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In vitro and *in vivo* assessment of the benzydamine-mediated interference with the hepatic S-oxidation of the anthelmintic albendazole in sheep

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ABSTRACT

The aim of this research was to investigate the influence of benzydamine (BZ) on the *in vitro* and *in vivo* hepatic metabolism of the anthelmintic albendazole (ABZ) in sheep. The enantioselective ABZ S-oxidation was assessed by the amount of its (−) and (+) ABZ-sulphoxide (ABZSO) enantiomers formed in sheep liver microsomes (*in vitro* work). In the *in vivo* trial, lambs received ABZ (5 mg/kg, intra-ruminal route) or ABZ (5 mg/kg) plus BZ (8 mg/kg, i.m., two doses 4 h apart). Incubated and plasma samples were analysed by HPLC. *In vitro*, BZ IC50s (the concentrations that produced a 50% decrease in ABZ S-oxidation) for the production of total ABZSO and (+)ABZSO were 71.0 ± 8.1 and 62.6 ± 8.1 μM, respectively. BZ showed a strong inhibitory potency over the flavin-monooxygenase (FMO)-dependent production of (+)ABZSO compared to the cytochrome P450 (CYP)-mediated production of (−)ABZSO. *In vivo*, co-administration of BZ with ABZ did not change the pharmacokinetic parameters of ABZSO and $ABZSO_2$ with the exception of significantly higher ($p < 0.01$) formation half-lives ($t_{1/2for}$) for (−)ABZSO (3.24 ± 1.03 h *vs.* 6.19 ± 2.18 h) and (+)ABZSO (3.87 ± 1.20 h *vs.* 7.21 ± 2.46 h). BZ inhibited the hepatic FMO and CYP-dependent S-oxidation of ABZ *in vitro*. However, the metabolic interaction between ABZ and BZ was not observed in the *in vivo* pharmacokinetic trial. Hence, further work using a different dosing scheme or pharmaco-technical preparation of BZ may be required to observe *in vivo* the metabolic interference clearly shown under *in vitro* conditions.

1. Introduction

Helminth parasites are a major cause of productivity losses worldwide due to clinical and subclinical losses, and the cost of implementing control programmes in ruminant livestock (Waller, 2003; Jasmer et al., 2003). Although several strategies are currently available to control parasitism in domestic animals (*i.e.*: pasture management, biological control, *etc.*), the use of antiparasitic drugs is still the mainstay of nematode control (Mitreva et al., 2007). Several chemical families have been used for effective parasite control, including the benzimidazole anthelmintic compounds, which are still widely used in both human and veterinary medicine to control nematode, cestode and trematode infections. Their intrinsic anthelmintic action,

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based on the disruption of basic cell functions depending on the integrity of the microtubule system, requires a sustained presence of the active drug at the site of parasite location (Lanusse and Prichard, 1993). Consequently, the extension of the residence time of active benzimidazole moieties in the bloodstream is relevant for their anthelmintic efficacy (Lanusse and Prichard, 1993).

Albendazole (ABZ) [methyl-[(5-propylthio)-1H-benzimidazol-2-yl] carbamate] is a benzimidazole methylcarbamate derivative which has been largely used in our laboratory as a model compound to characterise different factors affecting the pharmacokinetic (PK) and biotransformation processes in ruminant species. The metabolism of ABZ takes place predominantly in the liver, and both flavin-monooxygenase (FMO) and cytochrome P450 (CYP) systems are involved in the production of its anthelmintically active metabolite albendazole sulphoxide (ABZSO). In fact, the participation of both enzyme systems on the hepatic S-oxidation of ABZ has been demonstrated in sheep liver (Galtier et al., 1986; Lanusse et al., 1993; Virkel et al., 2004). Albendazole sulphoxide has an asymmetric centre in the sulphur atom of its side chain, and consequently two ABZSO enantiomers have been identified (by chiral separation) in the plasma of sheep after administration of ABZ (Delatour et al., 1991). In ruminants, the (+)ABZSO enantiomer has been shown to be mainly produced by the FMO system, while its (−) antipode almost completely formed in a CYP-dependent reaction (Virkel et al., 2004), thought to be catalysed by a CYP1A isoenzyme (Benoit et al., 1992; Capece et al., 2009). In addition, ABZSO undergo a second, slower and irreversible oxidative step leading to the production of an inactive sulphone metabolite ($ABZSO_2$). This metabolite is thought to be predominantly produced by a CYP1A isoenzyme, being (−)ABZSO the primary source for this metabolic reaction (Benoit et al., 1992; Souhaili-El Amri et al., 1988).

In terms of parasite uptake/accumulation (Alvarez et al., 2000) and mode of action (binding to tubulin) (Lubega and Prichard, 1991), ABZ parent drug is more efficient than its oxidised metabolite (ABZSO). Compared to the parent drug, ABZSO have less anthelmintic potency whereas $ABZSO_2$ is virtually inactive. Indeed, the oxidative metabolism in the liver results in a considerable reduction of ABZ anthelmintic efficacy. Therefore, *in vivo* interference with the liver FMO-mediated and/or CYP-mediated metabolism has resulted in pronounced modifications to the PK behaviour of anthelmintically active benzimidazole metabolites. Overall, it has been shown that co-administration of benzimidazole compounds with known metabolic inhibitors (methimazole, piperonyl butoxide) enhances the systemic availability of the parent drug and/or their metabolites (Lanusse and Prichard, 1992; Lanusse et al., 1995; Benchaoui and McKellar, 1996).

