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Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidized metabolites into *Fasciola hepatica*

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1. Triclabendazole (TCBZ) is an halogenated trematodicidal benzimidazole compound extensively used in veterinary medicine. It is active against immature and adult stages of the liver fluke *Fasciola hepatica*.

2. Free and conjugated TCBZ metabolites have been identified in the bile of treated sheep.

3. The experimental aims were to characterize the *in vitro* patterns of TCBZ biotransformation both in the animal host (sheep liver microsomes) and target parasite (*F. hepatica* microsomal preparation); and to compare the *ex vivo* diffusion of TCBZ parent drug and its oxidized metabolites (TCBZ sulphoxide [TCBZSO], TCBZ sulphone [$TCBZSO_2$], and TCBZ-hydroxy derivatives) into *F. hepatica*. Additionally, the octanol–water partition coefficients for TCBZ and all its metabolites were estimated as an indicator of the relationship between drug lipophilicity and diffusion into the target parasite.

4. Drug/metabolites concentrations were quantified by HPLC after sample clean up and a solvent-mediated chemical extraction.

5. Sheep liver microsomes metabolized TCBZ into its sulphoxide and sulphone metabolites after 30 min of incubation. The rate of TCBZ sulphoxidation in the liver was significantly greater ($p < 0.01$) than that observed for the sulphonation of TCBZSO.

6. The trematode parasite oxidized TCBZ into its sulphoxide metabolite after 60 min of incubation at a metabolic rate of 0.09 nmol min^{-1} mg $protein^{-1}$.

7. TCBZ and all its oxidized metabolic products were recovered from *F. hepatica* as early as 15 min after their *ex vivo* incubation in a Kreb's Ringer Tris buffer. However, the diffusion of the hydroxy-derivatives into the fluke was lower than that observed for TCBZ, TCBZSO and $TCBZSO_2$. There was a high correlation ($r = 0.82$) between drug lipophilicity (expressed as octanol–water partition coefficients) and drug availability measured within the parasite.

8. Unlike the uptake pattern previously observed for albendazole, the parent TCBZ and its sulphoxide and sulphone metabolites showed a similar ability to penetrate into the trematode parasite.

9. Understanding the relationship between TCBZ metabolism, the relative pharmacological potency of its metabolic products and their ability to reach the target parasite may be critical to optimize its flukicidal activity, particularly when TCBZ resistant flukes have been already isolated in the field.

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Introduction

Fascioliasis, caused by the trematode liver fluke *Fasciola hepatica*, is the cause of considerable loss in sheep and cattle production systems all over the world (Courtney and Roberson 1995). Human fascioliasis occurs as an accidental zoonotic disease in Africa, Western Europe and Latin America (Mas-Coma *et al*. 1999). The main strategy for the effective control of fascioliasis is based on chemotherapy. Most of the available flukicidal compounds, at their recommended therapeutic doses, have good activity against the mature stage of the liver fluke, but are not sufficiently effective against the pathogenic immature stages located in the liver parenchyma. Interestingly, triclabendazole (6-chloro-5(2-3-dichlorophenoxy)-2-methyl thio-benzimidazole) (TCBZ), an halogenated benzimidazole thiol derivative, shows excellent efficacy against both the mature and immature stages of the liver fluke in sheep and cattle, which is a differential feature compared to other available trematodicidal drugs (Boray *et al*. 1983).

Flukicidal drugs can reach the liver fluke either by oral ingestion of blood or by transtegumental diffusion. The high absorption surface of the trematode tegument may have a major role in drug diffusion from the surrounding medium. The entry of a drug into the parasite may mainly depend on the diffusion surface, the concentration gradient across the membrane, the pH/pK relationship and the lipophilicity of the molecule (Mottier *et al*. 2003). TCBZ is the most potent flukicidal compound, even though its *ex vivo* transtegumental diffusion into adult *F. hepatica* was shown to be significantly lower than that observed for albendazole (Alvarez *et al*. 2004). Although TCBZ mode of action remains unknown, its strong binding to various types of protein structures other than microtubules (the site of action for nematodicidal benzimidazole compounds), may suggest a multiplicity of targets for the trematodicidal activity of this drug (Bennett and Köhler 1987).

