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Assessment of the main metabolism pathways for the flukicidal compound triclabendazole in sheep

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Triclabendazole (TCBZ) is an halogenated benzimidazole (BZD) compound worldwide used to control immature and adult stages of the liver fluke *Fasciola hepatica*. The purpose of this investigation was to characterize *in vitro* the patterns of hepatic and ruminal biotransformation of TCBZ and its metabolites in sheep. TCBZ parent drug was metabolized into its sulphoxide (TCBZSO), sulphone ($TCBZSO_2$) and hydroxy derivatives by sheep liver microsomes. The same microsomal fraction was also able to oxidize TCBZSO into $TCBZSO_2$ and hydroxy-TCBZSO (HO-TCBZSO). TCBZ sulphoxidation was significantly ($P < 0.001$) inhibited after inactivation of the flavin-monooxygenase (FMO) system (77% inhibition) as well as in the presence of the FMO substrate methimazole (MTZ) (71% inhibition). TCBZ sulphoxidative metabolism was also reduced (24% inhibition, $P < 0.05$) by the cytochrome P450 inhibitor piperonyl butoxide (PB). The rate of TCBZSO conversion into $TCBZSO_2$ was also significantly inhibited by PB (55% inhibition), MTZ (52% inhibition) and also following FMO inactivation (58% inhibition). The data reported here indicate that the FMO is the main enzymatic pathway involved in TCBZ sulphoxidation (ratio FMO/P450 = 3.83 ± 1.63), although both enzymatic systems participate in a similar proportion in the sulphonation of TCBZSO to form the sulphone metabolite (ratio FMO/P450 = 1.31 ± 0.23). Additionally, ketoconazole (KTZ) did not affect TCBZ sulphoxidation but decreased (66% inhibition, $P < 0.05$) the formation of $TCBZSO_2$. Similarly, inhibition of $TCBZSO_2$ production was observed after incubation of TCBZSO in the presence of KTZ and erythromycin (ETM). Conversely, thiabendazole (TBZ) and fenbendazole (FBZ) did not affect the oxidative metabolism of both incubated substrates. The sheep ruminal microflora was able to reduce the sulphoxide (TCBZSO) into the parent thioether (TCBZ). The ruminal sulphoreduction of the HO-TCBZSO derivative into HO-TCBZ was also demonstrated. The rate of sulphoreduction of HO-TCBZSO was significantly ($P < 0.05$) higher than that observed for TCBZSO. The metabolic approach tested here contributes to the identification of the different pathways involved in drug biotransformation in ruminant species. These findings on the pattern of hepatic and ruminal biotransformation of TCBZ and its main metabolites are a further contribution to the understanding of the pharmacological properties of widely used anthelmintics in ruminants. Comprehension of TCBZ metabolism is critical to optimize its flukicidal activity.

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INTRODUCTION

Livestock animals are exposed to a variety of xenobiotic agents (e.g. veterinary drugs, feed-additives, pesticides, pollutants, etc.) during their production cycles. These compounds are likely to be metabolized by different enzymatic systems from both hepatic and extra-hepatic tissues. The metabolic activities of the hepatic mixed function oxidases, flavin-monooxygenase (FMO) and cytochrome P450 systems, play a major role in determining the persistence of therapeutically used drugs in target species. Metabolic interactions with either the FMO or cytochrome P450 enzymatic systems may drastically affect the disposition kinetics of different drugs used in livestock production.

In comparison to the liver where oxidative metabolism predominates, the gastrointestinal (GI) microflora is very active in reductive reactions of foreign compounds, particularly those containing -nitro (Acosta de Pérez *et al.*, 1992; Lanusse *et al.*, 1992a) and -sulphoxide (Renwick *et al.*, 1986; Rowland, 1986) groups. A large number of xenobiotics (feed contaminants/additives, mycotoxins, drugs, etc.) are likely to be metabolized by ruminal micro-organisms, with possible pharmaco-toxicological consequences. For instance, ruminal biodegradation of active drugs such as the anti-protozoan compound ronidazole (Vynckier & Debackere, 1993) and chloramphenicol (De Corte-Baeten & Debackere, 1978) leads to poor systemic bioavailability of these molecules after oral administration to cattle. The hepatotoxic pyrrolizidine alkaloids of *Senecio jacobaea* are metabolized (inactivated) by sheep ruminal micro-organisms prior to absorption and transport to the liver and the rumen therefore plays a protective role increasing the resistance of sheep to these toxic compounds (Craig *et al.*, 1986). The role of hepatic and extra-hepatic metabolic processes in the bioactivation and/or detoxication of different xenobiotics in domestic animals were thoroughly reviewed (Nebbia, 2001).

Benzimidazole (BZD) and pro-BZD anthelmintics are extensively metabolized in domestic animals and man. Their metabolic pattern and the resultant pharmacokinetic behaviour are relevant in the attainment of high and sustained concentrations of pharmacologically active drug/metabolites at the target parasite (Lanusse & Prichard, 1993). Both cytochrome P450 and FMO have been shown to be involved in the biotransformation of BZD anthelmintics in different animal species and man (Galtier *et al.*, 1986; Moroni *et al.*, 1995; Rawden *et al.*, 2000; Virkel *et al.*, 2004). Sulphoxidation, sulphonation and hydroxylation in position 5 of the BZD ring, are among the most relevant metabolic reactions. Additionally, the BZD sulphoxide metabolites are reduced back to their respective thioethers by the GI microflora. For instance, the ruminal sulphoreduction of albendazole sulphoxide and oxfendazole to albendazole and fenbendazole, respectively, is of great clinical relevance in sheep and cattle (Lanusse *et al.*, 1992b; Virkel *et al.*, 2002).