With the aim of searching for new potential pharmacological strategies to inhibit the metabolism of ABZ, the current research evaluated the effects of the FMO substrate benzydamine (BZ) on the hepatic S-oxidation of this anthelmintic in sheep under *in vitro* conditions. BZ, an indazole derivative with analgesic and antipyretic properties, therapeutically used in human and veterinary medicine, is metabolised to BZ N-oxide in a FMO-dependent reaction in the liver of different animal species (rat, hamster, rabbit, dog, swine, cattle) and human beings (Ubeaud et al., 1999; Fisher et al., 2002; Santi et al., 2002; Capolongo et al., 2010). The main objective of this research was to assess the influence of BZ on the hepatic metabolism of ABZ in sheep. The work was carried out under both *in vitro* and *in vivo* conditions. The *in vitro* assays included a comparison to the effects of the well known FMO substrate methimazole (MTZ), a strong inhibitor of the *in vitro* S-oxidation of this anthelmintic in sheep (Galtier et al., 1986; Lanusse et al., 1993; Virkel et al., 2004). In a forward step, an *in vivo* study was conducted to evaluate the effect of BZ on the plasma disposition kinetics of ABZ metabolites in sheep.

2. Materials and methods

2.1. Reagents

Reference standards (99% pure) of ABZ, ABZSO and $ABZSO_2$ were obtained from Sigma–Aldrich Chemical Company (St. Louis, USA). Stock solutions (5 mM) of each molecule were prepared in methanol (Baker Inc., Phillipsburg, USA). BZ (hydrochloride salt), oxibendazole (OBZ), nicotinamide adenine dinucleotide phosphate (NADP+), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5,5′-dithiobis nitrobenzoic acid (DTNB), dithiothreitol (DTT), tris base and tricine were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche Applied Science (Buenos Aires, Argentina). MTZ was a generous gift of Gador Argentina S.A. The solvents used for the chemical extraction and chromatographic analysis were HPLC grade (Baker Inc., Phillipsburg, USA). Buffer salts (KCl, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4 and CH_3COONH_4, $MgCl_2$) were purchased from Baker Inc. (Phillipsburg, USA).

2.2. Animals and preparation of subcellular fractions

Four healthy 2-year old Corriedale × Merino cross-breed rams were sacrificed to obtain samples of liver parenchyma (caudate lobe) for preparation of microsomes. Sheep were maintained under field conditions (grazed on a rye grass/white and red clover pasture) and received also a concentrate supplement (89.4% dry matter, 16.0% neutral detergent fibre, 11.2% crude protein, 74.2% dry matter digestibility and 2.68 Mcal/kg dry matter). Water was provided *ad libitum*. Animal procedures and management protocols were carried out according to internationally accepted animal welfare guidelines (AVMA, 2007) and approved by the Animal Welfare Committee of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina (Internal Protocol: FCV-BA-002-11; approval date: March 1st, 2012). Animals were stunned and exsanguinated immediately in agreement with these institutional and internationally accepted animal welfare guidelines.

After sacrifice, the abdomen was opened and the liver was removed. Samples were rinsed with ice-cold KCl 1.15%, stored in aluminium foils and chilled in ice. Tissue samples were brought to the laboratory for subsequent procedures, which started within 1 h from sample collection and were carried out between 0 and 4 °C. The preparation of liver microsomes was performed as described previously (Maté et al., 2008). Microsomal suspensions were stored at −70° C until being used in the different biotransformation assays.

2.3. Measurement of CYP content and FMO-dependent activity

The CYP content was determined as the carbon monoxide difference spectrum (450–490 nm) of sodium dithionite-reduced microsomal suspensions (Rutten et al., 1987). Methimazole S-oxidation was selected as a FMO-dependent specific pathway and assayed in pooled liver microsomes from four animals following the method described by Dixit and Roche (1984), using 0.12 mg of microsomal protein. Briefly, the enzyme activity was measured at 37 °C in the presence of 0.1 mM NADPH, 0.06 mM 5,5′-dithiobis nitrobenzoic acid (DTNB) and 0.02 mM

dithiothreitol (DTT) dissolved in 0.1 M tricine buffer (pH 8.4) containing 1 mM EDTA. The difference in absorbance between identical assay mixtures with and without MTZ was monitored at 412 nm for 5 min. Kinetic parameters for MTZ S-oxidation were obtained from individual experiments after the addition of increasing amounts of the substrate (0.125–5 mM) to the sample cuvette. Each substrate concentration was assayed in duplicate, either in the absence (control) or in the presence of BZ at 750 μM.

