In vitro ruminal biotransformation of benzimidazole sulphoxide anthelmintics: enantioselective sulphoreduction in sheep and cattle

G. VIRKEL
A. LIFSCHITZ
A. PIS &
C. LANUSSE

Laboratorio de Farmacología, Departamento de Fisiopatología, Facultad de Ciencias Veterinarias, UNCPBA, Campus Universitario, (7000) Tandil, Argentina

Virkel, G., Lifschitz, A., Pis, A., Lanusse, C. *In vitro* ruminal biotransformation of benzimidazole sulphoxide anthelmintics: enantioselective sulphoreduction in sheep and cattle. *J. vet. Pharmacol. Therap.* **25,** 15–23.

The comparative *in vitro* sulphoreduction of the (+) and (–) enantiomers of albendazole sulphoxide (ABZSO) and oxfendazole (OFZ) by ruminal fluid obtained from sheep and cattle, was investigated, under anaerobic conditions, in this study. Ruminal fluid samples were obtained from Holstein steers fitted with a permanent rumen fistula and from Corriedale lambs via an oesophageal tube. Albendazole sulphoxide, incubated as either the racemic (rac) mixture or as each individual enantiomeric form, was extensively sulphoreduced to form albendazole (ABZ) by ruminal fluid from both species. The concentrations of ABZ formed at different incubation times were between 55 and 158% greater after the incubation of cattle ruminal fluid with (+) ABZSO, compared with that produced when (–) ABZSO was the incubated substrate. Similarly, the concentrations of ABZ were 1.3–3.0-fold higher when (+) ABZSO was incubated with sheep ruminal fluid. Significantly higher rates of depletion were observed for the (+) enantiomeric form when ABZSO was incubated with ruminal fluid from both species. The rates of ABZ formation from both ABZSO enantiomeric forms were significantly higher in sheep compared with cattle ruminal fluid. Fenbendazole (FBZ) was the metabolite formed after the incubation of the racemic form of OFZ with ruminal fluid obtained from both species. The metabolic profile of both OFZ enantiomers followed a similar pattern to that observed for ABZSO enantiomers. A bi-directional chiral inversion of one enantiomer into its antipode was observed. The (+) enantiomer appeared in the incubation medium when (–) ABZSO was the incubated substrate, and also the (–) antipode was detected after (+) ABZSO incubation with ruminal fluid obtained from both species. The results reported here demonstrate an enantioselective ruminal sulphoreduction of ABZSO and OFZ (substrate enantioselectivity). These findings contribute to interpret the chiral behaviour of benzimidazole-sulphoxide anthelmintics.

(Paper received 30 March 2001; accepted for publication 4 September 2001)

Professor Carlos Lanusse, Laboratorio de Farmacología, Departamento de Fisiopatología, Facultad de Ciencias Veterinarias, UNCPBA, Campus Universitario, (7000) Tandil, Argentina. E-mail: clanusse@vet.unicen.edu.ar

INTRODUCTION

Studies on the metabolism and fate of drugs used in domestic animals are useful to understand differences in pharmacokinetic behaviour, clinical efficacy and levels of tissue residues. Biotransformation takes place predominantly in the liver, although metabolic activity is apparent in other tissues such as blood, lungs, kidneys and gastrointestinal (GI) tract. In comparison with the liver where oxidative metabolism predominates, the GI microflora is very active in reductive reactions of foreign compounds, particularly those containing -nitro (Acosta de Pérez *et al.*, 1992) and -sulphoxide (Renwick *et al.*, 1986; Rowland, 1986) groups. Drug metabolic processes taking place in the rumen are particularly important in ruminant therapeutics. Biodegradation of active drugs such as ronidazole (Vynckier & Debackere, 1993) and chloramphenicol (De Corte-Baeten & Debackere, 1978) by ruminal bacteria has been described, which can contribute to poor systemic bioavailability after oral administration to cattle.

Benzimidazole (BZD) anthelmintics were introduced into the animal health market primarily for the control of GI roundworms, lungworms, tapeworms and liver fluke in livestock

animals. The BZD-sulphoxide derivatives, albendazole sulphoxide (ABZSO) and oxfendazole (OFZ), are the main anthelmintically active metabolic products found systemically after administration of albendazole (ABZ) and fenbendazole (FBZ) to sheep (Marriner & Bogan, 1980; Hennessy *et al.*, 1989; Lanusse *et al.*, 1995; McKellar *et al.*, 1995) and cattle (Prichard *et al.*, 1985; Sánchez *et al.*, 1997). Albendazole sulphoxide and OFZ are also available for anthelmintic therapy in sheep and cattle, and are reduced by ruminal microflora to their parent sulphides (ABZ and FBZ, respectively) after oral/intraruminal treatment. No differences in the systemic availability of ABZSO were observed after administration of either ABZ or ABZSO to sheep (McKellar *et al.*, 1995). Furthermore, FBZ and its sulphoxide (OFZ) and sulphone ($FBZSO_2$) metabolites were recovered in equal proportions after the administration of both FBZ and OFZ to sheep (Lanusse *et al.*, 1995). Equivalent anthelmintic efficacy has been shown following administration of the sulphoxide derivatives compared with that observed after treatment with the parent sulphides (McKellar & Scott, 1990).

Albendazole sulphoxide and OFZ have an asymmetrical centre in the sulphur atom of the BZD side chain. This nucleophilic sulphur atom is attached to four different functional groups, which results in an asymmetric molecule. Two ABZSO and OFZ enantiomers have been identified (by chiral separation) in the plasma of sheep after administration of both ABZ and FBZ (pro-chiral molecules) (Delatour *et al.*, 1990). It has been demonstrated that (+) ABZSO is the main enantiomeric form in plasma (Delatour *et al.*, 1991) and in tissues of parasites location (Alvarez *et al.*, 2000; Cristofol *et al.*, 2001) in both sheep and cattle. *In vitro* sulphoreduction of ABZSO (Lanusse *et al.*, 1992; Virkel *et al.*, 1999) and OFZ (Beretta *et al.*, 1987) has been demonstrated but without chiral separation of the sulphoxide enantiomers and the relative contribution of each enantiomeric form to produce ABZ in the GI tract remains unknown. The studies reported here characterized the comparative *in vitro* sulphoreduction of the (+) and (–) ABZSO and OFZ enantiomers by ruminal fluid obtained from sheep and cattle.