Triclabendazole [2-methylthio-5(6)-chloro-6(5)-(2′,3′-dichloro) phenoxy-benzimidazole] (TCBZ) is an halogenated BZD anthelmintic worldwide used to control immature and adult stages of the liver fluke *Fasciola hepatica* (Boray *et al.*, 1983). There is only limited information available describing the plasma pharmacokinetic behaviour of TCBZ in ruminant species (Alvinerie & Galtier, 1986; Hennessy *et al.*, 1987; Bogan *et al.*, 1988). TCBZ parent drug was not detected in plasma after its oral administration to sheep, indicating it was completely removed from portal blood by the liver following absorption (Hennessy *et al.*, 1987). Overall, TCBZ is oxidized to form the sulphoxide and sulphone metabolites, triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$), respectively. Recent work carried out in our laboratory showed that both cytochrome P450 and FMO mixed function oxidases are involved in such metabolic reactions in sheep liver (Mottier *et al.*, 2004). Besides, TCBZ hydroxylation occurs at the 4′ position of the dichlorophenoxy ring, which gives rise to the corresponding hydroxylated metabolites, hydroxy-TCBZ (OH-TCBZ), hydroxy-TCBZSO (OH-TCBZSO) and hydroxy-$TCBZSO_2$ (OH-$TCBZSO_2$). Although the intrinsic mechanism of action of TCBZ and its metabolites remains to be fully understood, TCBZ-sulpho and -hydroxy derivatives are supposed to be less potent than the parent thioether. TCBZ metabolites are strongly bound to plasma proteins (≥90%), which could account for their long residence time in the animal's body (Hennessy *et al.*, 1987).

The metabolic pathways involved in the hepatic and extra-hepatic biotransformation of different BZD anthelmintics in ruminants have been studied in our laboratory. Considering the therapeutic relevance of TCBZ as the most used flukicidal drug worldwide, the purpose of the work described here was to gain further insight into the identification of the metabolic pathways involved in the formation of TCBZ sulpho- and hydroxy-metabolites in the host. Besides, the involvement of the GI microflora in the reduction of its sulphoxide derivatives was assessed. Overall, the assays described here contribute to characterize the patterns of hepatic and GI (ruminal) biotransformation of TCBZ and its metabolites in sheep, which is considered critical to optimize its flukicidal activity against immature and adult liver flukes.

MATERIALS AND METHODS

Chemicals

Reference standards (99% pure) of TCBZ and its -sulpho and -hydroxy metabolites were provided by Novartis Animal Health (Basel, Switzerland). Stock solutions (5000 nmol/mL) of each substance were prepared in methanol (Baker Inc., Phillipsburg, NJ, USA). The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Methimazole (MTZ) was a generous gift of Gador Argentina S.A. (a stock solution of 2000 nmol/mL was prepared in deionized water). Piperonyl butoxide (PB) (Sigma-Aldrich Chemical Company) stock solution (5000 nmol/mL) was prepared in methanol. Ketoconazole (KTZ), erythromycin (ETM), thiabendazole (TBZ) and fenbendazole (FBZ) were obtained from the local market. Stock solutions (5000 nmol/mL) of these drugs were also prepared in methanol. The solvents used for chemical extraction and chromatographic

analysis were HPLC grade (Baker Inc.). Buffer salts ($NaHCO_3$, Na_2HPO_4 and CH_3COONH_4) were purchased from Baker Inc.

Animals

Six healthy Corriedale lambs (males, 8–10 month old) were killed to collect liver tissue for preparation of the microsomal fraction. Four healthy Corriedale ewes were used as a source of ruminal fluid. Animals were fed with high quality lucerne hay and water *ad libitum*. The management of experimental animals was in agreement with institutional and internationally accepted welfare guidelines (American Veterinary Medical Association, 2001).

Preparation of microsomes

After killing, the abdomen was opened and the liver removed. Samples (approximately 2 cm × 2 cm × 2 cm) of liver parenchyma were rinsed with ice-cold KCl (1.15%) and then transported to the laboratory in phosphate buffer (0.1 M, pH 7.4) at 4 °C. All subsequent operations were performed between 0 and 4 °C. For each experimental animal, samples of 10 g of liver tissue were cut into small pieces with scissors and washed several times with the phosphate buffer (to remove haemoglobin). Then, tissue samples were homogenized in phosphate buffer with an Ultra-Turrax homogenizer (IKA Works Inc., Wilmington, NC, USA), centrifuged at 10 000 ***g*** for 20 min and the resulting supernatant at 100 000 ***g*** for 60 min. The pellet (microsomal preparation) was suspended in a 0.1 M phosphate buffer containing 0.1 mM of EDTA and 20% of glycerol and stored at −70 °C until used for incubation assays. An aliquot of the microsomal preparation was used to determine protein content using bovine serum albumin as a control standard (Smith *et al.*, 1985).

Enzyme assays

The metabolic activity was assessed by the rate (nmol/min/mg of microsomal protein) of each metabolite formed after either TCBZ or TCBZSO incubation in the presence of NADPH. A typical reaction mixture contained: 100 μL of NADPH solution prepared in phosphate buffer (0.1 M, pH 7.4), 100 μL of tissue preparation (0.5 mg of microsomal protein) and 20 nmol (40 μM final concentration) of the incubated substrates (TCBZ or TCBZSO) dissolved in 10 μL of methanol. The incubation mixture was adjusted to 500 μL with phosphate buffer. Thawed microsomal samples were diluted with the same phosphate buffer. The incubation mixture was allowed to equilibrate (5 min at 37 °C) and the reaction started with the addition of NADPH. Incubations (30 min at 37 °C) were carried out in glass vials in an oscillating water bath (Yamato Shaking Bath; Yamato Scientific Co., Tokyo, Japan) under aerobic conditions. The drug substrates were also incubated, under the same conditions, either without microsomes or without NADPH. These incubations were used as controls for possible nonenzymatic drug conversion. All reactions were stopped by the addition of 200 μL of ice-cold acetonitrile and stored at −20 °C until analysis. The oxidation of both TCBZ and TCBZSO was linear up to 60 min. The maximal rates of metabolic conversion were obtained using 1 μmol of NADPH per mg of microsomal protein.

Inactivation of FMO was performed by heating the diluted microsomal preparation (2 min at 50 °C) without NADPH, which was immediately chilled in ice (Dixit & Roche, 1984) and followed by incubation of the substrates in presence of the FMO substrate MTZ (100 μM) as previously shown by Virkel *et al.* (2004). The reaction started with the addition of NADPH.