The methylcarbamate benzimidazole (albendazole, fenbendazole, etc.) anthelmintics are extensively metabolized in all mammalian species studied (Gottschall *et al*. 1990, Lanusse and Prichard 1993). There is only limited information available describing the plasma pharmacokinetic behaviour of TCBZ in ruminant species (Alvinerie and 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). TCBZ is oxidized to form the sulphoxide and sulphone metabolites, triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$), respectively. Hydroxylation of TCBZ occurs at the 4′ position of the dichlorophenoxy ring, forming the corresponding hydroxylated metabolites, hydroxy-TCBZ (OH-TCBZ), hydroxy-TCBZSO (OH-TCBZSO) and hydroxy-$TCBZSO_2$ (OH-$TCBZSO_2$) (figure 1). Extremely low concentrations of TCBZ were recovered in bile. TCBZSO, $TCBZSO_2$ and the hydroxy derivatives were the major biliary metabolites identified in sheep bile (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 to almost half of the total conjugated metabolites. Hydroxylated TCBZ metabolites have not been detected in plasma, with TCBZSO and $TCBZSO_2$ being the only metabolites found in the bloodstream. TCBZ metabolites are strongly bound to plasma proteins ($\geq$90%) which could account for their long residence time in the animal body (Hennessy *et al*.

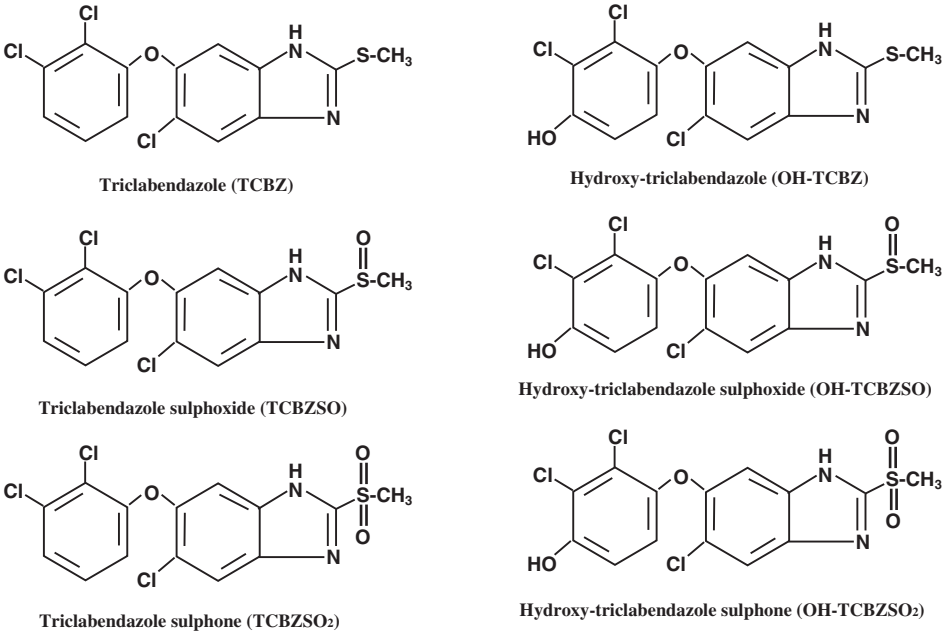

Figure 1. Chemical structures of the halogenated trematodicidal benzimidazole thiol molecules assayed.

1987). One of the purposes of the work reported herein was to provide detailed information on the biotransformation pathways involved on the formation of the TCBZ sulpho- and hydroxy- metabolites in the host liver and within its main target parasite.

To gain further insight on the relationship between the pharmacological behaviour and the flukicidal activity of TCBZ, the goals of the work reported here were: (1) to characterize *in vitro* the patterns of TCBZ biotransformation both in the animal host (sheep liver microsomes) and target parasite (*F. hepatica* microsomal preparation) and (2) to compare the *ex vivo* diffusion of TCBZ parent drug and its oxidized metabolites into *F. hepatica*. The data obtained from the transtegumental diffusion kinetic studies were correlated with molecular lipophilicity, estimated by lipid-to-water partition coefficients (PC), the most frequently used parameter for defining the lipophilic character of a given drug molecule.

Materials and Methods

Chemicals

Reference standards (97–99% pure) of TCBZ and its metabolites were provided by Novartis Animal Health (Basel, Switzerland). The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), tris[hydroxymethyl]aminomethane hydrochloride (Tris), methimazole (MTZ) and piperonyl butoxide (PB) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). The solvents used for the chemical extraction and chromatographic analysis were high-performance liquid chromatography (HPLC) grade (Baker, Inc., Phillipsburg, USA). Buffer salts ($NaHCO_3$, Na_2HPO_4 and CH_3COONH_4) were purchased from Baker.

