfendazole (OFZ), plus an internal standard mebendazole (MBZ). Liver tissue was selected for analysis because it contains the highest total residue levels and is the tissue from which residues deplete most slowly (European Medicines Agency, 2004).

The aims of this study were threefold: (1) to develop a simple and reliable method to quantify FBZ residues in grouse livers; (2) to assess variation in FBZ dosage among birds during treatment periods (December to March); and (3) to measure FBZ residue levels in samples from the shooting season (August 12 to December 10) in order to quantify residues entering food products.

Materials and methods

We obtained livers from 14 male red grouse aged at least 1 year from three grouse moors in the north of England, UK during August 2002 and March and August 2003 (Table 1). One moor was in Teesdale (T_{FBZ}, $n = 6$) and two were in North Yorkshire (Y_{FBZ}, $n = 5$; Y_{NEG}, $n = 3$). Subscripts indicate history of benzimidazole treatment: T_{FBZ} and Y_{FBZ} had 10 and 13 years, respectively, of consecutive treatment with FBZ-medicated grit prior to sampling while Y_{NEG} had never been treated, although situated adjacent to treated moors. The sampling dates of two Y_{FBZ} samples (August 2002) and two T_{FBZ} samples (August 2003) fell within the shooting season, five months after drug treatment had ceased. Males more than one year old, and therefore likely to have had access to medicated grit treatments earlier in the year, were selected at random from the grouse harvested that day from each site. The remaining 10 samples were collected from dead birds outside

54 the shooting season in March 2003. These samples were taken opportunistically, and we have no
55 information on self-medication frequency, dosage or proximity to medicated grit piles. We also
56 cannot assume that sampling was representative. Nevertheless, as medicated grit piles were
57 available to the grouse during this period, these samples were considered more likely to contain
58 detectable residues than those collected in August. After collection, liver samples were frozen on
59 dry ice (-78.5 °C) and transported to storage at -70 °C. Medicated grit was also collected from a grit
60 pile in March 2003 and stored at -70 °C.

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62 Frozen liver samples were thawed and 300 mg subsamples were removed to 50 mL centrifuge
63 tubes. To monitor variation introduced by the extraction procedure, two sub-samples were extracted
64 from each liver and each was analysed separately. Control (drug negative) livers from the untreated
65 site Y_{NEG} were selected for spiking with drug standards after we had checked that no detectable
66 drug residues were present. Sample and control liver samples were fortified with 100 ng of the
67 internal standard mebendazole (MBZ) to allow standardisation of recovery rates across HPLC-MS-
68 MS assays. Mean (±SD) recovery of the internal standard was 78 (±18) %. Control liver samples
69 were also fortified with FBZ at a range of concentrations to allow validation of the method. The
70 quantities added were 0, 0.05, 0.5, 5 and 50 ng, equivalent to 0, 0.167, 1.67, 16.7 and 167 μg kg^{-1} of
71 liver.

72

73 The residues to be quantified were FBZ and its metabolites oxfendazole (OFZ) and fenbendazole
74 sulphone ($FBZSO_2$) (Supplementary Figure 1). The presence of OFZ and $FBZSO_2$ are indicative of
75 FBZ intake (Danaher et al., 2007). FBZ, OFZ, $FBZSO_2$ and MBZ were identified by their
76 fragmentation pattern and by comparison of the peak retention times with that of previously run
77 drug standards (Supplementary Figure 2). Each extraction was HPLC-MS-MS-analysed twice. The
78 means of the resulting pair peak areas were interpolated onto calibration curves to calculate residue
79 concentration in μg kg^{-1}. A detailed description of the HPLC-MS-MS analysis is given in the

supplementary methods.

Results

FBZ was detected reliably in the three control livers: no FBZ was detected in either subsample from the negative control liver, and FBZ was detected in at least one subsample from each of the four spiked livers (Table 2). FBZ was also quantified accurately: measured concentration accurately reflected spiked concentration, as indicated by the closeness of the slope of the regression line to one (regression equation: ln measured concentration = 1.05 × ln spiked concentration; $r^2 = 0.986$). The limit of detection (LOD) for FBZ was 0.17 μg kg^{-1}, within the range reported in other studies using LCMS-MS (De Ruyck et al., 2002). The limits of quantification (LOQ) for all three residues were 0.7 μg kg^{-1} and 667 μg kg^{-1}.