Metabolic studies in presence of several enzyme inhibitors or substrates

The microsomal biotransformation of both TCBZ and TCBZSO was also studied in the presence of MTZ (FMO substrate), PB (cytochrome P450 inhibitor), FBZ (FMO/P450 substrate), TBZ (P450 1A substrate) and KTZ (P450 3A inhibitor). Besides, TCBZSO was incubated in the presence of the cytochrome P450 3A substrate ETM. Microsomal preparations containing MTZ (100 μM), PB (100 and 200 μM), FBZ (5, 10 and 40 μM), TBZ (5, 10 and 40 μM) or KTZ (40 μM) and one of each substrate assayed (TCBZ or TCBZSO) were maintained 5 min at 37 °C for system equilibration and then each reaction was initiated by the addition of NADPH. Incubation mixtures containing ETM (40 and 250 μM) were pre-incubated during 30 min at 37 °C in the presence of NADPH, following the methodology reported by Zweers-Zeilmaker *et al.* (1999). The reaction started with the addition of TCBZSO. MTZ was dissolved in 25 μL of distilled water. The other enzyme inhibitors/substrates were dissolved in 10 μL of methanol and parallel control tubes contained the same volume of the solvent. Control tubes for nonenzymatic conversion of TCBZ or TCBZSO contained all components of the reaction mixture, except NADPH, with the highest concentration of a given inhibitor or substrate. These incubations were also useful to ensure that the presence of these inhibitors in the incubation mixture did not interfere with the chromatographic determination of TCBZ and its metabolites.

Incubation assays with sheep ruminal fluid

Samples of ruminal fluid were collected by an oesophageal tube. Each ruminal fluid sample collected was hand mixed thoroughly and aliquots were kept at 38 °C in 50 mL tubes, transported to the laboratory and then processed for incubation within 2 h. Briefly, samples were filtered through a hydrophilic gauze to remove solid material and the filtrate kept saturated with N_2 in 50 mL tubes at 38 °C. Aliquots of ruminal fluid were incubated with either TCBZSO or HO-TCBZSO as follows: 20 μL of the substrate (stock solution) was added to 1980 μL of the mixture to reach a final drug concentration of 40 nmol/mL (40 μM). Samples were gently gassed with pure nitrogen (N_2) for 2 min at 38 °C and incubated in a thermostatic shaking water bath (Yamato Shaking Bath, Yamato Scientific Co.) at 38 °C under anaerobic conditions for 10, 20, 30 60, 120, 240 and 360 min. Blank samples of boiled ruminal fluid were added with both

substrates and incubated under the same conditions. At the end of the incubation period, samples were immediately frozen at −20 °C until analysis.

Drug/metabolites extraction

The internal standard (IS) mebendazole (MBZ) (5 nmol dissolved in 20 μL of methanol) was added to inactivated microsomal dilution mixtures. Samples (500 μL), fortified with TCBZ and its metabolites, were mixed with 200 μL of ice-cold acetonitrile followed by the addition of the IS. Experimental and fortified samples were mixed with 1.5 mL of ethyl acetate and shaken on a mechanical shaker (VWR Multi-tube Vortexer; VWR Scientific, Buffalo Grove, IL, USA) for 5 min. This extraction clean up step was repeated once and the combined ethyl acetate extracts were evaporated using an Automatic Environmental Speed Vac System (Savant, Holbrook, NY, USA). The same extraction procedure was used for ruminal fluid samples except that the volume of ethyl acetate used in each step was 3 mL. The dry residue was re-dissolved in 300 μL mobile phase and 50 μL was injected into the HPLC system (Shimadzu Corporation, Kyoto, Japan).

Chromatographic analysis

Samples were analysed for TCBZ and its metabolites. Fifty microlitres of each extracted sample was injected through an autosampler (Shimadzu SIL 10 A Automatic Sample Injector) into a Shimadzu 10 A HPLC system (Shimadzu Corporation) fitted with a Betasil C_{18} (5 μm, 250 mm × 4.60 mm) reverse-phase column (Thermo Electron Corporation, Bellefonte, PA, USA) and UV detector (Shimadzu, SPD-10A UV detector) reading at 292 nm. The mobile phase was an acetonitrile/ammonium acetate (0.025 M, pH 6.6) elution gradient. The chromatographic conditions were as previously reported (Mottier *et al.*, 2004) with slight modifications in 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 (Class LC 10, Shimadzu Corporation) of the HPLC system.

Drug/metabolites quantification

Validation of the analytical procedures for extraction and quantification of TCBZ and its metabolites was performed before starting the analysis of the experimental samples from the incubation assays. Known amounts of each analyte (1–60 nmol/mL) were added to aliquots of boiled (inactivated) microsomal preparations or ruminal fluid samples, extracted and analysed by HPLC (triplicate determinations) to obtain calibration curves and percentages of recovery. Calibration curves were analysed using the least squares linear regression analysis (Instat 3.00, Graph Pad Software, Inc., San Diego, CA, USA) of HPLC peak area ratios of analytes/IS and nominal concentrations of spiked samples. Correlation coefficients (r) for the different analytes ranged between 0.995 and 0.999. 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. Absolute recoveries were established by comparison of the detector responses (peak areas) obtained for spiked microsomal and ruminal fluid samples and those of direct standards prepared in mobile phase. Absolute recoveries from microsomal samples were 96–99% (OH-TCBZSO), 91–99% ($TCZSO_2$), 74–87% (OH-TCBZ), 84–98% (TCBZSO) and 89–92% (TCBZ). Absolute recoveries from ruminal fluid samples were 94–98% (OH-TCBZSO), 94–99% ($TCZSO_2$), 82–89% (OH-TCBZ), 95–99% (TCBZSO) and 66–71 % (TCBZ). Inter-assay precision coefficients of variation (CVs) were <15% and relative error (accuracy) values were below 20%.