Collection of parasite material

Eight parasite-free Corriedale weaned lambs (males, 8–10 months old) were infected with 200 metacercariae of *F. hepatica* (provided by Instituto DILAVE, 'Miguel C. Rubino', Montevideo,

Uruguay), given in a gelatine capsule by the oral route. Sixteen weeks after infection, the animals were stunned and exanguinated immediately, according to internationally accepted animal welfare guidelines (AVMA 2001). Adult specimens of *F. hepatica* were recovered from the liver, common bile ducts and the gall-bladder of each lamb. The parasite specimens were rinsed extensively with saline solution (NaCl 0.9%) (37°C) to remove bile and/or adhering materials.

TCBZ biotransformation by sheep liver microsomes and F. hepatica *microsomal fraction*

Microsome preparation. Two healthy (uninfected) Corriedale lambs (males, 8–10 months old) were sacrificed to be used as a source of liver material for preparation of microsomes. The microsomal fraction from pooled *F. hepatica* specimens was also prepared. Briefly, samples (approximately $2 \times 2 \times 2$ cm) of liver and parasite specimens (1 g) were rinsed with cold KCl (1.15%) and then transported to the laboratory kept in flasks filled with phosphate buffer (0.1 M, pH 7.3) at 4°C. All subsequent operations were performed between 0 and 4°C. Liver samples were cut into small pieces with scissors and washed several times with the phosphate buffer (to remove haemoglobin). Then, tissue samples and parasite material were homogenized in the above phosphate buffer using a Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, DE, USA), centrifuged at 10 000*g* for 20 min and the resulting supernatant was further centrifuged at 100 000*g* for 60 min. The pellet (microsomal preparation) was suspended in phosphate buffer 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. Metabolic activity was assessed by the amount of TCBZSO and $TCBZSO_2$ formed in the presence of NADPH. A typical reaction mixture contained 250 µl NADPH solution prepared in phosphate buffer (0.1 M, pH 7.3), 100 µl tissue preparation (0.5 mg microsomal protein) and 15 nmol TCBZ (sheep liver and parasite microsomes) or TCBZSO (liver microsomes) dissolved in 10 µl methanol. The incubation mixture was adjusted to a final volume of 500 µl with phosphate buffer (0.1 M, pH 7.3). The initial concentration of the incubated substrates (TCBZ and TCBZSO) was 30 µM. Incubations (30 min for sheep liver and 60 min for parasite microsomes) were carried out at 37°C in glass vials in an oscillating water bath under aerobic conditions. The drug substrates were also incubated, under the same conditions, either with inactivated (boiled) microsomes or without NADPH. These incubations were used as controls for possible non-enzymatic drug conversion. All reactions were stopped by the addition of 200 µl ice-cold acetonitrile and stored at −20°C until analysis. The liver microsomal oxidation of both incubated substrates was linear up to 30 min. The maximal oxidation rates of TCBZ and TCBZSO were obtained using 1 µmol (sheep liver microsomes) and 0.5 µmol (*F. hepatica* microsomes) NADPH (table 1).

Inactivation of the FMO system was performed by heating the diluted microsomal preparation (2 min at 50°C) without NADPH, which was immediately chilled in ice and followed by pre-incubation (5 min at 37°C) with 100 µM of the FMO substrate methimazole (MTZ) (Dixit and Roche 1984). Then, the incubated substrate (TCBZ or TCBZSO) was added and the reaction started with the addition of NADPH. MTZ was dissolved in 25 µl distilled water.

The oxidation of TCBZ and TCBZSO was also studied in the presence of 100 µM piperonyl butoxide (PB), a well known cytochrome P450 inhibitor. Incubation mixtures containing PB were pre-incubated for 5 min at 37°C without NADPH followed by the addition of TCBZ or TCBZSO. The reaction started with the addition of NADPH. The inhibitor (PB) was dissolved in 10 µl methanol and parallel control tubes contained the same volume of the solvent. Both MTZ and PB were

Table 1. Comparative sulphoxidation of triclabendazole by sheep liver and *Fasciola hepatica* microsomal fractions: effect of the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) concentration on the rate of triclabendazole sulphoxide (TCBZSO) formation after 60 min of incubation.

NADPH ($\mu mol\,ml^{-1}$)	TCBZSO rate of formation ($nmol\,min^{-1}\,mg^{-1}$)	
	Sheep liver microsomes	*F. hepatica* microsomes
0	0.006	0.002
0.125	0.140	0.020
0.250	0.240	0.040
0.500	0.350	0.100
1.000	0.380	0.100
2.000	0.370	0.100

Data are derived from six (sheep liver microsomes) and five (*F. hepatica* microsomal fraction) determinations.

also incubated in the absence of the substrates under the same conditions to ensure that their presence in the incubation mixture did not interfere with the chromatographic determination of TCBZ and its metabolites. All the incubated microsomal mixtures have the same volume (20 µl) of methanol.