MATERIALS AND METHODS

Animals and collection of ruminal fluid

Two healthy Holstein steers, fitted with a permanent rumen fistula and three Corriedale lambs were used as a source of ruminal fluid. Animals were fed with high quality lucerne hay and water was provided *ad libitum*. Ruminal fluid was obtained through the rumen fistula (cattle) and by an oesophageal tube (sheep). Aliquots of the collected fluid were kept at 38°C, transported to the laboratory and processed for incubation within 2 h of their collection. Samples were filtered through a hydrophilic gauze to remove solid material and the filtrate kept saturated with pure N_2 at 38°C until the incubation assays were carried out.

Incubation assays

The substrates incubated with cattle ruminal fluid were: racemic (rac) ABZSO, rac OFZ, (+) ABZSO and (–) ABZSO. Aliquots of sheep ruminal fluid were incubated with (+) ABZSO, (–) ABZSO and rac OFZ. Twenty microlitres of each substrate (prepared in methanol solution, 356 nmol/mL) were added to 1980 μL of the ruminal fluid filtrate to reach a final drug concentration of 7.1 nmol/mL of incubation mixture. Each incubation mixture was gently gassed with pure N_2 for 5 min at 38°C. Incubation assays were performed by triplicate in a thermostatic shaking water bath (Yamato Shaking Bath, Yamato Scientific Co., Tokyo, Japan) at 38°C under anaerobic conditions for up to 360 min. Blank samples of boiled ruminal fluid were prepared and incubated under the same conditions. Immediately after the incubation period, the samples were frozen at –20°C.

Drugs/metabolites extraction

Oxibendazole (OBZ) (0.5 μg in 5 μL) as internal standard, was added to an aliquot (500 μL) of ruminal fluid incubation mixture. The products (metabolites) formed after each incubation period were extracted using ethyl acetate following a technique adapted from that described by Alvinerie and Galtier (1984). Briefly, incubation samples were mixed with 2.5 mL of ethyl acetate and shaken on a mechanical shaker for 20 min. This extraction step was repeated once and the combined ethyl acetate extracts were evaporated under a stream of N_2. The dry residue was redissolved in 300 μL of mobile phase and 50 μL were injected into the HPLC system.

Chromatographic analysis

Incubation samples were analysed for ABZ, ABZSO, ABZ sulphone ($ABZSO_2$), FBZ, OFZ and FBZ sulphone ($FBZSO_2$) by HPLC. Fifty microlitres (50 μL) of each extracted sample were injected into a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a Selectosil C_{18} (5 μm, 250 mm × 4.60 mm) reverse-phase column (Phenomenex, Torrance, CA, USA) and UV detector (Shimadzu, SPD-10 A UV detector, Shimadzu Corporation, Kyoto, Japan) reading at 292 nm. Chromatographic conditions for ABZ, ABZSO and $ABZSO_2$ were adapted from that previously reported (Lanusse & Prichard, 1990). The solvent system was acetonitrile/ammonium acetate (0.025 M) gradient in the following proportions: 27:73 (initial conditions), 50:50 (from 5 to 12 min) and 27:73 (from 13 to 16 min), with a flow rate of 1.2 mL/min. The analytes were identified with the retention times of 96–99% pure reference standards (Schering Plough, Kenilworth, NJ, USA). Under these chromatographic conditions the mean retention times were 4.85 min (ABZSO), 6.25 min ($ABZSO_2$), 9.40 min (OBZ, internal standard) and 11.26 min (ABZ). The acetonitrile/ammonium acetate gradient was modified for the HPLC analysis of FBZ, OFZ and $FBZSO_2$: the initial conditions (27:73) progressed exponentially over a period of 6 min to 50:50, which was maintained for 5 min prior to returning to the initial solvent

ratio (at 12 min) over 6 min for column equilibration. The mean retention times were 6.82 min (OFZ), 8.35 min ($FBZSO_2$), 9.94 min (OBZ) and 13.67 min (FBZ).

During the reverse phase HPLC analysis, the ABZSO and OFZ chromatographic peak fractions were collected into a glass tube. The collected fraction was evaporated to dryness under a N_2 stream and redissolved with 150 μL of chiral mobile phase (1% 2-propanol in 0.008 M of Na_2HPO_4 buffer, pH 6.9). Fifty microlitres (50 μL) of each sample were injected into the same 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 the methodology described previously (Delatour *et al.*, 1990). Albendazole sulphoxide and OFZ enantiomers were identified after chromatographic analysis of a 99% pure racemic standard of each molecule. Retention times were: 3.20 min [(–) ABZSO], 4.15 min [(+) ABZSO], 7.25 min [(–) OFZ] and 9.52 min [(+) OFZ]. The relative proportions (%) of each antipode were obtained using the integrator software (Class LC 10, Shimadzu Corporation, Kyoto, Japan) of the HPLC system. The solvents (Baker Inc., Phillipsburg, NJ, USA) used during the extraction and drug chromatographic analysis were HPLC grade.