2.4. Enzyme assays

Albendazole S-oxidation was assessed in sheep liver microsomes by the amount of ABZSO formed in the presence of a NADPH generating system. A typical reaction mixture contained (in a final volume of 0.5 mL): phosphate buffer 0.1 M (pH 7.4), 0.5 mg of microsomal protein diluted in 100 μL of the same buffer, NADPH-generating system (0.32 mM $NADP^+$, 6.4 mM glucose-6-phosphate, 5 mM $MgCl_2$, 0.8 mM EDTA and 1.25 U of glucose-6-phosphate dehydrogenase in phosphate buffer) and 50 μM of ABZ dissolved in 10 μL methanol. To assess the concentrations of BZ or MTZ that produced a 50% decrease in the production of ABZSO, the substrate was incubated either in the absence (control assays) or in the presence of variable concentrations (12.5 to 1000 μM) of BZ (dissolved in 5 μL of methanol) or MTZ (dissolved in 20 μL of water). Control incubations contained also the same amount of methanol or water. Thawed microsomal suspensions were diluted in the phosphate buffer followed by the addition of the NADPH-generating system. These incubation mixtures were allowed to equilibrate (3 min at 37 °C) before the addition of BZ or MTZ. Immediately after, the reaction started with the addition of ABZ. All incubations (15 min at 37 °C) were performed by duplicate in glass vials in an oscillating water bath under aerobic conditions.

Inactivation of FMO was performed by heating the diluted microsomal preparation without NADPH (2 min at 50 °C), which was immediately chilled in ice (Dixit and Roche, 1984). Then, the NADPH-generating system was added and the reaction started with the addition of the substrate (Virkel et al., 2004). Blank samples were also incubated in all metabolic assays, containing all components of the reaction mixture except the NADPH-generating system. These incubations were used as controls for possible non enzymatic drug conversion. All reactions were stopped by the addition of 0.2 mL of ice-cold acetonitrile and stored at −20 °C until analysis.

2.5. Pharmacokinetic trial

Eight healthy Corriedale × Merino cross-breed lambs (24.9 ± 2.4 kg) were randomly allocated into two treatment groups of four animals each and placed in indoor facilities during the experiment. Sheep received the same diet as stated above and also had free access to water. Animals' health was clinically monitored prior and throughout the entire trial. Lambs were parasite free, in optimal body condition and no clinical evidences of disease (*i.e.*: hyperthermia, dyspnoea, diarrhoea) were observed. Physiologic average values were measured for rectal temperature (39.6 °C), cardiac frequency (76 beats/min) and respiratory frequency (19 breaths/min). Mucous membranes' colour was normal.

A cross over design with a wash out period of 15 days was employed. In phase 1, four lambs were treated with a commercial suspension (38 mg/mL) of ABZ (Baxen®, Tecnofarm, Argentina) at 5 mg/kg by the intra-ruminal (i.r.) route (control group), and four animals received the same ABZ suspension at the same dose rate (i.r.) plus two equimolar doses (8 mg/kg each other) of an aqueous solution (50 mg/mL) of BZ (hydrochloride salt) *via* intramuscular (i.m.). The two doses of BZ were administered 30 min before and 3.5 h after the anthelmintic treatment. Because, to the best of our knowledge, this anti-inflammatory drug is not marketed for use in sheep nor there is any available dose recommended for this species, an equimolar dose (to ABZ dose rate) was empirically used. In addition, in a preliminary assay, BZ administered at 8 mg/kg was clinically safe (data not shown). In phase 2, drug treatments were reversed for each group.

Jugular blood samples were taken into vacutainer tubes containing heparin prior to and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48 and 54 h post-treatment. The collected blood samples were centrifuged at 2000 *g* during 15 min and plasma stored at −20 °C until the time of analysis.

2.6. Chromatographic analysis

Both incubated and plasma samples were analysed for ABZ and its metabolites. Twenty microlitre of 0.25 mM OBZ (internal standard solution in methanol) were added to the inactivated microsomal incubation mixture. Then, samples were shaken during 10 s and centrifuged at 10 000 × *g* for 15 min at 10 °C. Supernatants were directly injected into the HPLC system. Aliquots of the collected plasma (0.5 mL) were also supplemented with 20 μL of OBZ solution (25 μg/mL methanol). Then, ABZ and its metabolites were extracted using disposable C18 columns for solid-phase extraction (Strata® C18-T, Phenomenex, Torrance, USA) following previously described procedures (Alvarez et al., 2000). Extracted plasma samples were evaporated to dryness in a Savant Automatic SpeedVac® System (Thermo Savant, NY, USA). The dry residue was dissolved in the mobile phase (300 μL) and injected into the HPLC system.

Fifty microlitre of supernatants (microsomal incubations) or evaporated plasma samples dissolved in mobile phase were directly injected through an autosampler (Shimadzu SIL 10 A Automatic Sample Injector) into a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a Kromasil C18 (5 μm, 250 mm × 4.60 mm) reverse-phase column (Eka Chemicals, USA) and UV detector (Shimadzu, SPD-10A UV detector) reading at 292 nm. The mobile phase was an acetonitrile/ammonium acetate (25 mM, pH 6.6) elution gradient. The chromatographic conditions were as previously reported (Virkel et al., 2004), with slight modifications made to the elution gradient. The analytes were identified with the retention times of pure reference standards. Chromatographic peak areas of the analytes were measured using the integrator software (LC Solution, Shimadzu Corporation, Kyoto, Japan) of the HPLC system.

Albendazole sulphoxide chromatographic peak fractions were collected into a glass tube by using a fraction collector (FRC-10A Shimadzu Corporation, Kyoto, Japan). The collected fractions were evaporated to dryness and redissolved with 2% acetonitrile in water (150 μL). Fifty microlitre of each sample were injected into the HPLC system fitted with a chiral stationary phase column (5 μm, 100 mm × 4.0 mm) (Chiral-AGP column, ChromTech, Hägersten, Sweden). This chiral chromatographic method was adapted from a methodology described previously for the chiral separation of ABZSO (Delatour et al., 1991). Albendazole sulphoxide enantiomers were identified after the chromatographic analysis of a pure racemic standard of this metabolite. The relative proportions (%) of each enantiomeric form were obtained using the integrator software of the HPLC system. These proportions were used to calculate the rates of formation of each enantiomer.