Drug diffusion assays

The collected flukes were maintained for 2 h before starting the incubation process in a Kreb's Ringer Tris (KRT) buffer (pH 7.4) at 37°C (McCraken and Lipkowitz 1990). Two fluke specimens (approximately 0.2 g) were incubated at 37°C in the KRT buffer (2 ml), containing either TCBZ, TCBZSO, $TCBZSO_2$, OH-TCBZ, OH-TCBZSO or $OH\text{-}TCBZSO_2$ at a final concentration of 5 nmol ml^{-1}. This is a pharmacologically relevant concentration obtained from previously reported work where TCBZ concentrations in bile and plasma were measured after conventional treatments in ruminants (Hennessy *et al*. 1987). Times of incubations were 15, 45, 60, 90 and 180 min. There were four replicate incubation assays for each drug at each incubation time. Blank samples containing parasite material and incubation medium without drug, and drug-spiked medium without parasite material were incubated during the same time intervals. Once the incubation time elapsed, the flukes were rinsed thoroughly with saline solution, blotted on coarse filter paper and stored at −20°C until their preparation for HPLC analysis to measure drug concentrations. The parasite material was processed within 2–4 h after the incubation assays.

Sample preparation, extraction and analytical procedures

HPLC analysis for TCBZ and its metabolites was carried out as described (Mottier *et al*. 2004).

Enzyme assays. The internal standard (IS) mebendazole (MBZ) (5 nmol dissolved in 20 µl methanol) was added to inactivated incubation mixtures. Spiked samples (500 µl), fortified with TCBZ and its metabolites, were mixed with 200 µl cold acetonitrile followed by the addition of the IS. Experimental and fortified samples were mixed with 1.5 ml ethyl acetate and shaken on a mechanical shaker 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, USA). The dry residue was re-dissolved in 300 µl mobile phase and 50 µl were injected into the HPLC system (Shimadzu Corporation, Kyoto, Japan). 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^{-1}) were added to aliquots of boiled (inactivated) microsomal preparations, 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 samples and those of direct standards prepared in mobile phase. Drug/metabolite absolute recoveries were 93–99% ($OH\text{-}TCBZSO_2$), 96–99% (OH-TCBZSO), 91–99% ($TCZSO_2$), 74–87% (OH-TCBZ), 84–98% (TCBZSO) and 89–92% (TCBZ). Inter-assay precision coefficients of variation (CVs) were $< 15\%$ and the limits of quantification (nmol ml^{-1}) were 0.07 ($OH\text{-}TCBZSO_2$), 0.18 (OH-TCBZSO), 0.39 ($TCZSO_2$), 0.32 (OH-TCBZ), 0.37 (TCBZSO) and 0.38 (TCBZ).

Drug diffusion assays. Drug concentrations are expressed as nmol 100 mg trematode $protein^{-1}$. The determination of parasite protein concentrations was carried out according to the methodology described by Smith *et al*. (1985). Protein concentration in *F. hepatica* ($n = 5$) was 183.7 ± 20 mg g^{-1} trematode parasite. Drug-free *F. hepatica* material (0.1 g) was spiked with each target molecule (TCBZ, TCBZSO, $TCBZSO_2$, OH-TCBZ, OH-TCBZSO, $OH\text{-}TCBZSO_2$) to achieve the following final concentrations: 0.27, 0.54, 1.36, 2.72, 5.44, 10.89, 16.33 nmol 100 mg $protein^{-1}$, and with MBZ (IS) (10 µl, stock solution of 500 µM). Validation of the analytical procedures for extraction and quantification of TCBZ and its metabolites from trematode material was carried out as described (Mottier *et al*. 2004).

Octanol–water partition coefficients (PC). The octanol–water PC (log P) was used as an indicator of lipid solubility of the drug/metabolites used in the current experiments. The methodology used to calculate this parameter was adapted from Péhourcq *et al*. (2000).

Analysis of the data

The data for the enzyme assays are expressed as mean (±SD) of six determinations (three incubations with the liver microsomal preparation obtained from each animal). Metabolic rates of the

products formed are expressed in nmol min^{-1} mg^{-1} of microsomal protein. Statistical comparisons were carried out using analysis of variance (ANOVA) (Instat 3.0 Software, Graph Pad Software). The Tukey's range test was used to indicate the order of significance when a significant F was obtained. A value of $p < 0.05$ was considered statistically significant. Statistical comparison between the oxidation rates obtained for TCBZ and TCBZSO in the liver microsomal fraction was performed using the Mann–Whitney U-test. The same non-parametric test was used for the statistical comparison between TCBZSO and $TCBZSO_2$ rates of formation following TCBZ incubation with sheep liver microsomes.

