Fenbendazole as a possible marker of supplement intake in sheep

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ABSTRACT

This study aimed to determine the dose-dependent relationship between oral doses of fenbendazole (FBZ) and the plasma concentration of its metabolites, oxfendazole (OFZ) and fenbendazole-sulphone (SUL). Twenty five, two year-old, Merino wethers were equally allocated to treatment groups of different oral dose rates of FBZ (n = 5) and housed in individual pens. Treatment groups were designed to provide daily oral doses of 0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg live weight of FBZ, suspended in water, for six days. Blood samples were collected from each animal at 48, 96, and 144 hours after administration of FBZ. Plasma was equally combined within each animal and analysed to determine concentrations of FBZ, OFZ and SUL. There was a positive linear relationship between FBZ dose rate and FBZ metabolite plasma concentration (R^2 = 0.991, P <0.001). Mean separation of plasma concentrations indicated significant differences (P <0.05) between treatments designed to provide 1.0, 2.0 or 4.0 mg/kg/day FBZ. Plasma concentrations of animals which received 0.25 or 0.50 mg/kg/day FBZ were significantly lower than other treatments (P <0.05). The results from this experiment provide preliminary support for the investigation of FBZ as a useful marker to estimate supplement intake of grazing animals.

Keywords: fenbendazole; oxfendazole; sheep; fenbendazole-sulphone; supplement intake.

INTRODUCTION

Estimating individual supplement consumption and its variability between animals within a flock is an important, but difficult to measure, requirement for determining product efficacy under grazing conditions. Established techniques to measure individual intake of supplements have a number of restrictions, making them undesirable for long-term supplement studies in grazing animals. Tritiated water has been used to estimate supplement intake (Nolan *et al*., 1975) but is constrained by its radioactive nature, associated with the ß-emitting unstable ^{3}H isotope, with concerns for operator and animal safety and the limitations it places on the sale of animals (Dixon *et al*., 2003). The digesta marker chromic oxide (Corbett *et al.,* 1958) requires complete faecal collection and digestibility assumptions (Langlands, 1975; Dixon *et al*., 2003) and is useful for estimates of short-term total intake rather than supplement intake. Plant wax alkanes (Dove & Mayes, 1991) recovered from faeces have been used to estimate forage and forage-based supplement intake, but the predictive capacity of this approach diminishes with increased botanical diversity (Lee & Nolan, 2003) and it is not used to measure intake of non-forage multi-ingredient supplements. Lithium chloride has been used to estimate supplement intake of grazing animals (Kahn, 1994) but excessive intake can lead to feed aversion (Burritt & Provenza*,* 1989), while plateau blood concentrations only last for approximately 10 hours, rendering it unsuitable for long-term studies.

Fenbendazole (FBZ) is a member of the benzimidazole anthelmintic class and has been reported to have a prolonged appearance in plasma following oral administration (Prichard & Hennessy *et al.*, 1978). This attribute suggests that blood FBZ may be a useful marker of supplement intake in a way that is independent of its anthelmintic activity and avoids the need for faecal collection.

FBZ is stable over a range of environments, has a wide margin of safety in mammals and is detectable in blood for approximately five days after dosing (Duwel, 1977). Hepatic metabolism of FBZ in sheep leads to the formation of two metabolites, oxfendazole (OFZ) and fenbendazole-sulphone (SUL) both of which can be detected in plasma with relative ease and accuracy using high pressure liquid chromatography (HPLC) (Bogan & Marriner, 1979; Boisvenue & Colestock *et al*., 1988; Prichard *et al.*, 1978). Knox et al. (1995) demonstrated that the plasma concentration of FBZ and OFZ in housed sheep increases in a linear manner with increased intake of a molasses supplement block, containing FBZ, and with intraruminal FBZ infusion.

This experiment was designed to determine (i) the relationship between repeated graded oral doses of FBZ and the corresponding plasma concentration of its metabolites OFZ and SUL; and (ii) the minimum detectable level of FBZ dose.

MATERIAL AND METHODS

Experimental design

The experiment was a one way randomized block design. Blocks (n = 5) were based on position in animal house and treatments were different oral dose rates (n = 5) of FBZ. Sheep were stratified on live weight and randomly allocated to each treatment with five replicate animals per treatment. Animals were maintained in single pens under natural lighting conditions with free access to water.