FBZ or its metabolites OFZ and $FBZSO_2$ were detected in eight of the eleven livers from the two FBZ-treated estates (Table 2) and none of the three livers from the untreated estate. Measured levels of FBZ averaged across each pair of subsamples ranged from below 0.17 μg kg^{-1} (the lower LOD) to 1.3 μg kg^{-1}. Levels of OFZ ranged from 1.3–1916 μg kg^{-1} (median 29 μg kg^{-1}), with the highest being values considerably above the LOQ of 667 μg kg^{-1}. $FBZSO_2$ ranged from 1.7–530 μg kg^{-1} (median 13 μg kg^{-1}).

Among the eight birds where residues were detected — indicating exposure to FBZ — there was a strong tendency for the concentrations of both of the FBZ metabolites, OFZ and $FBZSO_2$, to be higher than FBZ itself. This result agrees with data from sheep liver, where FBZ depleted ten times more quickly than OFZ over an 8 day period (Blanchflower et al., 1994). Levels of the three residues tended to covary. Log concentrations of OFZ and $FBZSO_2$ were strongly positively correlated among the eight livers ($r = 0.96$, $n = 8$). Log concentrations of OFZ and $FBZSO_2$ were also correlated with FBZ but less strongly (FBZ-OFZ $r = 0.86$, $n = 4$; FBZ-$FBZSO_2$ $r = 0.78$, n =

4). From site Y_{FBZ}, the samples collected during the shooting season ($Y_{FBZ}1$–$Y_{FBZ}2$) tested positive for FBZ metabolites (Table 2), whereas the samples collected during the period of medicated grit treatment (Spring 2003) showed no detectable levels of FBZ or its metabolites. FBZ residues were generally at a higher level in March (during the treatment period) than in August (Table 2), although these differences were significant only for FBZ ($P = 0.02$, Wilcoxon rank sum test).

No FBZ was found in the medicated grit sample, although its metabolites OFZ and $FBZSO_2$ were detected at high levels relative to those observed in the liver samples (Table 2). The peak areas for these metabolites were above the LOQ, considerably so in the case of the active metabolite OFZ, and therefore the amount of active anthelmintic remaining of the 1000 μg kg^{-1} FBZ in fresh grit could not be quantified.

Discussion

When parasite control in free-living animals involves medicated grit or feed instead of direct drug application, managers need simple and efficient tools to monitor temporal and spatial variation in drug uptake. Our method for detecting the anthelmintic fenbendazole and its metabolites in liver tissue of game birds provides such a tool. The sensitivity of the method is demonstrated by the low LOD and by the detection of benzimidazole residues four months after the withdrawal of medicated grit piles. The lack of false positives among negative controls and samples from the untreated moor indicate high specificity. All but one of the pairs of replicate samples agreed in sensing the presence or absence of BZ residues, indicating that the technique is reliable but that running duplicate samples is advisable. The simplicity and speed of the method would allow it to be offered to estate managers at a cost that is low compared to the cost of treatment.

Our method could also aid the prevention of the development of anthelmintic resistance. Since the pace and extent of anthelmintic resistance evolution is greatly affected by variation in drug dosage (Smith et al., 1999), managers should consider using the technique presented here to monitor individual variation in drug uptake to identify conditions and methods that delay the onset of drug resistance among the parasites for as long as possible. Our method may also be applicable to other game bird species such as pheasants (Draycott et al., 2006), and with the necessary modifications, to free-living mammals such as bighorn sheep (Miller et al., 2000; Schmidt et al., 1979) where medicated feeds are used and where drug uptake may vary greatly among individuals.