Validation of the analytical procedures for quantification of ABZ and its metabolites was performed before starting the analysis of the experimental samples from the incubation assays. These procedures were performed as described earlier (Virkel et al., 2004). Briefly, known amounts of each analyte were added to aliquots of boiled (inactivated) microsomal preparations or plasma samples from untreated animals to obtain calibration standards (microsomes: 2–100 μM; plasma: 0.05–2 μg/mL) which were analysed by HPLC (triplicate determinations). Calibration curves were prepared using the least squares linear regression analysis (Instat 3.00, Graph Pad Software, Inc., San Diego, USA) of HPLC peak area ratios of analytes/internal standard and nominal concentrations of spiked samples. A lack-of-fit test was also carried out to confirm the linearity of the regression line of each analyte. The concentrations in the experimental samples were determined following interpolation of peak area ratios of analytes/internal standard into the standard curves. Square correlation coefficients (R^2) were 0.998–0.999 and a lack-of-fit test was also carried out to confirm the linearity of the regression line of each analyte. The concentrations in the experimental samples were determined following interpolation using the standard curves. For each analyte, inter-day precision coefficients of variation (CVs) and relative error (accuracy) values were below 15%. Drug/metabolites limits of detection (LOD) and quantification (LOQ) were as follows: 0.006 and 0.040 μg/mL (ABZSO); 0.009 and 0.030 μg/mL ($ABZSO_2$); 0.040 and 0.110 μg/mL (ABZ). The concentrations of each ABZSO enantiomer were calculated by means of the enantiomeric proportion (%) found in each incubation/plasma sample and the actual concentration of total ABZSO in the same experimental sample.

2.7. Data, pharmacokinetic and statistical analyses

The reported data are expressed as mean ± SD. Metabolic rates are expressed in nmol of metabolic products formed per min mg^{-1} of

microsomal protein (nmol/min mg). A non-linear regression model was used to calculate enzyme kinetic parameters for MTZ S-oxidation (FMO-dependent specific activity). For this propose, the maximal velocity of the enzyme reaction (V_{max}) and the substrate concentration giving half maximal velocity (K_m) were obtained by means of the following equation:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where v is the reaction velocity and [S] is the substrate concentration.

The concentrations of BZ or MTZ that produced a 50% decrease in the production of ABZSO (IC50s) were determined from plots of the percentage of inhibition *versus* the natural logarithm of inhibitor concentration.

The concentration *versus* time curves for ABZ and its metabolites in plasma for each individual animal after the different treatments were fitted with PK Solution 2.0 (SUMMIT Research Services, Ashland, USA). The following equation (Notari, 1987) was used to describe the biexponential concentration–time curves for ABZSO and $ABZSO_2$ after the i.r. administration of ABZ:

$$C_p = Be^{-\beta t} - Be^{-kt}$$

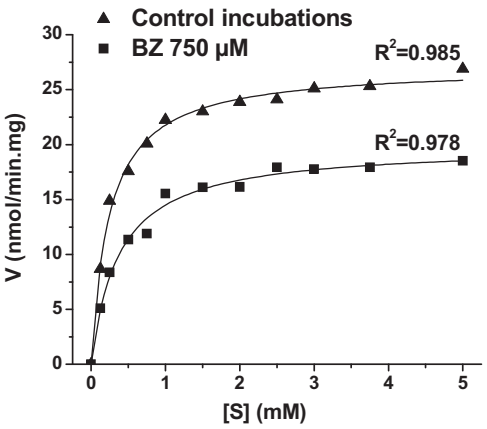
where C_p = concentration in plasma at time t after administration (μg/mL); B = concentration at time zero extrapolated from the elimination phase (μg/mL); e = base of the natural logarithm; β = terminal slope (h^{-1}); and k is the slope obtained by feathering which represents the first order metabolite formation rate constant (k_{for}) (h^{-1}). The elimination half-life ($t_{1/2}\beta$) and metabolite formation half-lives ($t_{1/2for}$) were calculated as $\ln 2/\beta$ and $\ln 2/k$, respectively. The peak concentration (C_{max}) and time to peak concentration (T_{max}) were displayed from the plotted concentration–time curve of each analyte. The area under the concentration time curve (AUC) was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope (β). The mean residence time (MRT) was determined as AUMC/AUC (Perrier and Mayersohn, 1982) where AUMC is the area under the curve of the product of time and the plasma drug concentration *vs.* time (Gibaldi and Perrier, 1982) and AUC is as defined above.

IC50s and PK parameters were compared by means of the Student t-test with Welch correction using the Instat 3.00 software (Graph Pad Software, Inc., San Diego, USA). P values less than 0.05 were considered significant.

3. Results

The CYP content in sheep liver microsomes was 0.7 ± 0.2 nmol per mg^{-1} of microsomal protein. The FMO-dependent specific activity (MTZ S-oxidation) was measured in pooled liver microsomes. When the inhibitory effect of BZ (250–1500 μM) was tested, the maximum percentage of inhibition was obtained at 750 μM (data not shown). In a further step, kinetic constants were calculated after the incubation of variable substrate concentrations (0.125–5 mM) both in absence (control) and in presence of BZ at 750 μM (Fig. 1).