Once the concentration values (expressed as nmol 100 mg $protein^{-1}$) for each compound within the target parasites were determined for each individual incubation assay, the area under the concentration–time curve (AUC) was calculated by the trapezoidal rule method (Gibaldi and Perrier 1982), using the PkSolution 2.0 program (Summit Research Services, Ashland, OH, USA). The AUC (nmol min 100 mg $protein^{-1}$) was considered as an indicator of the total drug availability within the trematode parasite. The individual concentrations and AUCs are presented as mean ± SD (four replicates). The individual concentrations, AUC and log P values obtained for each molecule assayed were also compared by ANOVA.

Results

Both the sheep liver and *F. hepatica* microsomal fractions were capable of oxidizing TCBZ into its sulpho-metabolites. A negligible sulphoxidation activity was observed in the absence of NADPH, whereas the production of TCBZSO was related to the NADPH concentration added to the incubation mixture. The relationship between the required NADPH concentrations and the rates of TCBZ sulphoxidation in both liver and trematode parasite microsomal fractions is shown in table 1. Also, there was no measurable oxidation activity in control incubations with inactivated (boiled) microsomes (data not shown).

Although both TCBZSO and $TCBZSO_2$ were produced after 30 min incubation of TCBZ with sheep liver microsomes, the rate of production of the sulphoxide metabolite was significantly higher ($p < 0.001$). $TCBZSO_2$ was the major metabolite formed following TCBZSO incubation with sheep liver microsomes. Only trace amounts of OH-TCBZSO were recovered in sheep liver microsomal incubations after incubations with TCBZ and TCBZSO. The rates (nmol min^{-1} mg^{-1}) of OH-TCBZSO production by sheep liver microsomes ranged between 0.006–0.009 (TCBZ incubations) and 0.01–0.02 (TCBZSO incubations).

The rate of TCBZ sulphoxidation by sheep liver microsomes was 0.38 ± 0.06 nmol TCBZSO formed per min^{-1} mg^{-1} microsomal protein. Sheep liver microsomes were also able to metabolize TCBZSO into the sulphone metabolite ($TCBZSO_2$) at a rate of 0.12 ± 0.01 nmol min^{-1} mg^{-1}. Thus, the rate of TCBZ sulphoxidation was significantly higher ($p < 0.01$) compared with that observed for the sulphonation of TCBZSO. On the other hand, the mean rate of TCBZ conversion into $TCBZSO_2$ by sheep liver microsomes was 0.028 ± 0.005 nmol min^{-1} mg^{-1}. Inhibition of FMO in the presence of MTZ reduced TCBZSO production (62%, $p < 0.01$) following TCBZ incubation with sheep liver microsomes (figure 2). Similarly, inhibition of the cytochrome P450 system by PB decreased (51%, $p < 0.05$) the sulphoxidation of TCBZ to TCBZSO. Formation of the sulphone metabolite was not observed when either the FMO or cytochrome P450 were inhibited (data not shown). Inhibition of liver microsomal FMO also inhibited (47%, $p < 0.001$) the sulphonation of TCBZSO (figure 3). Similarly, a significant ($p < 0.001$) reduction (69%) in the rate of $TCBZSO_2$ production was observed after TCBZSO incubation in the presence of PB, a cytochrome P450 inhibitor.