Our study was based on samples collected from wild grouse for which we had no information on proximity to medicated grit piles, dosing frequency, length of time since the last dose, or how representative samples were of the grouse moor populations. Thus, the livers we analysed may therefore have been at different stages and levels of fortification. Despite these caveats, we found striking differences among birds in levels of the active anthelmintics FBZ and OFZ during the treatment period. FBZ was detected in only four of the seven samples taken from treated sites

during the treatment period (Table 2). Indeed, one of the treated sites (Y_{FBZ}) exhibited no measurable FBZ residues from any of the three birds sampled during the treatment period. These differences in residue level may reflect variation in the time since the last ingestion of medicated grit. However it is also possible that these differences reflect long term variation among birds in FBZ uptake caused by differential access to medicated grit piles in conjunction with territorial behaviour (Mougeot et al., 2003). Either way, our results suggest that there is substantial room for improvement to enable more birds to receive adequate FBZ dosage to control *T. tenuis* parasites.

None of the four livers collected during the shooting season in August had any detectable FBZ, but FBZ metabolites tested were detected in all four of the samples. Maximum Residue Limits (MRLs) are established for anthelmintic residues in products for human consumption from most livestock species and these tend to be in the order of 10 $\mu g\ kg^{-1}$ for milk and 500 $\mu g\ kg^{-1}$ for liver in bovine, ovine, porcine and equine species (European Commission, 1990). The levels measured in our study are well below these limits, and therefore our data provide no evidence that the 19-week withdrawal period is insufficient to prevent human consumption of harmful levels of anthelmintic residues.

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References

Blanchflower, W.J., Cannavan, A., Kennedy, D.G., 1994. Determination of fenbendazole and oxfendazole in liver and muscle using liquid chromatography–mass spectrometry. Analyst 119, 1325-1328.

Danaher, M., De Ruyck, H., Crooks, S.R.H., Dowling, G., O'Keeffe, M., 2007. Review of methodology for the determination of benzimidazole residues in biological matrices. J. Chromatogr. B 845, 1-37.

De Ruyck, H., Daeseleire, E., De Ridder, H., Van Renterghem, R., 2002. Development and

validation of a liquid chromatographic–electrospray tandem mass spectrometric multiresidue method for anthelmintics in milk. J. Chromatogr. A 976, 181-194.

Draycott, R.A.H., Woodburn, M.I.A., Ling, D.E., Sage, R.B., 2006. The effect of an indirect anthelmintic treatment on parasites and breeding success of free-living pheasants *Phasianus colchicus*. J. Helminthol. 80, 409-415.

European Commission, 1990. Council regulations 2377/90/EC of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal of the European Union L224, 1-124.

European Medicines Agency 2004. Fenbendazole summary report (4) EMEA/MRL/866/03-Final (Committee for Medicinal Products for Veterinary Use, Veterinary Medicines and Inspections, London, UK).

Hudson, P.J., Dobson, A.P., Newborn, D., 1998. Prevention of population cycles by parasite removal. Science 282, 2256-2258.

McKellar, Q.A., Scott, E.W., 1990. The benzimidazole anthelmintic agents - a review. J. Vet. Pharmacol. Ther. 13, 223-247.

Miller, M.W., Vayhinger, J.E., Bowden, D.C., Roush, S.P., Verry, T.E., Torres, A.N., Jurgens, V.D., 2000. Drug treatment for lungworm in bighorn sheep : Reevaluation of a 20-year-old management prescription. J. Wildl. Manag. 64, 505-512.

Mougeot, F., Redpath, S.M., Moss, R., Jason, M., Hudson, P.J., 2003. Territorial behaviour and population dynamics in red grouse *Lagopus lagopus scoticus*. I. Population experiments. J. Anim. Ecol. 72, 1073-1082.

Newborn, D., Foster, R., 2002. Control of parasite burdens in wild red grouse *Lagopus lagopus scoticus* through the indirect application of anthelmintics. J. Appl. Ecol. 39, 909-914.

Schmidt, R.L., Hibler, C.P., Spraker, T.R., Rutherford, W.H., 1979. An evaluation of drug treatment for lungworm in bighorn sheep. J. Wildl. Manag. 43, 461-467.

Smith, G., Grenfell, B.T., Isham, V., Cornell, S., 1999. Anthelmintic resistance revisited: under-dosing, chemoprophylactic strategies, and mating probabilities. Int. J. Parasitol. 29, 77-91.