Animals and management

Twenty five two-year-old Merino wethers weighing an average of 38.3 kg (standard deviation = 2.2 kg), were sourced from the University of New England's rural property (Animal Ethics approval number AEC10/029). They were drenched at recommended rates with Virbamec LV® (0.2 mg/ kg abamectin; Virbac, Peakhurst, NSW, Australia) and Rotate® (3.6 mg/ kg albendazole oxide, 7.0 mg/kg levamisole hydrochloride; Novartis animal Health Australasia, North Ryde, NSW, Australia) before being transported to the animal house. Faecal samples were collected 10 days later and the concentration of nematode eggs in each sample counted. The counts indicated that the wethers were free of endoparasites prior to the start of the experiment. They were fed a ration of chaffed lucerne (20%) and oaten (80%) hay based on fresh weight and allowed to adapt to pens over a two week period. Feed was offered at 1 kg/day (fresh weight) throughout the experiment. There were no refusals. A single animal was removed from the experiment because of inappetence.

Treatment and administration

The treatment groups received oral doses of FBZ, suspended in water, daily for six days. The treatment groups were 0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg live weight of FBZ. The FBZ doses were prepared by weighing the necessary mass of FBZ into a dry, preweighed, 20 mL syringe, drawing water into the syringe and shaking to emulsify the contents prior to oral delivery of the FBZ dose. The syringe was twice filled with water and administered to the animal to maximise delivery of FBZ to the sheep. To determine the amount of FBZ administered, the syringe was later dried in a 60°C oven for 48 hours and weighed to determine the amount of residue.

Measurements and sampling

Blood samples were collected via jugular venipuncture (10 mL onto EDTA as an anticoagulant) from each animal at 48, 96, and 144 hours after administration of FBZ. The sampling frequency was established from earlier work (F. Fishpool, Unpublished results) where FBZ dose discrimination was possible for up to 48 hours after administration. Blood samples were centrifuged, plasma removed into 2 x 2 mL aliquots and stored at -20°C. At time of processing, plasma samples were combined within each animal and the composite plasma sample was then analysed using high performance liquid chromatography (HPLC). The purpose of the composite sample was to increase the efficiency of sample analysis and had previously been shown to provide an accurate estimate of the mean plasma concentration of FBZ metabolites in separate plasma samples from sheep (F. Fishpool, Unpublished results).

HPLC analysis

FBZ and its metabolites were extracted from plasma prior to HPLC analysis. A C18-E SepPak (Phenomenex, Lane Cove, NSW, Australia, product code: 8B-S001-HCH) was prepared by flushing with 5 mL of methanol followed by 5 mL of ammonium carbonate buffer (0.05 M, pH = 8.0). Plasma (1 mL) was then applied to the SepPak and successively washed through with 20 mL of deionised water, 0.5 mL of 30% methanol in deionised water and 5 mL of 100% methanol.

FBZ, OFZ and SUL were eluted in the final 5 mL of 100% methanol and collected and evaporated to dryness using a rotary evaporator with an ACTEvap attachment. Dry residues were then reconstituted in 0.3 mL of 100 % methanol. A further 0.2 mL of the ammonium carbonate buffer was added to create a 60:40 solution similar to the solvent used during chromotography. The sample was then filtered through a 4 µm diameter nylon syringe filter (Phenomenex, Lane Cove, NSW, Australia, product code: AF3-3107-52).

The Waters HPLC system was comprised of a Waters 1525 Binary pump, Waters 712 WISP sample processor, C18 Luna 10µm column (Phenomenex, Lane Cove, NSW, Australia, product code: 00F-4253-E0) with a C18 security guard column (Phenomenex, Lane Cove, NSW, Australia, product code: AJ0-4287), column heater set at 30°C and Waters 2996 Photodiode array detector set at 292 nm. Empower Pro 2 software (Waters Associates, Rydalmere, NSW, Australia) was used to collect and analyse the chromatographs.

The operating conditions consisted of a constant 1.5 mL per minute flow rate which produced a column pressure of 1,300 – 1,600 psi. Solvent A was a 50:50 methanol, ammonium carbonate (0.05M) solution and Solvent B was a 90:10 solvent solution. The gradient programme was 100% Solvent A (0-7 minutes), 75% A and 25% B (7-17 minutes), Solvent B (17-29 minutes) and re-equilibration with Solvent A (29-36 minutes) before injection of the next sample.

Extraction recovery of OFZ and SUL standards added to plasma (n = 12) was 72.4%, with a precision of 8.8%. The accuracy of the HPLC

TABLE 1: Planned and actual fenbendazole (FBZ) oral doses (arithmetic means ± standard error of mean) given to sheep over a six day period.