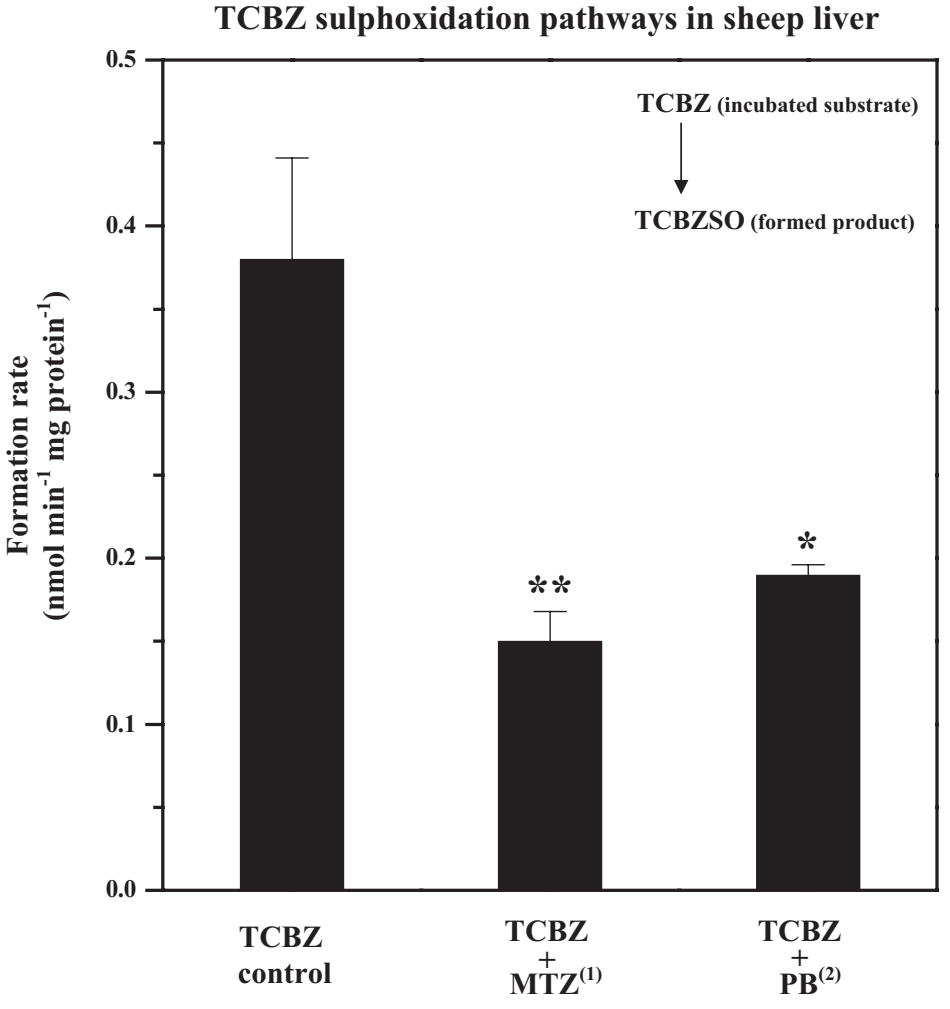

Figure 2. *In vitro* biotransformation of triclabendazole (TCBZ) by sheep liver microsomes. Effects of FMO inhibition (methimazole, MTZ) and piperonyl butoxide (PB)-mediated inhibition of the cytochrome P450 system on the sulphoxidation of TCBZ. Data (nmol min^{-1} mg^{-1} microsomal protein) are the mean (± SD) of six determinations. Values are significantly different from control incubations at $*p < 0.05$ and $**p < 0.01$. (1)Inactivation of the FMO system was carried out by heat pre-treatment (2 min at 50°C) of the microsomal preparation followed by pre-incubation with MTZ (100 µM) (see the Materials and methods). The initial TCBZ concentration was 30 µM. (2)Inhibition of cytochrome P450 was carried out with 100 µM PB and the initial TCBZ concentration was 30 µM.

Representative chromatograms of TCBZ and TCBZSO analysis by HPLC obtained after incubation of TCBZ with *F. hepatica* microsomes are shown in figure 4. There was not a measurable conversion of TCBZ to TCBZSO in the inactivated (boiled) parasite microsomal sample (figure 4a). Conversely, TCBZ was oxidized to TCBZSO after 60 min incubation in presence of NADPH (figure 4b). The rate of TCBZ sulphoxidation by the parasite microsomal fraction was 0.09 ± 0.009 nmol TCBZSO formed min^{-1} mg^{-1} microsomal protein. Only trace amounts of $TCBZSO_2$ were recovered in some parasite microsomal incubations.