206 **Tables**

207 **Table 1.**

208 Details of medicated grit use and sample size for each site.

Site	FBZ grit use (years)	Number of birds sampled		
		Treatment period	Hunting season	Total
T_{FBZ}	10	4	2	6
Y_{FBZ}	13	3	2	5
Y_{NEG}	None	3	0	3

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212 **Table 2** Concentration (μg kg^{-1}) of residues of fenbendazole (FBZ) and its metabolites oxfendazole
213 (OFZ) and fenbendazole sulphone ($FBZSO_2$) in eight red grouse livers collected from the BZ-
214 treated estates, T_{FBZ} and Y_{FBZ}. Results are also shown from two samples of medicated grit and 5
215 control livers spiked with FBZ. No residues were detected in an additional six livers collected in
216 March 2003, three from Y_{FBZ} and three from the untreated estate Y_{NEG} (data not shown). ND: not
217 detected.

			Residue concentration (μg kg^{-1})		
Sample ref.	Sample date	Sub-sample	FBZ	OFZ	$FBZSO_2$
$T_{FBZ}1$	Mar-03	A	0.8	2063*	486
		B	1.2	1770*	391
$T_{FBZ}2$	Mar-03	A	0.2*	1.9	1.6
		B	0.2*	1.1	1.7
$T_{FBZ}3$	Mar-03	A	1.1	863*	533
		B	1.5	833*	528
$T_{FBZ}4$	Mar-03	A	0.2*	78	165
		B	0.1*	103	92
$T_{FBZ}5$	Aug-03	A	ND	1.4	2.5
		B	ND	1.2	1.8
$T_{FBZ}6$	Aug-03	A	ND	38	9.9
		B	ND	65	15
$Y_{FBZ}1$	Aug-02	A	ND	2.2	1.4
		B	ND	2.4	4.2
$Y_{FBZ}2$	Aug-02	A	ND	6.0	15
		B	ND	5.6	12
T_{FBZ} (medigrit)	Mar-03	A	ND	16770*	1620*
		B	ND	16592*	1168*
FBZ-spiked (0 μg kg^{-1})	-	A	ND	-	-
		B	ND	-	-
FBZ-spiked (0.17 μg kg^{-1})	-	A	0.10*	-	-
		B	0.099*	-	-
FBZ-spiked (1.7 μg kg^{-1})	-	A	0.89	-	-
		B	1.1	-	-
FBZ-spiked (17 μg kg^{-1})	-	A	17	-	-
		B	ND	-	-
FBZ-spiked (167 μg kg^{-1})	-	A	175	-	-
		B	256	-	-

218 ***Concentration outside limits of quantification (0.7–667 μg kg^{-1})**

Supplementary material

Quantifying fenbendazole and its metabolites in self-medicating wild red grouse *Lagopus lagopus scoticus* using an HPLC-MS-MS approach

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Supplementary methods

Subsamples of 300 mg of each liver were allowed to stand for 30 min before being frozen at -70 ºC prior to freeze-drying for 36-48 hr. On removal samples were homogenised with a mortar and pestle and dry weights were taken. The standard deviation of the dry weights of the samples was 13% of the mean. Methanol (20 mL) was added to the ground tissue and each sample was vortexed for 5 min before centrifugation at 4000 rpm for 20 min at 4 °C in a cooling centrifuge. The supernatant was removed and partitioned with an equal volume of hexane. Partitioning was repeated before the methanol portion was removed to a quick-fit flask and evaporated to dryness using a rotary evaporator. The dried residue was resuspended in 1 mL methanol and vortexed. Samples were centrifuged in a sample concentrator for 1 hr 30 min and the pellet resuspended in 100 μl methanol. The extractions were centrifuged for a further 30 min at 13000 rpm to remove any remaining impurities. Finally, the purified supernatant was removed and used for HPLC-MS-MS analysis. Medicated grit samples were extracted in the same way as livers, with the exception that 1 g of grit was used.

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, diode array absorbance detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C. HPLC separation was carried out using a 4 μm, 250 × 2.0 mm i.d. Synergi RP-Max column with a 2-mm guard column maintained at 65 ºC, eluted with a gradient over 30 min of 25-55% acetonitrile in 0.1% formic acid at a flow rate of 200 mL/min and maintained at 65 °C. After passing through the flow cell of the diode array detector the column eluate was directed to an LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface controlled by Xcalibur software version 1.2.