Purification of albendazole sulphoxide enantiomers

Albendazole sulphoxide enantiomers were purified to be used as substrates in the incubation trials. The purification of each ABZSO enantiomer was carried out using a stock solution of rac ABZSO (prepared in chiral mobile phase, 200 μg/mL). Fifty microlitres (50 μL) of the stock racemic solution were injected several times into the HPLC system fitted with the chiral stationary phase column. The (–) and (+) ABZSO peak fractions were collected into individual 50 mL polypropylene tubes and stored at 4°C. Aliquots (10 mL) of the collected peak fractions were injected into preconditioned (5 mL methanol + 5 mL water) SepPak Cartridges (Waters Corporation, Milford, MA, USA) and eluted with methanol. All the eluted fractions were evaporated under a N_2 stream and redissolved in 1 mL of methanol. Quantification was performed by reverse phase chromatography using a calibration curve for ABZSO (concentration range: 0.25–4 μg/mL). The efficiency of the purification method ranged between 87% [(–) ABZSO] and 81% [(+) ABZSO] and was calculated using the following equation:

$$[\text{amount of each enantiomer quantified } (\mu g)] \times 100 \;/[\text{theorical (total) amount of each enantiomer injected } (\mu g)]$$

Representative chromatograms of purified (–) and (+) ABZSO fractions are shown in Fig. 1.

Drugs/metabolites quantification

Validation of the analytical procedures for extraction and quantification of ABZ, ABZSO and $ABZSO_2$ was performed as previously reported (Virkel *et al.*, 1999) before starting analysis of experimental samples from the incubation trials. For FBZ, OFZ and $FBZSO_2$, known amounts of each analyte (0.25–5 μg/mL) were added to aliquots of ruminal fluid, extracted and analysed by HPLC to obtain calibration curves and percentages of recovery. Calibration curves were constructed using the least squares linear regression analysis of HPLC peak area ratios of unknown analytes/internal standard and nominal concentrations of spiked samples. Correlation rates of elimination (r) were 0.996 (FBZ), 0.999 (OFZ) and 0.998 ($FBZSO_2$). Absolute recoveries were established by comparison of the detector responses obtained for ruminal fluid samples and those of direct standards prepared in mobile phase. Drug/metabolite recoveries from ruminal fluid were 65% (ABZ), 80% (ABZSO), 99% ($ABZSO_2$), 87% (FBZ), 83% (OFZ) and 91% ($FBZSO_2$).

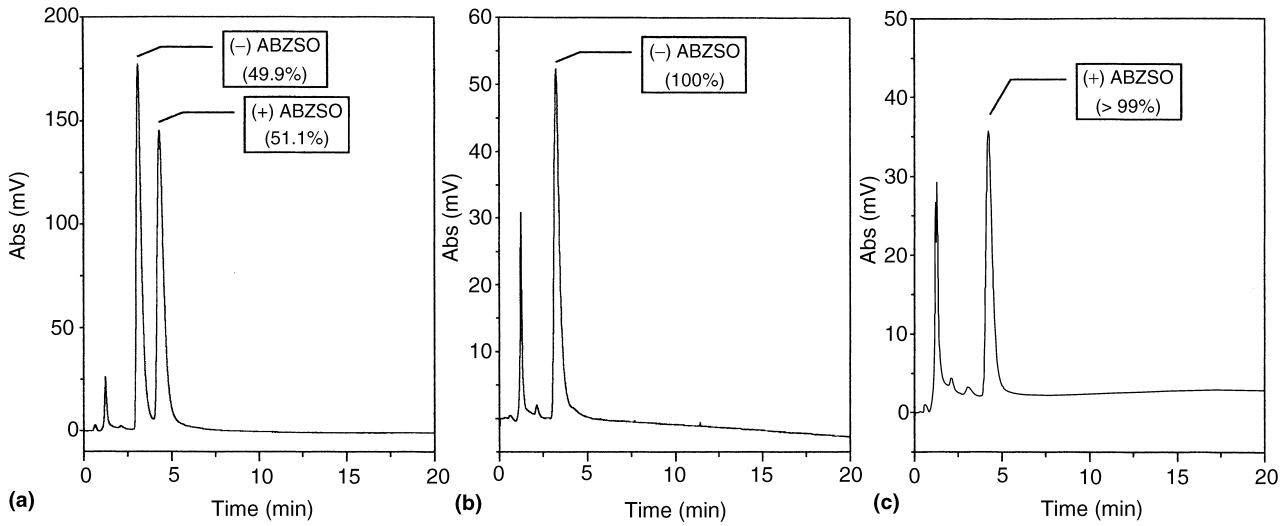

Fig. 1. Representative chromatograms obtained after the chiral analysis of a racemic standard of albendazole sulphoxide [(rac) ABZSO] (a) and ruminal fluid blank samples fortified with (–) albendazole sulphoxide [(–) ABZSO] (b) and (+) albendazole sulphoxide [(+) ABZSO] (c). A small chromatographic peak of (–) ABZSO (<1%) can be observed in chromatogram (c).

Ruminal fluid blanks ($n = 5$) fortified with the internal standard were analysed and the baseline noise at the time of retention of each analyte was measured to establish the limits of detection and quantification. The limits of detection [0.06 μg/mL (ABZ), 0.08 μg/mL (ABZSO), 0.05 μg/mL ($ABZSO_2$), 0.08 μg/mL (FBZ) 0.12 μg/mL (OFZ) and 0.03 μg/mL ($FBZSO_2$)] were calculated as the mean baseline noise at the retention time of each analyte plus three standard deviations. The mean baseline noise plus 10 standard deviations was defined as the quantification limit of each analyte under investigation.

Data and statistical analyses

The results are presented as mean (±SD) values of at least six (cattle) or nine (sheep) determinations. A lack of fit test (Instat 3.00, Graph Pad Software, Inc., San Diego, CA, USA) was performed to establish the period of incubation time in which the sulphoreduction reaction was linear. Metabolic rates (nmol/min) were calculated using the following equations:

Rate of ABZ or FBZ formation:

$$\text{Formation rate (nmol/min)} = \frac{\text{total amount of product formed (nmol)}}{\text{incubation time (min)}}$$

where the total amount of the product is the concentration (nmol/mL) at a given incubation time multiplied by the total volume (mL) of the incubation medium.