Data and statistical analysis

The reported data are expressed as mean ± SD. Metabolic rates are expressed in nanomole of metabolic products formed per min/mg of microsomal protein. Statistical comparisons were carried out using the Instat 3.00 software (Graph Pad Software, Inc.). Metabolic rates were compared using the Kruskal–Wallis test (nonparametric ANOVA). Where significant overall differences ($P < 0.05$) were observed, further analysis among individual incubation sets was performed using the Dunn's test. The concentrations of the metabolites formed after either TCBZSO or HO-TCBZSO incubations with ruminal fluid are expressed in nmol/mL and as percentage of total drug/metabolites recovered from the incubation medium. A lack of fit test (Instat 3.00, Graph Pad Software, Inc.) was performed to establish the period of incubation time at which the sulphoreduction reaction was linear. Then, metabolic rates (nmol/min) were calculated using the following equations:

Rate of TCBZ or HO-TCBZ 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. Comparisons between drug/metabolite concentrations and between the rates of metabolites formation were performed using the Student's t-test with Welch correction. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Liver microsomal biotransformation

TCBZ was mainly metabolized into its pharmacologically active sulphoxide (TCBZSO) metabolite by sheep liver microsomes. The sulphone metabolite was also produced following the incubations with the parent compound. $TCBZSO_2$ was the major metabolite formed following TCBZSO incubation with the liver microsomal fraction. Only trace amounts of OH-TCBZSO were

recovered after the incubation of both substrates. The rates of HO-TCBZSO production by sheep liver microsomes were 0.009 ± 0.005 nmol/min/mg (TCBZ incubations) and 0.010 ± 0.006 nmol/min/mg (TCBZSO incubations). Any measurable nonenzymatic conversion did occur in blank incubations either without microsomes or in the absence of NADPH.

The maximal rate of TCBZ sulphoxidation by sheep liver microsomes was 0.85 ± 0.18 nmol of TCBZSO formed per min/mg of microsomal protein. Sheep liver microsomes metabolized TCBZSO into $TCBZSO_2$ at maximal rate of 0.16 ± 0.06 nmol/min/mg. Thus, the rate of TCBZ sulphoxidation was 5.3-fold higher ($P < 0.001$) compared to that observed for the sulphonation of TCBZSO. $TCBZSO_2$ production from TCBZ had a maximal rate of 0.061 ± 0.038 nmol/min/mg.

The patterns of TCBZ and TCBZSO microsomal biotransformation in the presence of PB (a cytochrome P450 inhibitor), MTZ (a FMO substrate) as well as following the inactivation of the FMO system are presented in Figs 1 & 2. When TCBZ was the incubated substrate (Fig. 1), competitive inhibition of the cytochrome P450 and FMO systems reduced the production of both TCBZSO (PB = 24% inhibition, MTZ = 71% inhibition) and $TCBZSO_2$ (PB = 66% inhibition, MTZ = 70% inhibition). The production of both TCBZ metabolites was also inhibited (TCBZSO = 77% inhibition, $TCBZSO_2$ = 71% inhibition) after the inactivation of the microsomal FMO system. Besides, the production of $TCBZSO_2$ after TCBZSO incubation was inhibited in the presence of PB (55% inhibition), methimazole (52% inhibition) as well as following FMO inactivation (58% inhibition). Likewise, on the assumption that inactivation of FMO leaves the cytochrome P450 system able to metabolize TCBZ and TCBZSO, the relative involvement of both enzymatic systems on the liver biotransformation of both substrates was estimated (Table 1).

Figure 3 compares the effects of several substrates or inhibitors of either cytochrome P450 or FMO enzymatic systems on TCBZ biotransformation by sheep liver microsomes. Total

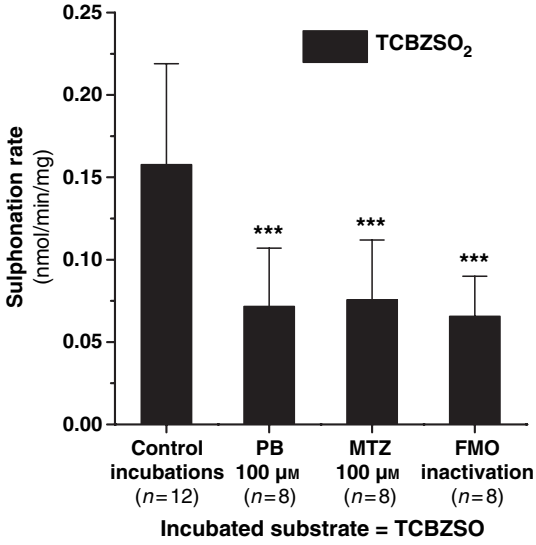

Fig. 2. *In vitro* biotransformation of triclabendazole sulphoxide (TCBZSO) to triclabendazole sulphone ($TCBZSO_2$) by sheep liver microsomes. The effects of cytochrome P450 inhibition and flavin-monooxygenase (FMO) inhibition/inactivation are shown. Cytochrome P450 and FMO were inhibited by pyperonyl butoxide (PB) and methimazole (MTZ), respectively. Inactivation of FMO was carried out by heating (2 min at 50 °C) the microsomal preparation followed by incubation of the substrate in presence of MTZ (see Materials and methods). The initial TCBZSO concentration was 40 µM. Data (mean ± SD) are expressed in nmol/min/mg of microsomal protein. The number of determinations in each incubation assay is shown in brackets. Values are significantly different from control incubations at (***) $P < 0.001$.

oxidation of TCBZ (sum of the metabolites formed) was inhibited by MTZ and PB. Increasing the concentration of PB from 100 to 200 µM did not enhance the inhibition of total TCBZ oxidation or the production of TCBZSO. KTZ did not affect the total oxidation of TCBZ or the production of TCBZSO, but decreased (66%