Analyses utilized the positive ion mode as this provided the best limits of detection. Analysis was performed using selected reaction monitoring carried out over 3 segments. For segment 1 from 0 to 14 min the parent ion at *m/z* 316 was fragmented with a relative collision energy of 35% and the product ions scanned from *m/z* 125 to 316. For segment 2 from 14.1 to 23.5 min the parent ions were *m/z* 296 and 332, the relative collision energy was 35%, and the product ions scanned from *m/z* 125 to 296 and *m/z* 125 to 332 respectively. For segment 3 from 23.6 to 30 min the parent ion was *m/z* 300, relative collision energy 35% and product ions scanned from *m/z* 125 to 300. In all cases the ion chosen for quantification was the fragment ion $[M-32+H]^+$ resulting from the loss of CH_3O (Supplementary Figure 1). As mebendazole and fenbenzadole sulphone elute within 2 minutes of each other, we used a single segment (segment 2) to facilitate integration of the peaks. Capillary temperature was 275 °C, sheath gas and auxiliary gas were 40 and 10 units respectively and source voltage was 4.5 kV.

Solvents used were HPLC grade methanol, hexane and acetonitrile (Rathburn Chemicals Ltd), and analytical grade formic acid (Fisher Scientific UK). The analytical standards were FBZ, oxfendazole (QMX Laboratories Limited), fenbendazole sulphone (kindly donated by Intervet International) and HPLC grade dimethyl sulfoxide (Sigma-Aldrich Company Ltd), and the internal standard was mebendazole (Sigma-Aldrich Company Ltd). All water used was of HPLC grade.

Stock standard solutions of FBZ, OFZ, $FBZSO_2$ and internal standard mebendazole (MBZ) were prepared at a concentration of 1 mg mL^{-1} in dimethyl sulfoxide + methanol (9/1, v/v). The internal standard was chosen due to its structural similarities to the compound under investigation and was used to control for extraction, HPLC and ionization variability. These solutions were stored at 5 ºC and showed no significant changes after one month, when they were replaced by fresh solutions. Working standard solutions were prepared at concentrations ranging from 5–50,000 ng mL^{-1} by dilution with dimethyl sulfoxide + methanol (9/1, v/v). A standard solution, 100 ng mL^{-1} was used for tuning the mass spectrometer. Linear calibration curves were generated by plotting ln(peak area) against ln(working standard solution concentration). r^2-values for the calibration curves were 0.99806 (MBZ), 0.99998 (FBZ), 0.99833 (OFZ) and 0.99983 ($FBZSO_2$).