Depletion rates applied to rac ABZSO, (+) ABZSO, (–) ABZSO and rac OFZ:

$$\text{Depletion rate (nmol/min)} = \frac{\text{substrate initial amount (nmol)} - \text{remaining amount (nmol)}}{\text{incubation time (min)}}$$

The percentages of chiral inversion between enantiomeric forms were calculated over the total amount (nmol) of products recovered (both ABZSO enantiomers plus ABZ) as follows:

$$\text{Chiral inversion from (+) ABZSO into (–) ABZSO} = \frac{\text{amount of } (-)\text{ABZSO}_{\text{(formed after (+)ABZSO incubation)}}}{\text{total amount of products recovered}} \times 100$$

$$\text{Chiral inversion from (–) ABZSO into (+) ABZSO} = \frac{\text{amount of } (+)\text{ABZSO}_{\text{(formed after (–)ABZSO incubation)}}}{\text{total amount of products recovered}} \times 100$$

Mean results obtained after rac ABZSO, (–) ABZSO and (+) ABZSO incubations with ruminal fluid obtained from cattle, were statistically compared using the Kruskal–Wallis test (nonparametric ANOVA) (Instat 3.00, Graph Pad Software, Inc.). Where significant overall differences ($P < 0.05$) were obtained, further analysis among individual groups was performed using the Dunn's test. The results obtained after (–) ABZSO and (+) ABZSO incubations with sheep ruminal fluid were compared by the Mann–Whitney *U*-test. The same nonparametric test was used to compare the concentrations of ABZ and FBZ formed from rac ABZSO and rac OFZ, respectively. A value of $P < 0.05$ was considered statistically significant.

RESULTS

There was no metabolic conversion neither for ABZSO nor for OFZ in the incubations with boiled ruminal fluid. Albendazole sulphoxide, incubated either as the racemic (rac) mixture (50% of each enantiomer) or as each individual enantiomeric form, was extensively metabolized to ABZ by sheep and cattle ruminal fluids, being the metabolic reaction linear for up to 60 min. Albendazole sulphone was not detected after incubations of ABZSO with ruminal fluid from both animal species.

The concentrations of ABZ formed following the incubations of cattle ruminal fluid with rac ABZSO, (–) and (+) ABZSO enantiomers are compared in Fig. 2. The concentrations of ABZ produced were between 55 and 158% higher following incubation of the (+) ABZSO enantiomer compared with those measured when (–) ABZSO was the substrate. The comparison of the concentrations of ABZ formed from 10 to 360 min after the incubations of the (–) and (+) ABZSO enantiomers with sheep ruminal fluid are shown in Fig. 3. As shown for cattle ruminal fluid, significantly higher concentrations of ABZ were formed from (+) ABZSO between 10 and 120 min of incubation, compared with those measured after (–) ABZSO incubation. Significantly higher rates of ABZ production were observed after (+) ABZSO incubation with ruminal fluid of both species, which agree with the higher depletion rates observed for the (+) enantiomer compared with those observed for its (–) antipode (Table 1).

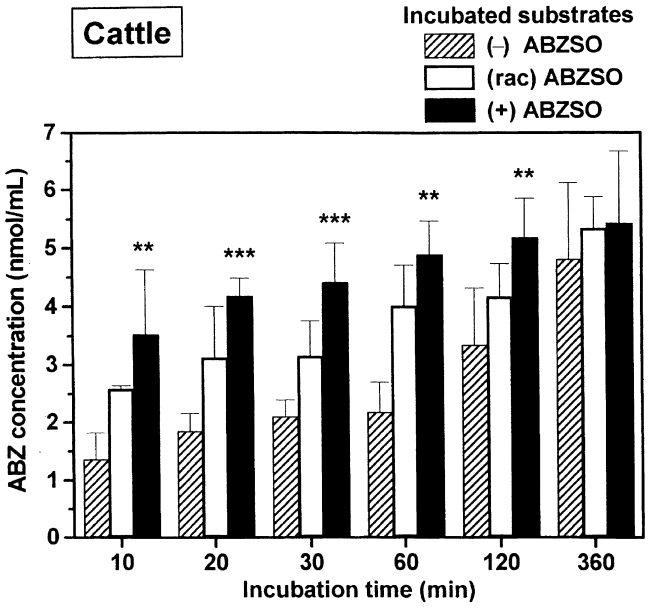

Fig. 2. Albendazole (ABZ) concentrations (nmol/mL) formed after the anaerobic incubation of either the racemic [(rac) ABZSO] or each enantiomeric form of albendazole sulphoxide [(–) ABZSO and (+) ABZSO], with ruminal fluid obtained from cattle. Values obtained after (+) ABZSO incubation are statistically different from those observed after incubation with (–) ABZSO at $^{**}P < 0.01$ and $^{***}P < 0.001$. Data represent the mean (±SD) of six determinations.