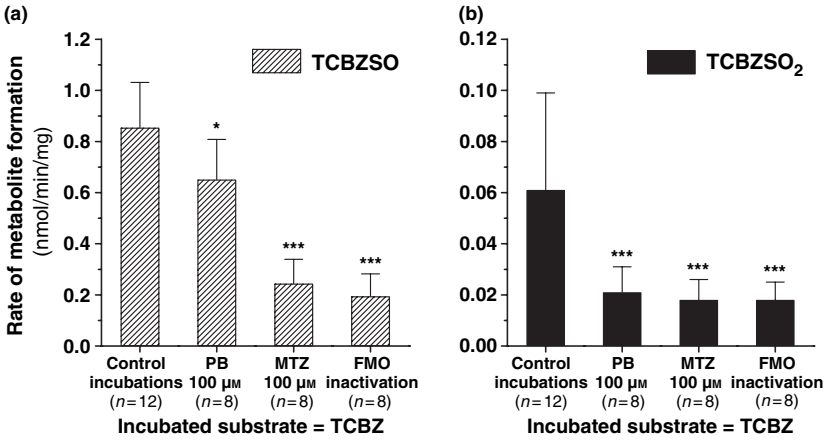

Fig. 1. *In vitro* biotransformation of triclabendazole (TCBZ) to (a) triclabendazole sulphoxide (TCBZSO) and (b) triclabendazole sulphone ($TCBZSO_2$) by sheep liver microsomes. The effects of cytochrome P450 inhibition and flavin-monooxygenase (FMO) inhibition/inactivation are shown. Cytochrome P450 and FMO were inhibited by pyperonyl butoxide (PB) and methimazole (MTZ), respectively. FMO inactivation was carried out by heating (2 min at 50 °C) the microsomal preparation followed by incubation of the substrate in the presence of MTZ (see Materials and methods). The initial TCBZ concentration was 40 µM. Data (mean ± SD) are expressed in nmol/min/mg of microsomal protein. The number of determinations in each incubation assay is shown in brackets. Values are significantly different from control incubations at (*) $P < 0.05$ and (***) $P < 0.001$.

Table 1. *In vitro* biotransformation of triclabendazole (TCBZ) and triclabendazole sulphoxide (TCBZSO) to their respective -sulpho metabolites, triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$), by sheep liver microsomes.. The involvement of the flavin-monooxygenase (FMO) and cytochrome P450 systems was estimated by the difference between the metabolic rates observed in control incubations and those observed after inactivation of the FMO system. The relative involvement of both enzymatic systems was estimated on the assumption that FMO inactivation leaves the P450 system able to metabolize the incubated substrate (TCBZ or TCBZSO)

Incubated substrate	Formed metabolite	Control incubations	FMO inactivation†	Estimated involvement		Ratio FMO/P450‡
				FMO	P450	
TCBZ	TCBZSO	0.839 ± 0.143	0.194 ± 0.094 ***	0.645 ± 0.131 (76.9%)	0.194 ± 0.094 (23.1%)	3.83 ± 1.63
TCBZSO	$TCBZSO_2$	0.152 ± 0.056	0.066 ± 0.026 ***	0.086 ± 0.031 (56.4%)	0.066 ± 0.026 (43.6%)	1.31 ± 0.23*

Values are expressed in nmol/min/mg of microsomal protein. Metabolic rates (mean ± SD) were obtained from four different liver microsomal preparations.

Values are significantly different from control incubations at (***) $P < 0.001$. *The ratio FMO/P450 for TCBZSO sulphonation was significantly different ($P < 0.05$) from that observed for TCBZ sulphoxidation.

†FMO inactivation was carried out by heating (2 min at 50 °C) the microsomal preparation followed by incubation in the presence of MTZ (see Materials and methods).

‡The mean (±SD) value of the ratio between the rate of TCBZSO or $TCBZSO_2$ production estimated for each enzymatic system in each microsomal preparation ($n = 4$).

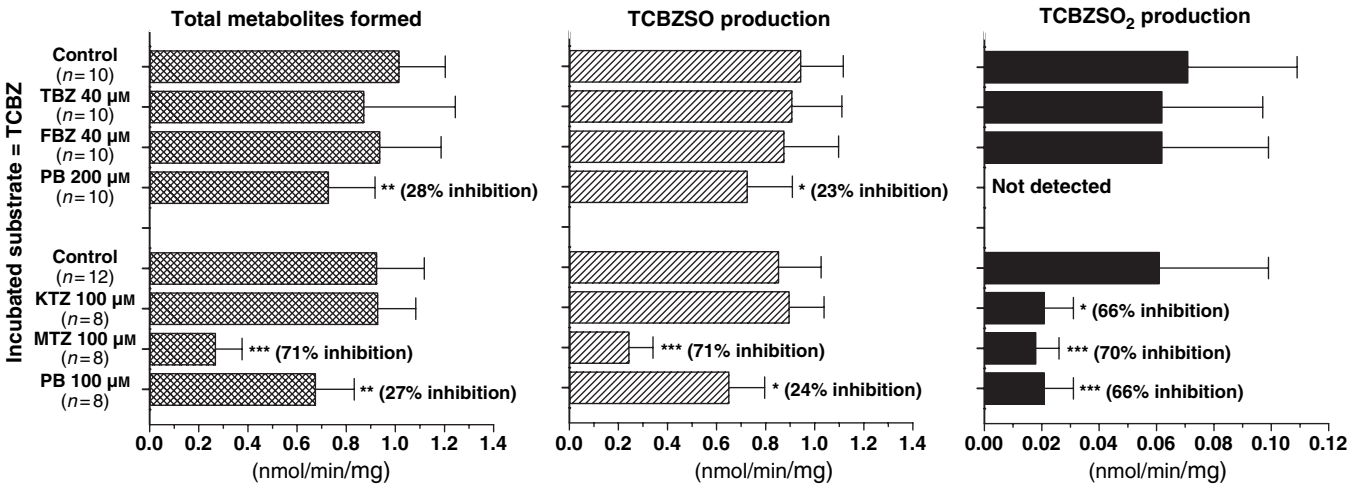

Fig. 3. Effects of several substrates and/or inhibitors of either the cytochrome P450 or flavin-monooxygenase systems on triclabendazole (TCBZ) biotransformation by sheep liver microsomes. The initial TCBZ concentration was 40 µM. Data (mean ± SD) are expressed in nmol of either total metabolites (▩), triclabendazole sulphoxide (TCBZSO ▨) and sulhpone ($TCBZSO_2$ ■) formed per min/mg of microsomal protein. The number of determinations in each incubation assay is shown in brackets. Values are significantly different from control incubations at (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$.

inhibition) the formation of $TCBZSO_2$. The presence of TBZ and FBZ in the incubation media did not modify the oxidative metabolism of TCBZ at any assayed concentrations (5, 10 and 40 µM). Similar findings were observed after the incubation of TCBZSO in presence of the same enzymatic substrates/inhibitors (Fig. 4). Thus, inhibition of $TCBZSO_2$ production was observed after incubation of TCBZSO in the presence of PB, MTZ, KTZ and ETM. The results obtained after the incubations of TCBZ and TCBZSO in presence of 5 and 10 µM of TBZ and FBZ are not shown.