Albendazole was metabolised into its pharmacologically active sulphoxide metabolite (ABZSO) by sheep liver microsomes. Only trace amounts of the $ABZSO_2$ metabolite were recovered in some of the incubation mixtures. The maximal rate of S-oxidation was 2.1 ± 0.2 nmol of total ABZSO formed per min mg^{-1} of microsomal protein. The (+)ABZSO/(−)ABZSO ratio and the enantiomeric excess (%) were 5.5 ± 1.2 and 68.2 ± 6.8, respectively. FMO inactivation decreased 65.6 ± 3.9% ($p < 0.001$) the total S-oxidation of ABZ (from 2.1 ± 0.2 to 0.7 ± 0.1 nmol/min mg). The relative contribution of both enzyme systems to the hepatic biotransformation of the anthelmintic drug was estimated on the assumption that FMO inactivation leaves



	CONTROL	BZ 750 μM
Vmax (nmol/min.mg)	27.1	19.9
Km (μM)	246	373
Cl_{int} (μL/min.mg)	110	53.4

Fig. 1. Kinetics of the flavin-monooxygenase-mediated S-oxidation of methimazole by pooled sheep liver microsomes. Incubations were carried out either in the absence (control) or presence of benzydamine (BZ) 750 μM. K_m: substrate concentration giving half-maximal velocity; V_{max}: maximal velocity of the metabolic reaction; Cl_{int} = intrinsic clearance (V_{max}/K_m).

the CYP system able to metabolise ABZ. Therefore, of the total ABZSO production, the FMO system produced 65.6% (1.4 ± 0.1 nmol/min mg), whereas the CYP system 34.4% (0.7 ± 0.1 nmol/min mg).

Both BZ and MTZ inhibited ABZ S-oxidation in sheep liver microsomes. The percentage of inhibition of total ABZSO and (+)ABZSO production *versus* the natural log of inhibitor concentration are plotted in Fig. 2. Methimazole IC50s for the production of total ABZSO and (+)ABZSO were lower ($p < 0.001$) compared to BZ IC50s (Fig. 2, inserted tables). Besides, both BZ and MTZ were able to inhibit the production of (−)ABZSO, but their estimated IC50s were around the highest concentration (1000 μM) used in the incubation assays (data not shown). Percentages of inhibition for the production of (−)ABZSO were 57.3 ± 6.4 (BZ) and 48.0 ± 19.0 (MTZ) when both metabolic inhibitors were incubated at 1000 μM.

Both ABZSO enantiomers and the sulphone metabolite ($ABZSO_2$) were detected in plasma after the i.r. administration of ABZ, either alone (control group) or co-administered with BZ. The mean (±SD) plasma concentration profiles for total ABZSO and its (−) and (+) enantiomers in both experimental groups are shown in Fig. 3. Biexponential plasma disposition profiles were observed for total ABZSO and both enantiomeric forms. Table 1 summarises the mean (±SD) PK parameters for total ABZSO and its (−) and (+) enantiomers obtained for both anthelmintic treatments. Within each experimental group, the mean (±SD) AUC and C_{max} values of (+)ABZSO were significantly higher ($p < 0.001$) compared to those observed for its antipode. Besides, the mean (±SD) (+)ABZSO/(−)ABZSO AUC and C_{max} ratios after the administration of ABZ alone ($AUC_{(+)ABZSO/(-)ABZSO}$: 8.0 ± 4.3; $C_{max(+)ABZSO/(-)ABZSO}$: 5.5 ± 1.8) were similar

Table 1
Comparative plasma disposition kinetics of albendazole sulphoxide (ABZSO) enantiomers in sheep after the intraruminal administration of albendazole (ABZ) parent drug (5 mg/kg) either alone or co-administered with the metabolic inhibitor benzydamine (BZ).

PK parameter	(+)ABZSO		(−)ABZSO	
	ABZ alone	ABZ + BZ	ABZ alone	ABZ + BZ
$t_{1/2for}$ (h)	3.9 ± 1.2	7.2 ± 2.5**	3.2 ± 1.0	6.2 ± 2.2**
C_{max} (μg/mL)	0.6 ± 0.2(a)	0.6 ± 0.1(a)	0.1 ± 0.0	0.1 ± 0.0
T_{max} (h)	11.5 ± 6.4	14.3 ± 6.0	8.6 ± 4.8	10.5 ± 3.7
$t_{1/2}\beta$ (h)	8.1 ± 3.4	8.4 ± 2.0	5.5 ± 3.4	6.3 ± 1.7
AUC_{0-t} (μg h/mL)	12.7 ± 3.3(a)	14.3 ± 2.3(a)	1.8 ± 0.5	2.0 ± 0.8
MRT (h)	19.2 ± 3.9	19.3 ± 2.1	14.4 ± 4.7	14.9 ± 2.3

Data are expressed as mean ± SD ($n = 8$ animals).
C_{max}, peak plasma concentration; T_{max}, time to peak plasma concentration; $t_{1/2for}$, metabolite formation half-life; $t_{1/2}\beta$, elimination half-life; AUC_{0-t}, area under concentration–time curve from time 0 to the last plasma concentration detected; MRT, mean residence time. Values are statistically different (**$p < 0.01$) with respect to ABZ control. (a) Statistically different ($p < 0.001$) with respect to (−)ABZSO in the same experimental group.
BZ (hydrochloride salt) was administered as an aqueous solution (50 mg/mL) by the intramuscular route (two doses of 8 mg/kg each other).