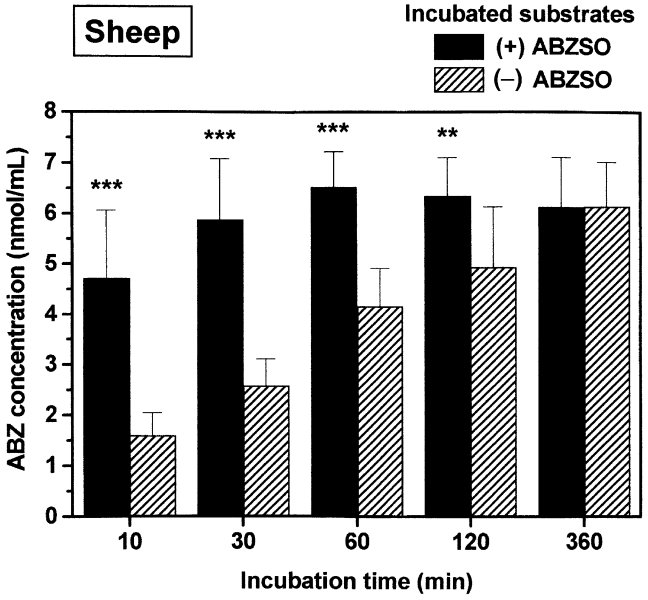

Fig. 3. Albendazole (ABZ) concentrations (nmol/mL) formed after anaerobic incubation of both albendazole sulphoxide enantiomers [(–) ABZSO and (+) ABZSO] with sheep ruminal fluid. Values are statistically different from those obtained after incubation with (–) ABZSO at $^{**}P < 0.01$ and $^{***}P < 0.001$. Data represent the mean (±SD) of nine determinations.

The metabolic profiles of each enantiomeric form in cattle ruminal fluid, observed after incubation of the racemic mixture of ABZSO (rac ABZSO) and each individual enantiomer separately, are shown in Fig. 4. After 10 min incubation with rac ABZSO, the concentration of (+) ABZSO (1.13 ± 0.47 nmol/mL) was significantly lower ($P < 0.01$) compared with that observed for its (–) antipode (2.89 ± 0.67 nmol/mL). Then, the concentration of the (+) enantiomer decreased a 41% between 10 and 60 min of incubation, while the concentration of the (–) isomer declined only 21% during the same time period. This drug depletion pattern was corroborated when both (–) ABZSO and (+) ABZSO were incubated as individual substrates: a 70% reduction (from 4.08 ± 1.41 to 1.25 ± 0.27 nmol/mL) in the concentration of (+) ABZSO, compared with a 26% reduction (from 5.88 ± 1.36 to 4.35 ± 0.63 nmol/mL) in the amount of (–) ABZSO, was observed between 10 and 60 min of incubation. Following incubation of both enantiomeric forms (as individual substrates) with sheep ruminal fluid, the concentration of (+) ABZSO (3.25 ± 1.23 nmol/mL) was significantly lower ($P < 0.001$) compared with that observed for its antipode (5.71 ± 0.77 nmol/mL) after 10 min of incubation. After 60 min of incubation, the concentration of the (+) enantiomer (1.08 ± 0.44 nmol/mL) was significantly lower ($P < 0.001$) than that observed for (–) ABZSO (3.95 ± 1.12 nmol/mL). Thus, a 67% reduction in the concentration of (+) ABZSO was observed between 10 and 60 min of incubation with sheep ruminal fluid, compared with a 31% reduction in the concentration of its (–) antipode during the same time period. Metabolic rates of depletion of the incubated substrates are shown in Table 1.

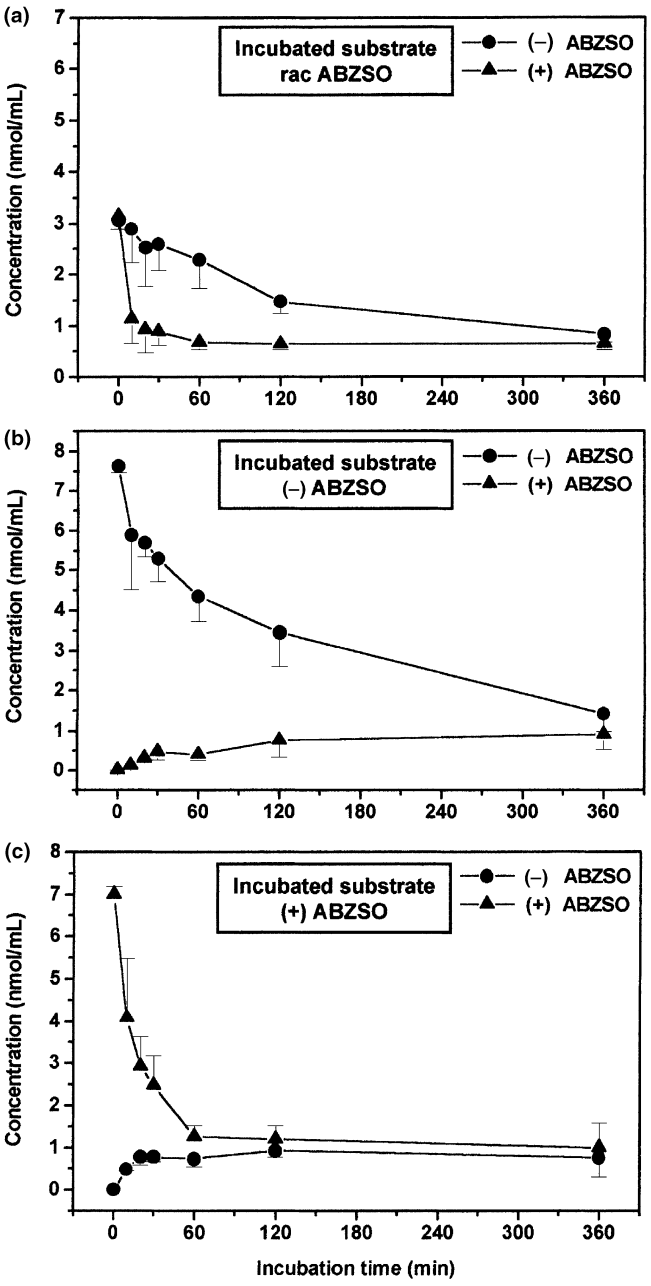

Fig. 4. Concentrations of albendazole sulphoxide enantiomers [(–) ABZSO and (+) ABZSO] recovered after the anaerobic incubation of racemic (rac) ABZSO (a), (–) ABZSO (b) and (+) ABZSO (c) with ruminal fluid obtained from cattle. Data represent the mean (±SD) of six determinations.