Ruminal metabolism

Neither TCBZSO nor HO-TCBZSO were metabolically changed after their incubation with boiled ruminal fluid. TCBZSO was extensively metabolized to TCBZ by sheep ruminal fluid under anaerobic conditions. The thioether hydroxy-TCBZ (HO-TCBZ) was the metabolic product formed after the ruminal sulphoreduction of HO-TCBZSO. The sulphoreductive metabolic reactions for both sulphoxide substrates were linear for up 120 min incubation. No other TCBZ metabolite was detected in these metabolic assays. The concentrations of each thioether metabolic product formed after ruminal fluid incubations with TCBZSO and HO-TCBZSO are shown in Fig. 5. Significantly ($P < 0.01$) higher HO-TCBZ concentrations were formed from HO-TCBZSO, compared to those of TCBZ produced from TCBZSO, at all the assayed incubation times. After 120 min incubation, the rate of HO-TCBZ formation was 65% higher ($P < 0.05$) compared to that estimated for the TCBZ production (Table 2). At the same incubation time, the concentration ratio thioether/sulphoxide

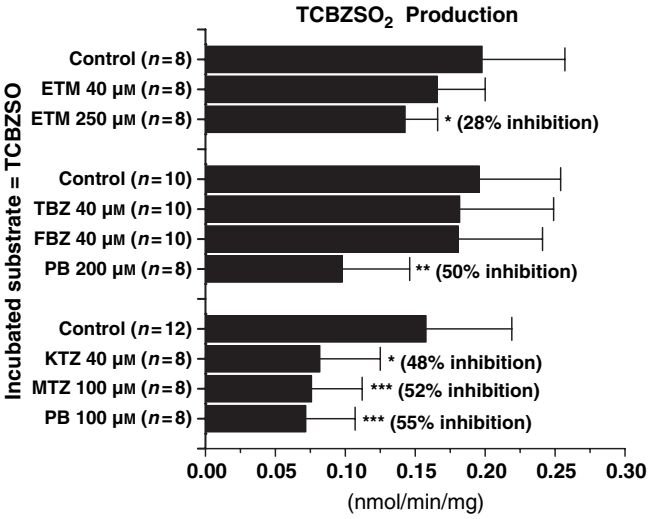

Fig. 4. Effects of several substrates and/or inhibitors of either the cytochrome P450 or flavin-monooxygenase systems on the biotransformation of triclabendazole sulphoxide (TCBZSO) by sheep liver microsomes. The initial TCBZSO concentration was 40 µM. Data (mean ± SD) are expressed in nmol of triclabendazole sulhpone ($TCBZSO_2$) formed per min/mg of microsomal protein. The number of determinations in each incubation assay is shown in brackets. Values are significantly different from control incubations at (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$.

collected after HO-TCBZSO incubation was 12.7-fold higher ($P < 0.01$) compared to that observed when TCBZSO was the incubated substrate.

DISCUSSION

The BZD anthelmintics require extensive hepatic oxidative metabolism to achieve sufficient polarity for excretion (Hennessy *et al.*, 1993). The plasma pharmacokinetic profile of TCBZ has been described in many species, including cattle (Bogan *et al.*, 1988), sheep (Hennessy *et al.*, 1987; Bogan *et al.*, 1988), goats, horses, ponies, donkeys, pigs and humans (Bogan *et al.*, 1988). Sulphoxidation and sulphonation appear to be the main metabolic reactions involved in TCBZ hepatic biotransformation in sheep. Extremely low concentrations of TCBZ parent compound were recovered in bile whereas TCBZSO, $TCBZSO_2$ and the hydroxy derivatives were the major biliary metabolites measured in bile from TCBZ-treated sheep (Hennessy *et al.*, 1987). TCBZ biliary metabolites were recovered in their unconjugated and conjugated forms. The major metabolite found in bile was conjugated OH-TCBZSO and contributed almost half of the total conjugated metabolites. Hydroxylated TCBZ metabolites have not been detected in plasma, whereas TCBZSO and $TCBZSO_2$ are the only metabolites detected in the bloodstream. We have

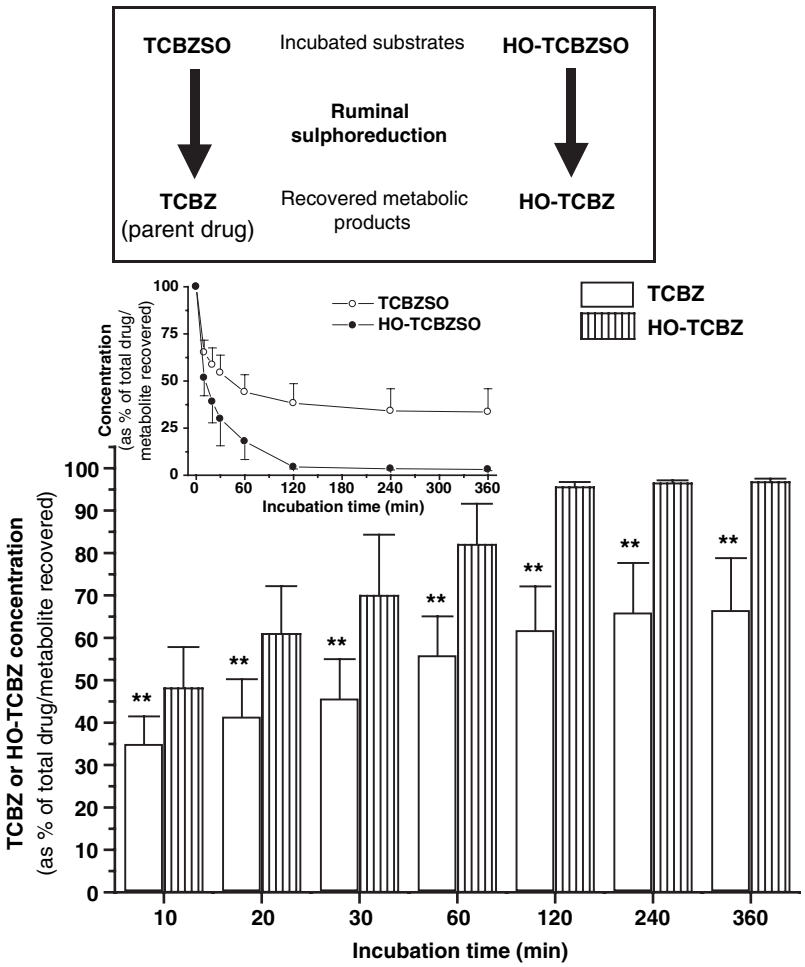

Fig. 5. Ruminal sulphoreduction of triclabendazole sulphoxide (TCBZSO) and hydroxy-triclabendazole sulphoxide (HO-TCBZSO) into their respective thioeters, triclabendazole (TCBZ) and hydroxy-triclabendazole (HO-TCBZ) under anaerobic conditions. The inserted plot shows the disappearance of the incubated substrates (TCBZSO and HO-TCBZSO) from the incubation medium over the time. Each value (mean ± SD) was obtained after the incubation of both substrates (triplicate determinations) with ruminal fluid samples taken from 4 animals. The concentrations of TCBZ formed are significantly different (**) $P < 0.01$ compared to those measured for HO-TCBZ.