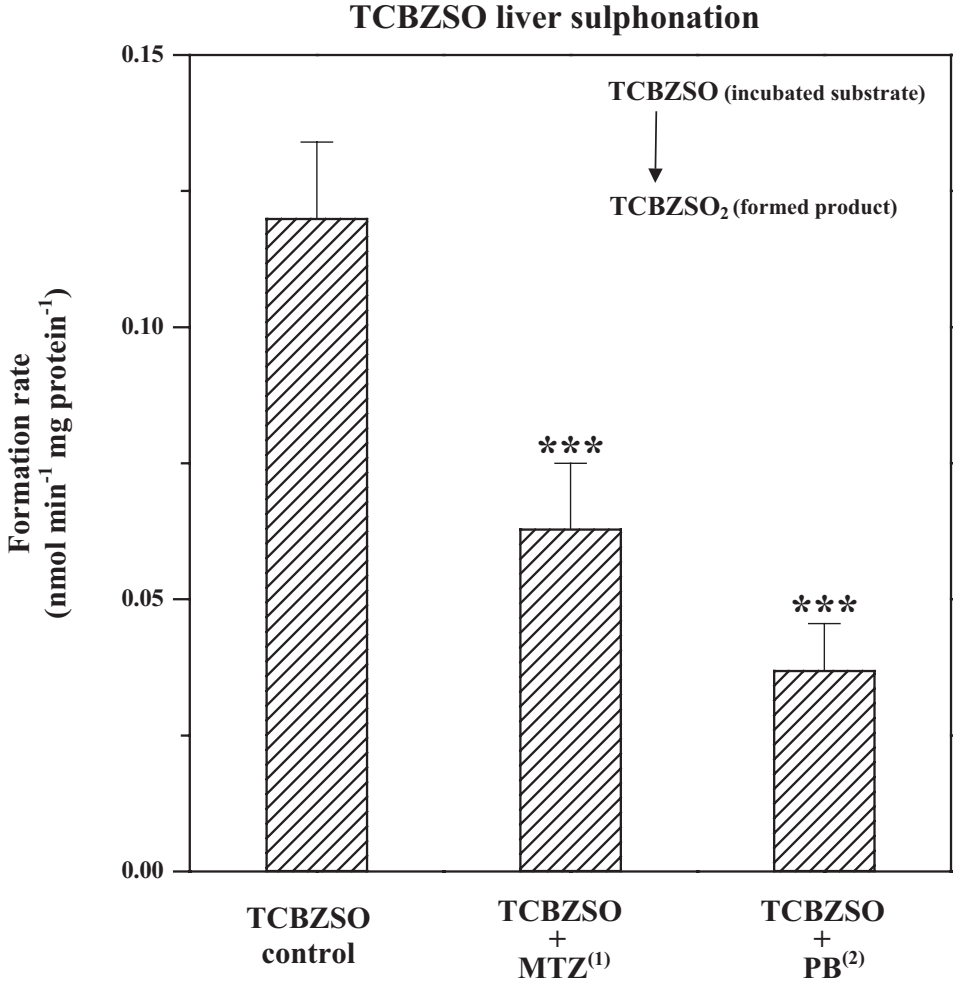

Figure 3. *In vitro* metabolism of triclabendazole sulphoxide (TCBZSO) by sheep liver microsomes. Effects of FMO inhibition (methimazole, MTZ) and piperonyl butoxide (PB)-mediated inhibition of the cytochrome P450 system on the sulphonation of TCBZSO. Data (nmol min^{-1} mg^{-1} microsomal protein) are the mean (± SD) of six determinations. Values are significantly different from control incubations at ***$p < 0.001$. (1)Inactivation of the FMO system was carried out by heat pre-treatment (2 min at 50°C) of the microsomal preparation followed by pre-incubation with MTZ (100 μM) (see the Materials and methods). The initial triclabendazole (TCBZ) concentration was 30 μM. (2)Inhibition of the cytochrome P450 was carried out with 100 μM PB and the initial TCBZ concentration was 30 μM.

TCBZ and all its metabolic products were recovered from *F. hepatica* after their *ex vivo* incubation as early as 15 min post-incubation, demonstrating a fast diffusion of TCBZ-related molecules from the medium to the trematode parasite tissues. The concentrations (mean ± SD) of TCBZ, TCBZSO, $TCBZSO_2$, OH-TCBZ, OH-TCBZSO and $OH\text{-}TCBZSO_2$ measured in *F. hepatica* at different times post-incubation in a KRT medium are shown in table 2. The amount of TCBZ and TCBZSO recovered over time did not significantly increase throughout the incubation period. Only slight differences on the concentration of $TCBZSO_2$, OH-TCBZ, OH-TCBZSO and $OH\text{-}TCBZSO_2$