Fenbendazole was the product formed after the incubation of rac OFZ with ruminal fluid obtained from sheep and cattle; the reaction was linear for up to 60 min. The comparative sulphoreduction of rac OFZ by both sheep and cattle ruminal fluid is summarized in Table 2. No statistical differences were observed in the concentrations of FBZ formed after rac OFZ incubation with ruminal fluid from both species. The metabolic profile of both OFZ enantiomers followed a similar pattern to that

Incubated substrates	Cattle	Sheep
ABZ or FBZ formation rates (nmol/min)		
rac ABZSO	0.13 ± 0.02*	–
(−) ABZSO	0.07 ± 0.02	0.14 ± 0.03†
(+) ABZSO	0.16 ± 0.02*	0.22 ± 0.02*†
rac OFZ	0.14 ± 0.04	0.16 ± 0.02
Depletion rates (nmol/min)		
rac ABZSO	0.14 ± 0.02‡	–
(−) ABZSO	0.09 ± 0.02	0.11 ± 0.04
(+) ABZSO	0.20 ± 0.01*	0.20 ± 0.02*
rac OFZ	0.14 ± 0.02	0.12 ± 0.01

Table 1. Metabolic rates (nmol/mL) obtained after 60 min incubation of the racemic and enantiomeric forms of albendazole sulphoxide (ABZSO) and the racemic form of oxfendazole (OFZ) with cattle and sheep ruminal fluids

Data represent the mean (±SD) of six (cattle) and nine (sheep) determinations. Metabolic rates were calculated following the equations described in Materials and Methods.
*Statistically different ($P < 0.001$) from the value obtained after incubation with (−) ABZSO.
†Statistically different ($P < 0.001$) from the value obtained after incubation of the same enantiomer with cattle ruminal fluid.
‡Statistically different ($P < 0.01$) from the values obtained after incubation with (−) ABZSO and (+) ABZSO.

Table 2. *In vitro* sulphoreduction of oxfendazole (OFZ) by ruminal fluid obtained from sheep and cattle. Incubations were performed with the racemic form of OFZ at 7.11 nmol/mL of ruminal fluid

Incubation time (min)	Species	FBZ formed (nmol/mL)*	Remaining substrate (OFZ) (nmol/mL)†	(−) OFZ (%)‡	(+) OFZ (%)‡
10	Cattle	2.82 ± 1.64	4.53 ± 0.99	60.3	39.7
	Sheep	2.37 ± 0.26	5.40 ± 0.31	60.4	39.6
30	Cattle	2.77 ± 1.37	3.76 ± 0.58	62.5	37.5
	Sheep	3.67 ± 0.74	4.30 ± 0.49	70.9	29.1
60	Cattle	3.53 ± 1.10	2.78 ± 0.55	69.4	30.6
	Sheep	4.69 ± 0.63	3.48 ± 0.31	77.1	22.9
120	Cattle	4.22 ± 1.06	1.52 ± 0.49	73.7	26.3
	Sheep	5.57 ± 0.96	2.63 ± 0.56	80.5	19.5
360	Cattle	5.86 ± 1.17	0.42 ± 0.15	69.0	31.0
	Sheep	6.59 ± 2.35	0.93 ± 0.20	57.6	42.4

Data represent the mean (±SD) of six (cattle) and nine (sheep) determinations.
*Mean fenbendazole (FBZ) (product formed) concentrations measured at each incubation time.
†Mean remaining OFZ concentrations measured at each incubation time.
‡Mean (−) OFZ and (+) OFZ percentages of the remaining (total) OFZ concentrations measured at each incubation time.

observed for ABZSO enantiomers after incubation of the racemic form. The rates of depletion of (+) OFZ (0.09 ± 0.03 – cattle – and 0.09 ± 0.01 – sheep – nmol/mL) were significantly higher ($P < 0.01$) compared with those observed for (–) OFZ (0.05 ± 0.02 – cattle – and 0.03 ± 0.01 – sheep – nmol/mL) after 60 min incubation of rac OFZ with ruminal fluid from both species. Metabolic rates of FBZ production and rac OFZ depletion are shown in Table 1. No statistical differences were observed between the sulphoreduction of rac ABZSO and rac OFZ by cattle ruminal fluid.

Interestingly, the (+) enantiomer appeared in the incubation medium when (–) ABZSO was the incubated substrate, but also the (–) antipode was detected after (+) ABZSO incubation with ruminal fluid obtained from cattle (Fig. 4) and sheep. The concentrations of the (–) enantiomer formed from (+) ABZSO (incubated as individual substrate) were significantly higher than those measured for the (+) antipode formed from (–) ABZSO (Fig. 5). This pattern was observed for the ruminal fluid obtained from both animal species. Table 3 summarizes the percentages of chiral inversion obtained after incubation of both ABZSO enantiomeric forms with sheep and cattle ruminal fluid. The proposed metabolic pathways for ABZSO enantiomers, including the comparative metabolic rates obtained in ruminal fluid from sheep and cattle, are shown in Fig. 6.