Table 2. Rates of metabolic conversion obtained after incubation of either triclabendazole sulphoxide (TCBZSO) or hydroxy-TCBZSO (HO-TCBZSO) during 120 min with sheep ruminal fluid

Incubated substrate	Formed metabolite	Sulphoreduction rate (nmol/min)	Ratio thioether/ sulphoxide†
TCBZSO	TCBZ	0.17 ± 0.07*	1.80 ± 0.92
HO-TCBZSO	HO-TCBZ	0.28 ± 0.06	22.9 ± 5.70**

Each value (mean ± SD) was obtained after the incubation of both substrates (triplicate determinations) with ruminal fluid samples taken from four animals.

The rate of TCBZ formation was significantly higher (*) $P < 0.05$ compared to that observed for HO-TCBZ production. The ratio thioeter/ sulphoxide obtained after HO-TCBZSO incubation was significantly higher (**) $P < 0.001$ than that observed after incubation of TCBZSO.

†The mean (±SD) value of the ratio between the concentration (nmol/mL) of the thioether (TCBZ or HO-TCBZ) and its respective sulphoxide derivative (TCBZSO or HO-TCBZSO) measured in each individual incubation assay.

recently shown the involvement of both FMO and cytochrome P450 enzymatic systems on TCBZ oxidative metabolism (Mottier *et al.*, 2004). A detailed follow up study addressed to identify the metabolic pathways implicated on the extensive TCBZ biotransformation in sheep is described here.

It has been well documented that both FMO and cytochrome P450 systems are implicated in the liver sulphoxidation of albendazole (ABZ) in sheep (Galtier *et al.*, 1986; Lanusse *et al.*, 1993), pigs (Souhaili El Amri *et al.*, 1987), cattle (Lanusse *et al.*, 1993), rats (Moroni *et al.*, 1995) and humans (Rawden *et al.*, 2000). The FMO system accounts for approximately 30% of the ABZ sulphoxidation in human liver, while the cytochrome P450 system is the major contributor (approximately 70%) (Rawden *et al.*, 2000). Similarly, cytochrome P450 is primarily involved (approximately 60%) in ABZ hepatic sulphoxidation in rats, although the FMO enzymatic system is also implicated (Moroni *et al.*, 1995). Other *in vitro* studies showed the relative involvement of FMO (approximately 32%) and cytochrome P450 (approximately 68%) on liver sulphoxidation of FBZ in rats (Murray *et al.*, 1992). Conversely, it has been demonstrated that FMO is primarily involved on ABZ and FBZ hepatic sulphoxidation in sheep and cattle (Lanusse *et al.*, 1993; Virkel *et al.*, 2004). The involvement of both enzymatic systems in TCBZ sulphoxidation and TCBZSO sulphonation was shown in the current work after FMO inactivation and/or incubation of the substrates in presence of MTZ and PB (see Figs 1 & 2). FMO was inactivated by heat pretreatment of the microsomal preparation (Dixit & Roche, 1984) followed by incubation of the substrates in the presence of MTZ. The relative participation of both enzymatic systems may be estimated following the assumption that inactivation of FMO leaves the cytochrome P450 system as the main metabolic system able to metabolize TCBZ or TCBZSO. The data reported herein (Table 1) show that FMO is the main enzymatic pathway involved in the sulphoxidation of TCBZ (ratio FMO/P450 = 3.83 ± 1.63), although both enzymatic systems participate in a similar proportion in the sulphonation reaction to form the sulphone metabolite (ratio FMO/P450 = 1.31 ± 0.23). Thus, the FMO system produced approximately 77% of TCBZSO after TCBZ incubation and approximately 56% of $TCBZSO_2$ when TCBZSO was the incubated substrate. Altogether the available information clearly demonstrates the major involvement of the FMO system, compared with cytochrome P450, in the hepatic metabolism of the most important BZD anthelmintics used in ruminants. For this reason, metabolic interactions between BZD compounds and many other FMO substrates (e.g. sulphur-containing pesticides) to which animals could be exposed, may affect the disposition kinetics of these anthelmintic drugs. Besides, such drug to drug metabolic interactions may impact on the permanence of residual concentrations of different chemical agents in edible tissues, a major concern for public health and consumer's safety.

After an intra-ruminal treatment with TCBZ at 10 mg/kg in sheep, higher plasma concentrations of TCBZSO and $TCBZSO_2$ were observed compared to those measured for the sulphoxide and sulphone metabolites of ABZ and FBZ after their oral administrations at the same dosages in the same species (Hennessy *et al.*, 1987; Bogan *et al.*, 1988). It has been shown that TCBZSO and $TCBZSO_2$ are strongly bound to albumin (≥90%) and the relatively slow appearance of both metabolites in plasma because of the rate of release of the protein from the liver may have accounted for a higher total TCBZ metabolites area under curve (AUC; approximately 1000 μg/h/mL) than those observed for total ABZ and FBZ metabolites AUC values (approximately 150 and 50 μg/h/mL, respectively; Hennessy *et al.*, 1987). Moreover, the total TCBZ liver oxidation observed in the current trial (control incubations = 0.93–1.02 nmol/min/mg; see Fig. 3) seems to be higher than that reported for ABZ (0.54 nmol/min/mg) and FBZ (0.22 nmol/min/mg) in sheep (Virkel *et al.*, 2004). These studies were carried out under the same experimental conditions and the higher metabolic pattern observed for TCBZ, compared with ABZ and FBZ, is a clear outcome from these biotransformation studies in ruminant species. Indeed, a higher rate of TCBZ oxidation by the liver may also contribute to the observed differences between the pharmacokinetic profiles of TCBZ metabolites and those determined for ABZ and FBZ metabolites in sheep.