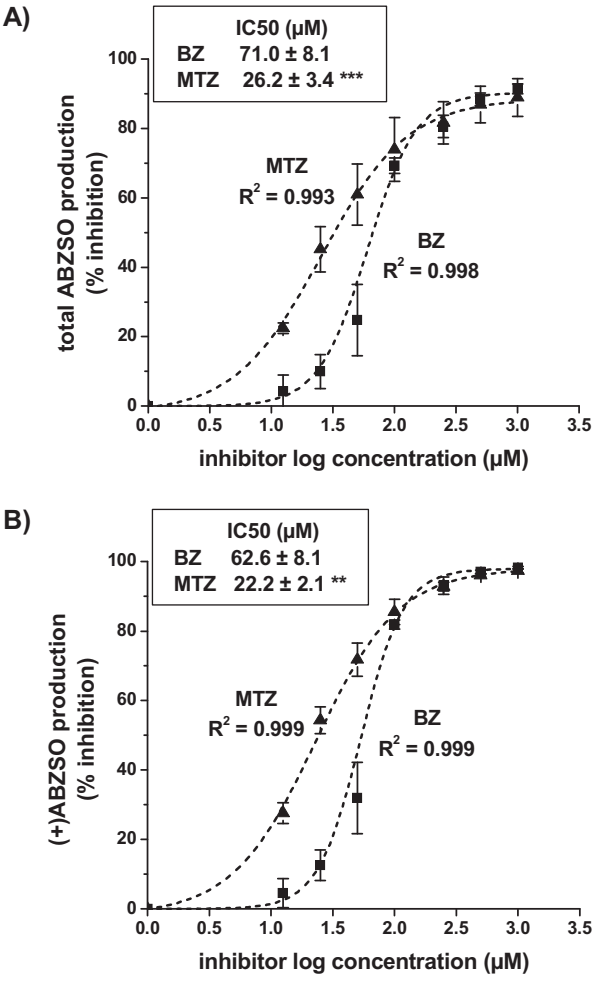


Fig. 2. Inhibitory effects of benzydamine (BZ) and methimazole (MTZ) on albendazole (ABZ) sulphoxidation by sheep liver microsomes. Data (mean ± SD) represent the percentages of inhibition of the production of total albendazole sulphoxide (ABZSO) (A) and the enantiomer (+)ABZSO (B) *versus* the natural logarithmic concentrations of each metabolic inhibitor. The inserted tables show the mean (±SD) IC50s of both metabolic inhibitors expressed in μM. Values are statistically different (**$p < 0.01$ and ***$p < 0.001$) with respect to BZ.

to those obtained after the co-administration of ABZ and BZ ($AUC_{(+)ABZSO/(-)ABZSO}$: 8.1 ± 2.5; $C_{max(+)ABZSO/(-)ABZSO}$: 5.7 ± 2.4). With the exception of significantly higher ($p < 0.01$) formation half-lives ($t_{1/2for}$) obtained for (−)ABZSO and (+)ABZSO when BZ was co-administered with the anthelmintic, no statistical differences were observed among PK parameters describing the disposition kinetics of ABZ metabolites after both experimental treatments. Mean (±SD) PK parameters for the sulphone metabolite are shown in Table 2.

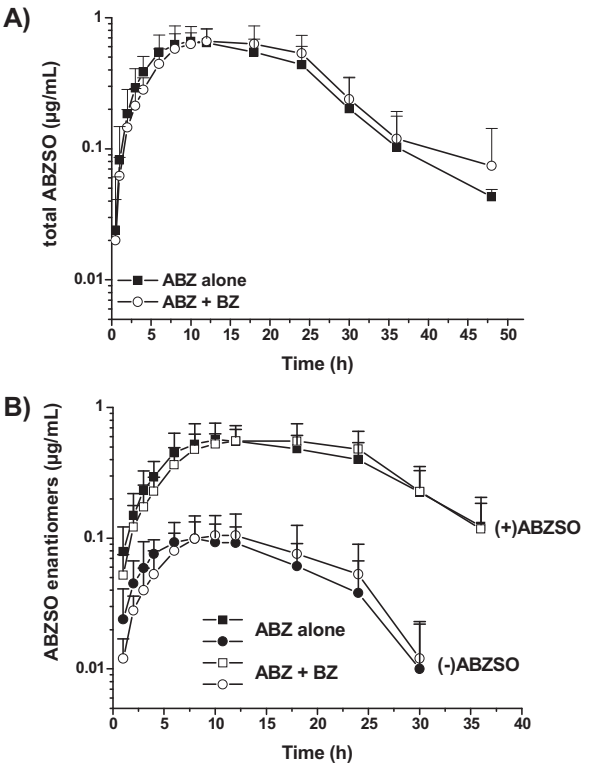


Fig. 3. Mean (±SD) plasma concentration profiles (μg/mL) of total albendazole sulphoxide (ABZSO) (A) and its (+) and (−) enantiomers (B) in sheep after the intraruminal administration of albendazole (ABZ) parent drug (5 mg/kg) either alone or co-administered with benzydamine (BZ).