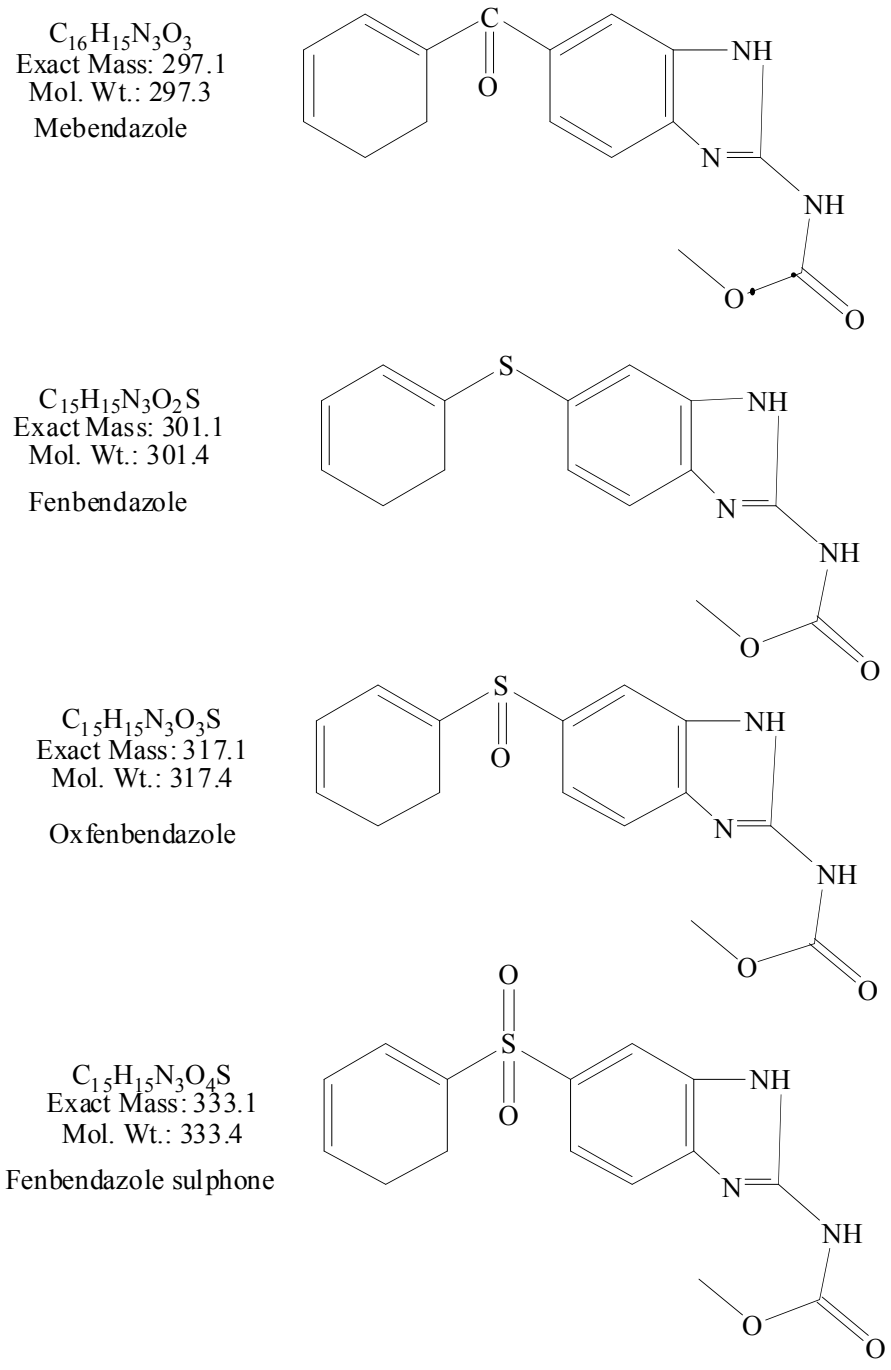
FBZ, OFZ, $FBZSO_2$ and MBZ were identified by their fragmentation pattern and by comparison of the peak retention times with that of previously run drug standards (Supplementary Figure 2). The four compounds under investigation all displayed a characteristic loss of CH_3O in the MS-MS spectra with no other significant ions being produced. The absence of any other ions in the MS-MS spectrum was used as additional proof of compound identity and peak purity. The use of Selected Reaction Monitoring using an ion trap mass spectrometer allows confirmation of the peak under investigation, by providing the full fragmentation spectra of the parent ion, without a loss of sensitivity. Only the major fragment ion formed from each parent was used in quantification.

The limit of detection (LOD) was determined at a signal to noise ratio of 3, as reported by the Xcalibur peak integration software and quantification was carried out on peaks that had a signal to noise ratio greater than 9. No false positive peaks were detected for any of the four compounds under investigation during the course of the study. Tissue drug residue concentration in $\mu g\ kg^{-1}$ (ppb) was calculated from sample drug concentrations (estimated from the calibration curve), sample solution volume and sample weight. Mean (±SD) recovery of the internal standard was 78 (±18) %; where recovery was less than 100%, residue concentration was adjusted by dividing by the recovery fraction.

The extraction method used in this analysis is much quicker and simpler than any reported for similar compounds (Danaher et al., 2007). Although the lack of clean up stages could result in more contaminants in the extract, no interfering peaks or false positives were seen in the analysis. The sensitivity and selectivity of the analysis allowed sample weights to be reduced to only 300 mg of liver. The low variation in the dry weights of these samples suggests that 300 mg samples can be weighed with a sufficient degree of accuracy for detection and quantification. However, it is likely that the accuracy of quantification would be further improved by increasing the sample weight.