DISCUSSION

Aromatic BZD derivatives such as FBZ and OFZ require more extensive hepatic oxidative metabolism than aliphatic derivatives (ABZ and ABZSO) to achieve sufficient polarity for excretion as previously suggested by Hennessy *et al.* (1993). As a consequence, longer plasma residence times and elimination half-lives for FBZ and its metabolites, compared with

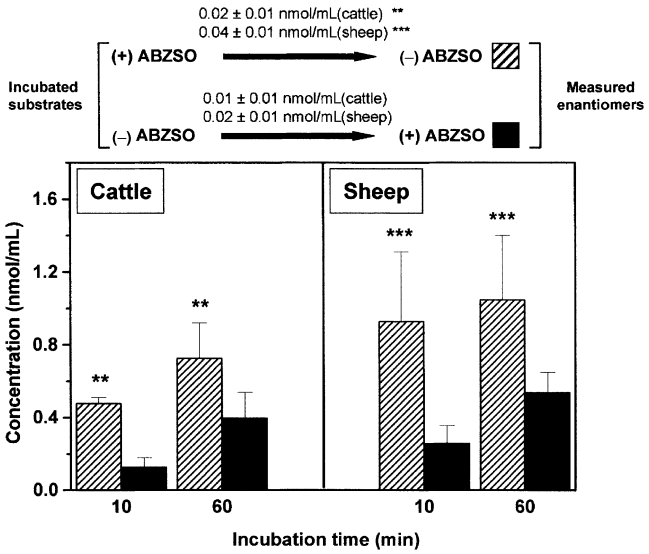

Fig. 5. Comparison of the chiral inversion of albendazole sulphoxide (ABZSO) enantiomers in ruminal fluid expressed as the concentration of the (–) enantiomer formed from its (+) antipode and that of (+) ABZSO formed from the (–) enantiomeric form. Rates of formation (nmol/min) of each enantiomer following 60 min incubation are shown in the top of the figure. Values are statistically different from those observed for (+) ABZSO at $^{**}P < 0.01$ and $^{***}P < 0.001$. Data represent the mean (±SD) of six (cattle) and nine (sheep) determinations.

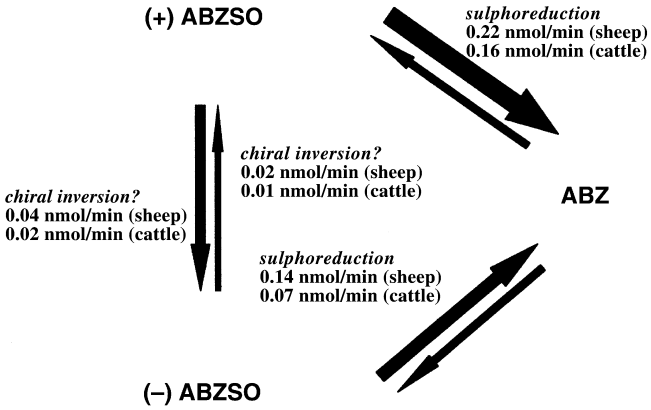

Fig. 6. Schematic representation of the ruminal biotransformation pathways proposed for albendazole sulphoxide (ABZSO) enatiomers. The width of the arrows indicates the magnitude of the metabolic reactions investigated in the current work, as shown by the conversion rates (nmol/mL) obtained after 60 min incubation of (–) ABZSO and (+) ABZSO with ruminal fluid obtained from both species.

those of ABZ metabolites, were observed in sheep (Lanusse *et al.*, 1995). Moreover, while FBZ is detected in plasma following its oral/intraruminal administration to cattle and sheep (Prichard *et al.*, 1985; Short *et al.*, 1987; Delatour *et al.*, 1990), ABZ is not detected in the bloodstream after its administration as parent drug (Marriner & Bogan, 1980; Hennessy *et al.*, 1989). The results obtained herein are consistent with those previous findings. There were no differences between the concentrations of ABZ and FBZ formed *in vitro* by cattle ruminal fluid (see Table 2) nor between the sulphoreduction rates of ABZSO and OFZ (see Table 1). Thus, the observed pharmacokinetic differences between ABZ and FBZ are not because of different ruminal biotransformation patterns of their sulphoxide metabolites, and are probably due to differences on the liver microsomal sulphoxidation of the parent sulphides (Virkel *et al.*, 2000a).

Higher FBZ concentrations have been measured in cattle compared with sheep ruminal fluid after OFZ incubation (Beretta *et al.*, 1987). Conversely, no differences in the ruminal sulphoreduction of OFZ were observed between sheep and cattle in the present study (see Table 2). While the concentrations of FBZ formed after 360 min of incubation ranged between 56% (cattle) and 34% (sheep) of the total products recovered in the earlier work (Beretta *et al.*, 1987), the concentrations of FBZ tended to be higher (94% – cattle – and 89% – sheep – of the total products recovered) after the same incubation time in the

Table 3. *In vitro* chiral inversion of albendazole sulphoxide (ABZSO) enantiomers by ruminal fluid obtained from sheep and cattle

		Chiral inversion (%)*	
Incubation time (min)	Species	From (+) ABZSO into (–) ABZSO	From (–) ABZSO into (+) ABZSO
10	Cattle	6.02 ± 0.33	1.84 ± 0.72**
	Sheep	10.3 ± 3.74	3.41 ± 1.27***
30	Cattle	10.1 ± 1.51	6.03 ± 2.23**
	Sheep	11.9 ± 2.66	4.49 ± 0.73***
60	Cattle	10.7 ± 2.47	5.70 ± 1.71**
	Sheep	13.0 ± 3.54	6.16 ± 0.48***
120	Cattle	12.7 ± 1.55	10.1 ± 5.10
	Sheep	13.0 ± 3.54	10.3 ± 2.63
360	Cattle	10.7 ± 6.03	12.7 ± 4.80
	Sheep	12.5 ± 2.85	10.2 ± 4.11

Data represent the mean (±SD) of six (cattle) and nine (sheep) determinations.
*The percentage of chiral inversion was calculated following the equations described in Materials and Methods.
Values are statistically different from those observed for the chiral inversion of (+) ABZSO into (–) ABZSO at: $^{**}P < 0.01$ and $^{***}P < 0.001$.

current experiments, which would indicate a greater metabolic conversion of OFZ under the experimental conditions described here.