Table 2
Comparative pharmacokinetic (PK) parameters for albendazole sulphone ($ABZSO_2$) in plasma obtained after the administration of albendazole (ABZ) parent drug (5 mg/kg) either alone or co-administered with the metabolic inhibitor benzydamine (BZ) to sheep.

PK parameter	$ABZSO_2$	
	ABZ alone	ABZ + BZ
$t_{1/2for}$ (h)	7.0 ± 1.9	5.6 ± 1.4
C_{max} (μg/mL)	0.2 ± 0.0	0.2 ± 0.0
T_{max} (h)	24.8 ± 2.1	24.8 ± 3.9
$t_{1/2}\beta$ (h)	13.9 ± 6.1	11.1 ± 4.1
$AUC_{0-36\ h}$ (μg h/mL)	5.6 ± 0.9	6.4 ± 1.1
MRT (h)	30.2 ± 7.8	28.5 ± 1.4

Data are expressed as mean ± SD ($n = 8$ animals).
C_{max}, peak plasma concentration; T_{max}, time to peak plasma concentration; $t_{1/2for}$, metabolite formation half-life; $t_{1/2}\beta$, elimination half-life; $AUC_{0-36\ h}$, area under concentration–time curve from time 0 to 36 h after drug treatment; MRT, mean residence time.
BZ (hydrochloride salt) was administered as an aqueous solution (50 mg/mL) by the intramuscular route (two doses of 8 mg/kg each other).

4. Discussion

The mammalian FMO system involves microsomal enzymes that catalyse the NADPH-dependent oxygenation of a large number of structurally diverse xenobiotics (*i.e.* drugs, chemicals and pesticides) containing nucleophilic atoms like nitrogen, phosphorus and sulfur (Ziegler, 2002). Five distinct functional FMO isoenzymes (FMO1 to 5) have been identified in mammals. In addition, mammalian FMOs have different but often overlapping substrate specificities, a similar feature to the CYP enzyme system (Ziegler, 2002). In this regard, both FMO1 and FMO3 metabolise MTZ and BZ, which have been used as marker substrates for the evaluation of the FMO-mediated metabolism in humans and laboratory animals (Ubeaud et al., 1999) as well as in species of veterinary interest (Longin-Sauvageon et al., 1998; Ubeaud et al., 1999; Capolongo et al., 2010). As FMO3 is the major flavin-containing isoenzyme expressed in sheep liver (Longin-Sauvageon et al., 1998), it would be the main isoform involved in the FMO-dependent oxidation of BZ, MTZ and sulfur-containing benzimidazole moieties in this species. On the other hand, FMO substrates may also compete for the active site of the FMO isoenzyme involved in their metabolism. For instance, MTZ inhibited the FMO-dependent production of BZ N-oxide in liver microsomes from cattle and rabbit (Capolongo et al., 2010). In the current work, MTZ S-oxidase was selected as a specific enzyme activity to test the FMO-dependent metabolism in sheep liver, and to investigate whether or not BZ is a suitable inhibitor for this metabolic reaction. Thus, in pooled sheep liver microsomes, BZ increased the K_m (from 246 to 373 μM) and decreased both the V_{max} (from 27.1 to 19.9 nmol/min mg) and the Cl_{int} (from 110 to 53.4 μL/min mg) of the MTZ S-oxidase activity (see Fig. 1). The observed changes on the kinetic constants justified the potential use of BZ as a suitable inhibitor of the hepatic metabolism of other FMO substrates such as ABZ, and likely other benzimidazole anthelmintics.

There are important species differences on the involvement of both FMO and CYP systems on ABZ hepatic S-oxidation. Thus, FMO accounted for ~30–40% of the ABZ S-oxidase activity in rats and humans, while the CYP system was found as the major contributor (~60–70%) (Moroni et al., 1995; Rawden et al., 2000). Conversely, it has been shown that FMO is the main enzyme system involved in ABZ hepatic S-oxidation in sheep and cattle (Virkel et al., 2004). The current work corroborated those previous findings in ruminants; the FMO system was found to participate in ~66% of ABZ S-oxidation, whereas ~34% of the total ABZSO formation depends on the CYP activity. Because FMO was found as the major contributor to the hepatic biotransformation of ABZ (and other benzimidazoles) in ruminants, any metabolic interference with this enzyme system may have a significant impact on the overall metabolic fate of these anthelmintic compounds.

Enantioselectivity of metabolic products occurs when chiral metabolites are generated differentially (in qualitative or quantitative terms) from a single achiral substrate (Testa and Mayer, 1988). Both FMOs and CYPs are known to be oppositely enantioselective when particular members within these enzyme systems metabolise the same substrate (Cashman, 1998). Therefore, the FMO activity accounted for 81% of (+)ABZSO production, whereas the CYP system was found to be mainly involved in the production of the (−) enantiomer in sheep liver (Virkel et al., 2004). The IC50s of both MTZ and BZ resulted extremely and significantly lower for the production of (+)ABZSO compared to (−)ABZSO. Indeed, this observation indicates a major effect of both metabolic inhibitors on the FMO-dependent S-oxidation of ABZ towards its (+)ABZSO metabolite. On the other hand, BZ IC50s for both the total S-oxidation and the production of (+)ABZSO were higher compared to those obtained for MTZ. This finding support the lower inhibitory potency of BZ compared to MTZ.