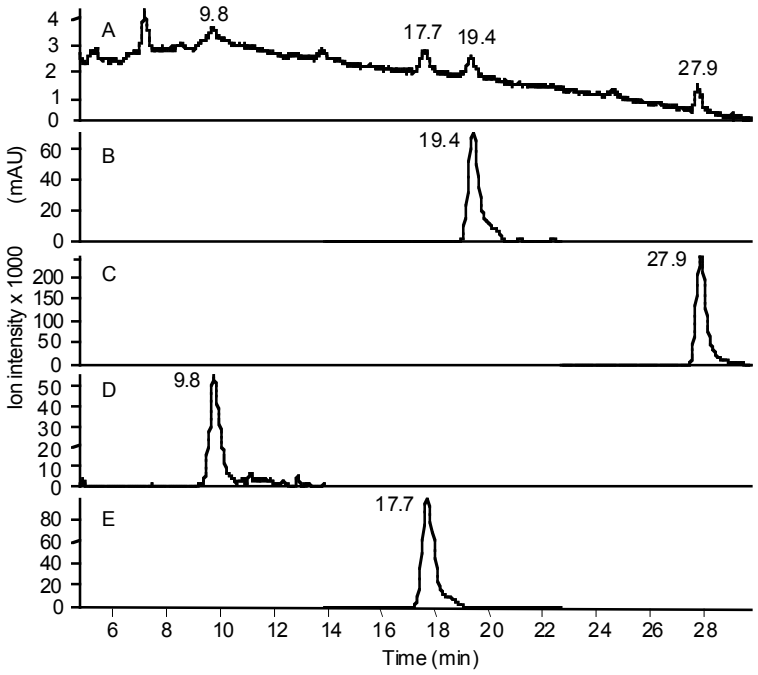
Reference

Danaher, M., De Ruyck, H., Crooks, S.R.H., Dowling, G., O'Keeffe, M., 2007. Review of methodology for the determination of benzimidazole residues in biological matrices. J. Chromatogr. B 845, 1-37.



Supplementary Figure 1 Molecular structures of the benzimidazoles investigated in this study.

I



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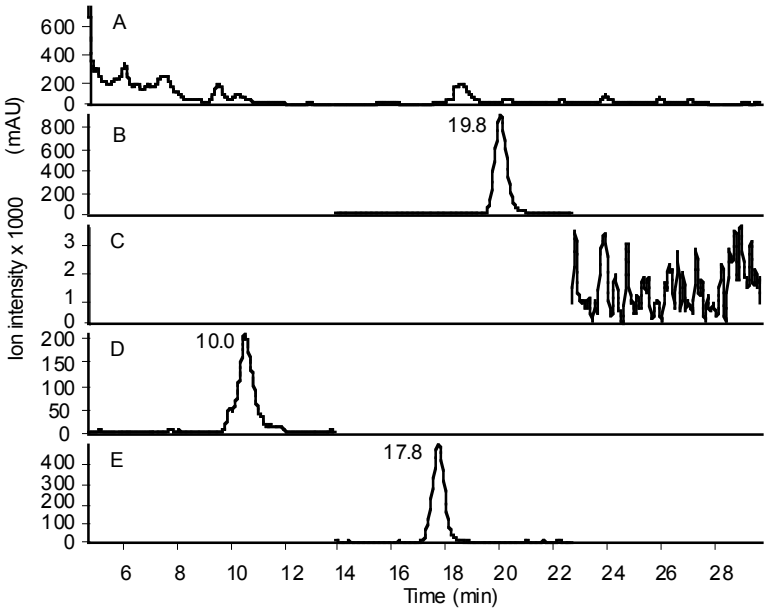

Supplementary Figure 2 Chromatograms of analytical standards (I) and a liver sample taken from sample $T^{+}6B$ (see Table 2) (II). A: absorbance at 290 nm. B: SRM m/z 298 > m/z 266 (mebendazole I.S.) C: SRM m/z 302 > m/z 270 (Fenbendazole). D: SRM m/z 318 > m/z 286 (oxfendazole.). E: SRM m/z 334 > m/z 302 (fenbendazole) sulphone.