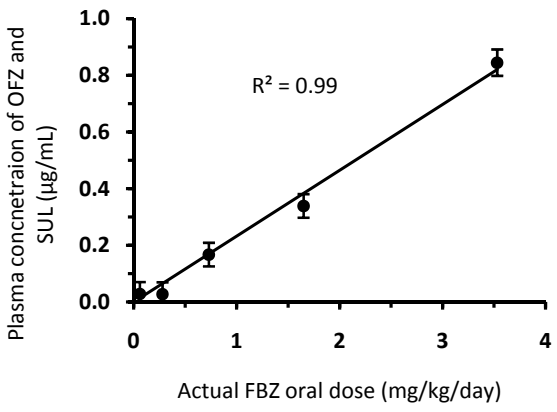
Planned FBZ oral dose (mg/kg/day)	Actual FBZ oral dose (mg/kg/day)
0.25	0.06 ± 0.01
0.5	0.28 ± 0.05
1.0	0.73 ± 0.01
2.0	1.65 ± 0.03
4.0	3.53 ± 0.01

analysis (n = 9) was 96.2% and precision was 3.9%. The repeatability of the HPLC analysis for individual samples (n = 3) processed three times was 1.4%.

Statistical analysis

The concentrations of OFZ and SUL were summed to provide a single estimate of the plasma concentration of FBZ metabolites (OFZ+SUL). The values of OFZ+SUL were not normally distributed and were transformed using $\log_e + 1$ prior to analysis. The effect of FBZ dose rate on OFZ+SUL was analysed using ANOVA and a protected student t-test was used to separate least square means. Significant differences were defined as $P < 0.050$. Block was not a significant factor in the model and was removed from the analysis. Data are presented as back transformed least square means ± 68% confidence intervals. Back transformed least square means with these confidence intervals are presented because they give a close approximation to standard errors which cannot be back transformed as they are asymmetrical about the mean.

FIGURE 1: Linear relationship (slope = 0.23 ± 0.01 (standard error)) between the actual fenbendazole (FBZ) oral dose (mean of treatment group) and mean plasma concentration of oxfendazole (OFZ) and fenbendazole-sulphone (SUL) (back-transformed means ± 68% confidence interval) in pooled plasma samples from individual sheep.



RESULTS

Actual FBZ oral doses were lower than the planned FBZ oral doses (Table 1). A larger proportion of FBZ remained undelivered to the animal as the dose rate decreased.

There was a positive linear relationship between FBZ dose rate and the combination of OFZ and SUL plasma concentrations ($R^2 = 0.99$; Figure 1). Mean separation of plasma concentrations indicated significant differences ($P < 0.05$) between each of the FBZ treatments which provided 0.73, 1.65 or 3.53 mg/kg/day FBZ. In addition, plasma concentrations of animals which received the lowest doses of 0.06 or 0.28 mg/kg/day FBZ were significantly lower ($P < 0.05$) than the other treatments but did not differ from each other. These plasma values are not likely to differ significantly from animals not dosed with FBZ and suggest a limit to detection.

DISCUSSION

The results from this experiment demonstrated a dose-dependent linear relationship between daily oral ingestion of FBZ and the combined plasma concentration of OFZ and SUL in sheep. This supports the observations of Knox *et al.* (1995) for unparasitised sheep that were fed a molasses supplement block containing FBZ, or that received an intraruminal FBZ infusion. From their work it can be calculated that the concentration of plasma OFZ and SUL increased by 0.31µg/mL (block) or 0.12 µg/mL (infusion) in response to a 1 mg/kg/day increase in FBZ. The response that we determined to this dose rate was 0.23 µg/mL which is mid-way between these earlier estimates.

The method of pooling blood samples taken at 48 hour intervals was effective for identifying differences in total ingestion of FBZ over a six-day period and reduced significantly the number of samples to be analysed. This sampling interval is considerably longer than the period between dose and sampling required for the lithium chloride technique (4-14 hours) (Kahn, 1994) which should lead to less disturbance of animals.

A lower limit of detection for plasma OFZ and SUL was identified as 0.03 µg/mL where, with the group sizes used in this experiment, this value did not differ from that expected for untreated animals. At this concentration, the noise to peak ratio limits the ability to confidently quantify OFZ and SUL plasma concentrations from the chromatograph. It can be calculated from the data used in Figure 1 that the FBZ daily dose needs to exceed 0.40 mg/kg to exceed the limit of detection and, with these group sizes, to differ significantly from zero. There was no evidence of a diminishing response in plasma OFZ and SUL concentrations with increasing FBZ dose.

Previous work in our laboratory with daily plasma samples showed that animals which had not received an oral dose of FBZ had no detectable levels of FBZ, OFZ or SUL in plasma.

In summary, plasma concentration of OFZ and SUL was linearly related with daily FBZ oral dose. The pooling of plasma from blood samples taken at 48 hour intervals was an effective and analytically-efficient way of detecting differences in FBZ intake which minimises animal disturbance. The relationship between FBZ dose and plasma metabolites requires verification with grazing animals where factors such as feed intake (Ali & Hennessy, 1995a), forage quality (Ali & Chick, 1995b) and parasitism (Marriner *et al.,* 1985) may diminish the usefulness of the technique. Nevertheless, these results encourage further investigation of FBZ as a marker to estimate supplement intake of grazing animals with a special role in longer-term observations.

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