The rates of (+) ABZSO depletion in the incubation assays were higher than that of (–) ABZSO (see Table 1), and the concentrations of ABZ formed from (+) ABZSO were also significantly higher. These results indicate that the (+) enantiomer may be the main substrate for the ruminal sulphoreduction to form ABZ (see Figs 2 & 3). In cattle ruminal fluid, the rate of depletion of rac ABZSO was higher than that observed after incubation of (–) ABZSO, and lower compared with that observed for the (+) enantiomer (see Table 1). This finding may indicate a competitive interference between both enantiomeric forms available in the incubation medium when rac ABZSO is the incubated substrate. As shown for ABZSO, the metabolic profile of OFZ enantiomers followed a similar pattern, with a higher rate of depletion of the (+) OFZ enantiomer. The concept of substrate enantioselectivity is used when two enantiomers of a chiral substrate are metabolized at different rates, in quantitative and/or qualitative terms and by the same biological system under identical experimental conditions (Testa & Mayer, 1988). Such situation observed *in vitro* for both ABZSO and OFZ enantiomers in this study, may occur after the enteral administration of a racemic BZD-sulphoxide anthelmintic in ruminants.

Albendazole sulphoxide is distributed from the plasma into the GI tract, allowing its microflora-mediated sulphoreduction into ABZ in the rumen and intestine (Lanusse *et al.*, 1993). On the other hand, the absence of ABZ in plasma has been attributed to a first-pass microsomal oxidation in the liver, which was shown to be enantioselective (Delatour *et al.*, 1991; Moroni *et al.*, 1995; Virkel *et al.*, 2000b). The (+) ABZSO enantiomer represents 91% of the total ABZSO plasma *AUC* in cattle and 86% in sheep (Delatour *et al.*, 1991). These differences between the plasma availabilities of (+) ABZSO and (–) ABZSO enantiomeric forms were attributed to the relative contribution of the FMO and cytochrome P450-dependent oxygenases to ABZ hepatic sulphoxidation. Moreover, it has been shown that (–) ABZSO rather than its (+) antipode, would be the substrate for the cytochrome P450-mediated formation of the inactive sulphone ($ABZSO_2$) metabolite (Delatour *et al.*, 1991; Benoit *et al.*, 1992). Altogether, these findings would indicate that (+) ABZSO, being predominant in the bloodstream, may be the main enantiomeric form available in the rumen. In fact, higher availabilities of the (+) antipode, compared with that of the (–) enantiomer, were observed in plasma and GI tract after the administration of rac ABZSO to cattle (Cristofol *et al.*, 2001). As ABZ is formed in rumen from ABZSO in a bacteria-mediated sulphoreduction, the (+) enantiomer would be the main source for ABZ formation, which is consistent with the higher depletion of this (+) antipode under the anaerobic *in vitro* conditions assayed here. Benzimidazole anthelmintics exert their antiparasite effects by binding to parasite tubulin, which produces the subsequent disruption of the tubulin-microtubule dynamic equilibrium (Lacey, 1990). It is well established that ABZ has a greater affinity for parasite tubulin than ABZSO (Lubega & Prichard, 1991). Although both enantiomeric forms are substrates for the formation of ABZ, the most efficient (enantioselective) sulphoreduction of (+) ABZSO to form a more pharmacologically potent product (ABZ), may greatly co-operate to the pattern of efficacy of these anthelmintics against GI parasites. Additionally, as pointed out by Delatour *et al.* (1991), a lower contribution of the (–) antipode to the overall efficacy of ABZ and related anthelmintics could be expected because this (–) enantiomer is a main substrate source to form the inactive $ABZSO_2$ metabolite.

Chiral inversion is the metabolic process by which one enantiomer is transformed into its antipode (Testa & Mayer, 1988). The presence of the (+) ABZSO enantiomer when (–) ABZSO was incubated as pure substrate is an interesting finding in the current assays. Moreover, (–) ABZSO appeared in the incubation medium when (+) ABZSO was the incubated substrate (see Figs 4 & 5). These observations may indicate that chiral inversion from (–) ABZSO into the (+) antipode is occurring, but also from (+) ABZSO into its (–) antipode. Thus, the reported data suggest that chiral inversion of ABZSO enantiomers is likely to be bi-directional, being predominant the conversion of the (+) ABZSO enantiomer into the (–) antipode (see Table 2). On the other hand, ruminal fluid is also capable to oxidize ABZ into ABZSO at very low rate (Lanusse *et al.*, 1992). Thus, after incubation of each ABZSO enantiomer as pure substrate, the sulphoxidation of the formed ABZ may produce both enantiomers. In conclusion, the chiral inversion process may depend on either the metabolic transformation of one enantiomer into its antipode or through the formation of ABZ as an intermediate metabolic product. A schematic representation of the proposed pathways for the enantioselective sulphoreduction and chiral inversion of ABZSO is shown in Fig. 6, where the metabolic rates for each pathway in both species are compared.

Finally, the work reported here describes the comparative *in vitro* sulphoreduction of ABZSO and OFZ enantiomers by ruminal fluid obtained from sheep and cattle. The sulphoreduction of both anthelmintic drugs is enantioselective (substrate enantioselectivity). Ruminal fluid from both species showed a higher capability to sulphoreduce the (+) enantiomeric form. The bi-directional chiral inversion of one enantiomer into its antipode was also characterized. These findings may contribute to understand the impact of chiral behaviour within the pharmacology of BZD-sulphoxide anthelmintics.

ACKNOWLEDGMENTS

The assistance received from Dr Alejandro Soraci (Laboratorio de Toxicología) in the development of the chiral analytical techniques and from members of the Laboratorio de Nutrición Animal (Dpto. Fisiopatología, Facultad Ciencias Veterinarias, UNCPBA) in the collection of ruminal fluid, is greatly acknowledged. Guillermo Virkel was a recipient of a fellowship from the Comisión de Investigaciones Científicas (CIC) de la Provincia de Buenos Aires (Argentina). Research at the Laboratorio de Farmacología is supported by CONICET (Argentina), Universidad

Nacional del Centro (Argentina) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 08–07277) (Argentina).

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