As a forward step, the work reported here included a PK trial performed in order to establish whether or not BZ would be a useful *in vivo* inhibitor of ABZ metabolism. The absence of ABZ parent drug in plasma after its i.r. administration, either alone (control group) or co-administered with BZ, reflects the extensive first-pass oxidation of the anthelmintic in sheep liver. The (+)ABZSO/(−)ABZSO C_{max} and AUC ratios observed after both experimental treatments were around 5 and 8, respectively. Indeed, these enantiomeric ratios indicate that (+)ABZSO predominates in sheep systemic circulation, as previously was demonstrated in this species and also in cattle and goats (Delatour et al., 1991). These observations are consistent to the results obtained *in vitro* and clearly reflect the relative contribution of the FMO and CYP enzyme systems to the enantioselective S-oxidation of ABZ. Moreover, ABZ sulphonation was found as a CYP-dependent reaction, and (−)ABZSO is thought to be the main substrate for the production of the inactive sulphone metabolite (Benoit et al., 1992).

In vivo interference with the hepatic metabolism of benzimidazole anthelmintics may delay the elimination and enhance the plasma levels of the parent drug and/or its active metabolite(s). This phenomenon may prolong their plasma-tissue recycling process and increase their concentrations at the most important tissues of parasite location (Lanusse and Prichard, 1993). As consequence of the enhanced levels, a significant parasite exposure to the active moieties may improve the clinical efficacy of the

administered compound. In sheep, co-administration of oxfendazole with MTZ (Lanusse et al., 1995) or PB (Sánchez et al., 2002) increased the concentrations of the active moieties (fenbendazole and oxfendazole itself) in the systemic circulation. Besides, MTZ and metyrapone, a potent inhibitor of the CYP system, improved the plasma availabilities of ABZ metabolites following the administration of netobimin (an albendazole prodrug) to sheep (Lanusse and Prichard, 1991, 1992). More than 3-fold increments in the AUC values of fenbendazole and its sulphoxide metabolite (oxfendazole) were observed following fenbendazole + PB administration to sheep and goats (Benchaoui and McKellar, 1996). Moreover, the concomitant administration of fenbendazole with PB markedly improved the efficacy against benzimidazole-resistant strains of *Ostertagia circumcincta* and *Haemonchus contortus* in sheep (Benchaoui and McKellar, 1996). On the other hand, co-administration of MTZ with netobimin or ABZ increased the efficacy against arrested larvae of *Ostertagia ostertagi* and against total adult gastrointestinal nematodes in naturally infected cattle (Lanusse and Prichard, 1993). All these previous results clearly demonstrate the practical relevance of the interference with the liver oxidative metabolism, which may represent a useful tool to increase the antiparasitic efficacy of benzimidazole anthelmintics in ruminants. Although BZ was able to inhibit ABZ S-oxidation *in vitro*, the drug did not modify the systemic exposure of ABZ metabolites in sheep. Significantly higher ($p < 0.01$) formation half-lives ($t_{1/2for}$) for (+)ABZSO and (−)ABZSO enantiomers (see Table 1) were evidenced after the co-administration of ABZ with BZ, being the unique pharmacokinetic modifications observed in the presence of this metabolic inhibitor. This observation may indicate a delayed *in vivo* hepatic ABZ S-oxidation in the presence of BZ, which did not induce changes on the plasma availability of ABZSO enantiomers. A clinically relevant metabolic interaction after the concomitant administration of two different drugs may occur if adequate concentrations are concurrently achieved at the site of biotransformation. Although there is not information available on the kinetic disposition of BZ in sheep, a high value of body clearance (~33 mL/min kg), a short half-life (~1.2 h) and a wide apparent volume of distribution (~3.5 L/kg) characterised its plasma disposition kinetics in dairy cows (Anfossi et al., 1993). Moreover, BZ was shown to be rapidly cleared from the liver (Fisher et al., 2002), the site where the metabolic interaction with the anthelmintic might have occurred. Although two doses of BZ (every 4 h) were administered in the current trial, the possible fast disposition and elimination of the inhibitor (compared to the anthelmintic drug) may have accounted for the lack of an *in vivo* metabolic interaction between ABZ and BZ under the experimental conditions described here. From a different point of view, the co-administration of BZ with ABZ seems to be safe as the anti-inflammatory drug does not affect the pharmacokinetic behaviour of the anthelmintic in this species.

Overall, the use of pharmacological strategies to increase the systemic exposure of anthelmintic drugs may be an alternative to optimise antiparasitic treatments, which is particularly relevant considering the widespread level of nematode resistance to benzimidazole anthelmintics in small ruminant production. The impairment of drug metabolism and excretion processes may give rise to increased drug exposure in tissues of parasite location and, therefore, to enhanced activity against resistant parasites. This research also highlights the importance of complementary *in vitro*/*in vivo* observations to characterise the metabolic fate of therapeutically used drugs. Further work using a different dosing scheme or pharmacotechnical preparation of BZ may be required to observe *in vivo* the metabolic interference clearly shown under *in vitro* conditions. Finally, the evaluation of other safe compounds to be used as *in vivo* metabolic inhibitors of benzimidazole anthelmintics remains as an open research area.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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