The metabolism of the BZD thioethers in the host leads to more polar and less active metabolites. In terms of parasite uptake (Alvarez *et al.*, 1999, 2000) and mode of action (binding to tubulin; Lubega & Prichard, 1991), the parent thioethers are more efficient than their respective -sulpho and -hydroxy metabolites. Therefore, the sequential oxidative steps, to which the BZD parent drugs are exposed, give rise to a considerable reduction in their ability to diffuse into the target parasite as well as in their anthelmintic efficacy. Several substrates or inhibitors of either cytochrome P450 or FMO enzymatic systems have been shown to interfere with the biotransformation of BZD anthelmintics either *in vivo* or *in vitro* (Lanusse & Prichard, 1993). Interference with the liver oxidative metabolism has resulted in pronounced modifications to the pharmacokinetic behaviour of active BZD parent drugs or their metabolites, which may improve their clinical efficacy. Thus, co-administration of oxfendazole (OFZ) with parbendazole (Hennessy *et al.*, 1992),

MTZ (Lanusse *et al.*, 1995) or PB (Sánchez *et al.*, 2002) increased the concentrations of the active moieties (FBZ and OFZ itself) in the systemic circulation in sheep. Besides, MTZ and metirapone (MTP), a potent inhibitor of the cytochrome P450 system, improved the plasma AUC values of ABZ metabolites following the administration of the pro-BZD netobimin (NTB) to sheep (Lanusse & Prichard, 1991, 1992). The *in vitro* liver microsomal sulphoxidation of ABZ was inhibited competitively by FBZ and noncompetitively by chlorpromazine in sheep (Galtier *et al.*, 1986). These authors also showed that chloramphenicol, ETM, nalidixic acid and hexobarbital are less potent inhibitors of the sulphoxidative pathway. Furthermore, other drugs such as the cytochrome P450 3A4 substrates KTZ and ritonavir inhibited ABZ sulphoxidation in human liver microsomes (Rawden *et al.*, 2000). In addition to MTZ and PB, other enzymatic substrates or inhibitors were tested for a possible interference with the oxidative metabolism of TCBZ and TCBZSO in the current trial. The BZD analogues FBZ and TBZ did not interfere with the oxidative metabolism of either substrates, whilst ETM and KTZ inhibited only the conversion of TCBZSO to the sulphone metabolite (Figs 3 & 4). Several experiments applying standardized models for the characterization of microsomal mixed function oxidases have shown that ruminants seem to express all major cytochrome P450 enzymes (Fink-Gremmels & van Miert, 1996). For example, cytochrome P450 enzymes with catalytic properties similar to those of the P450 3A subfamily of rats and humans were found in the liver of goat and cattle (van't Klooster *et al.*, 1993; Zweers-Zeilmaker *et al.*, 1996). Besides, a troleandomycin-inducible cytochrome P450 3A enzyme, purified from sheep liver, was further characterized as a rabbit cytochrome P450 3A6-like enzyme (Pineau *et al.*, 1990). In addition, cytochrome P450 3A substrates such as ETM and KTZ are useful tools to evaluate the participation of this isoenzyme in the *in vitro* biotransformation of a given xenobiotic (Newton *et al.*, 1995; Zweers-Zeilmaker *et al.*, 1999). ETM-mediated inhibition of cytochrome P450 3A is due to the formation of stable metabolic intermediate complexes (Zweers-Zeilmaker *et al.*, 1999). Although ETM is known as a weak inhibitor of cytochrome P450 3A in ruminants (Zweers-Zeilmaker *et al.*, 1999), this isoenzyme may be involved in the sulphonation of TCBZSO. On the other hand, KTZ concentrations higher than 10 μM (K_i value for cytochrome P450 3A inhibition) may inhibit the metabolic activities of other cytochrome P450 isoenzymes (Newton *et al.*, 1995). This observation precludes the consideration of KTZ as a specific cytochrome P450 3A inhibitor under the experimental conditions described here. Overall, the observed metabolic interference between TCBZ and ETM or KTZ should be carefully considered as the concurrent use of these drugs may occur in veterinary therapeutics.

The BZD sulphoxides are distributed from the plasma to the GI tract, allowing their microflora-mediated sulphoreduction into the parent thioethers in the rumen and intestine (Lanusse & Prichard, 1993). This metabolic sulphoreduction is pivotal for the action of BZD anthelmintics, as the thioethers have greater affinity for parasite tubuline than their respective sulphoxides (Lubega & Prichard, 1991). Although the flukicidal activity of TCBZ remains to be fully understood, there is data to support a possible microtubule-based action of this anthelmintic compound (Stitt & Fairweather, 1994, 1996). TCBZ parent drug is short lived and both TCBZSO and $TCBZSO_2$ are the main unconjugated analytes recovered in the bloodstream and bile (adult flukes are located in biliary ducts) of treated animals (Hennessy *et al.*, 1987). Consequently, flukicidal activity has been mainly related to TCBZSO systemic availability, although the sulphone metabolite may, at a much lower extent, contribute with some flukicidal activity (Büscher *et al.*, 1999). Therefore, the metabolic conversion of TCBZSO into TCBZ in the GI tract may give rise to a source of the main active metabolite against the fluke *F. hepatica* in the liver. From this point of view, the sulphoreduction of HO-TCBZSO into HO-TCBZ may be relevant as well, although the mode of action and/or the anthelmintic activity (if any) of these metabolites have not been established. Further work is required to understand the contribution of the hydroxylated metabolites of TCBZ to its overall anthelmintic efficacy.

The metabolic approach tested here contributes to the identification of the different pathways involved in drug biotransformation in ruminant species. The outcome of the current work on the characterization of the pattern of hepatic and ruminal biotransformation of TCBZ and its main metabolites is a further contribution to the understanding of the pharmacological properties of widely used anthelmintics in ruminants. Comprehension of TCBZ metabolism is critical to optimize its flukicidal activity and to delay development of resistance.

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