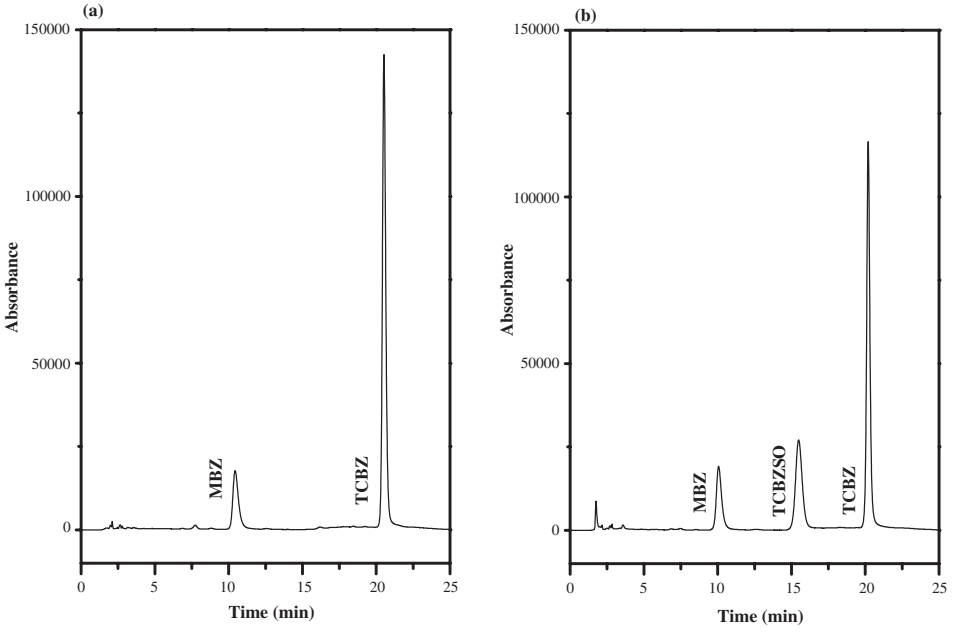

Figure 4. Oxidative metabolism of triclabendazole (TCBZ) in *Fasciola hepatica*. Chromatograms obtained from the HPLC analysis of the *F. hepatica* microsomal fraction incubated with TCBZ over 60 min: (a) inactivated (boiled) microsomes and (b) microsomes incubated in the presence of NADPH (0.5 µmol), where the formation of TCBZ sulphoxide (TCBZSO) was observed. Mebendazole (MBZ) was used as the internal standard.

Table 2. Mean concentrations (nmol 100 mg protein^{-1}) ($n = 4$, ± SD) of triclabendazole (TCBZ) and metabolites measured in *Fasciola hepatica* at different incubation times under *ex vivo* conditions. The area under the concentration versus time curve (AUC) (nmol min 100 mg protein^{-1}) ($n = 4$, mean ± SD) shown for each drug molecule is the total availability measured within the parasite after 180 min of incubation.

Incubation time (min)	TCBZ	TCBZSO	$TCBZSO_2$	OH-TCBZ	OH-TCBZSO	$OH\text{-}TCBZSO_2$
15	5.9 ± 1.5^{a}	4.0 ± 1.2^{a}	4.7 ± 0.6^{a}	2.8 ± 1.9^{a}	3.0 ± 0.5^{a}	2.6 ± 1.0^{a}
45	9.1 ± 2.2^{a}	6.5 ± 1.6^{a}	7.0 ± 1.1^{ab}	6.5 ± 3.1^{ab}	7.3 ± 1.1^{b}	5.7 ± 2.2^{ac}
90	12.8 ± 4.9^{a}	11.6 ± 3.9^{a}	13.2 ± 4.0^{bc}	9.9 ± 3.5^{b}	8.6 ± 0.8^{b}	8.2 ± 0.7^{bc}
120	8.8 ± 4.6^{a}	11.9 ± 5.6^{a}	12.6 ± 2.7^{bc}	9.3 ± 1.5^{ab}	5.0 ± 1.9^{ab}	9.9 ± 3.6^{bc}
180	10.5 ± 5.7^{a}	13.4 ± 8.1^{a}	14.0 ± 3.4^{c}	3.4 ± 2.6^{a}	6.0 ± 2.3^{ab}	6.7 ± 1.9^{ac}
AUC	1665 ± 228^{a}	1708 ± 106^{a}	1852 ± 256^{a}	1198 ± 106^{b}	1100 ± 90^{b}	1222 ± 178^{b}

[a–c]Means not having a common superscript within a column are significantly different at $p < 0.05$, except for the AUCs, were the statistical differences are within the row.

were observed between different incubation times. Drug diffusion into the parasite occurred as a slowly accumulative process up to 90 min of incubation, when drug concentrations measured in the parasite became statistically higher compared with those measured during the first 15 min of incubation. After 90 min of incubation, the concentration of $TCBZSO_2$ measured inside the fluke remained stable (table 2). Conversely, the concentration of the hydroxy-metabolites recovered inside the parasite tended to decrease. The concentration values measured in the parasite for the different drug molecules at each incubation time did not show

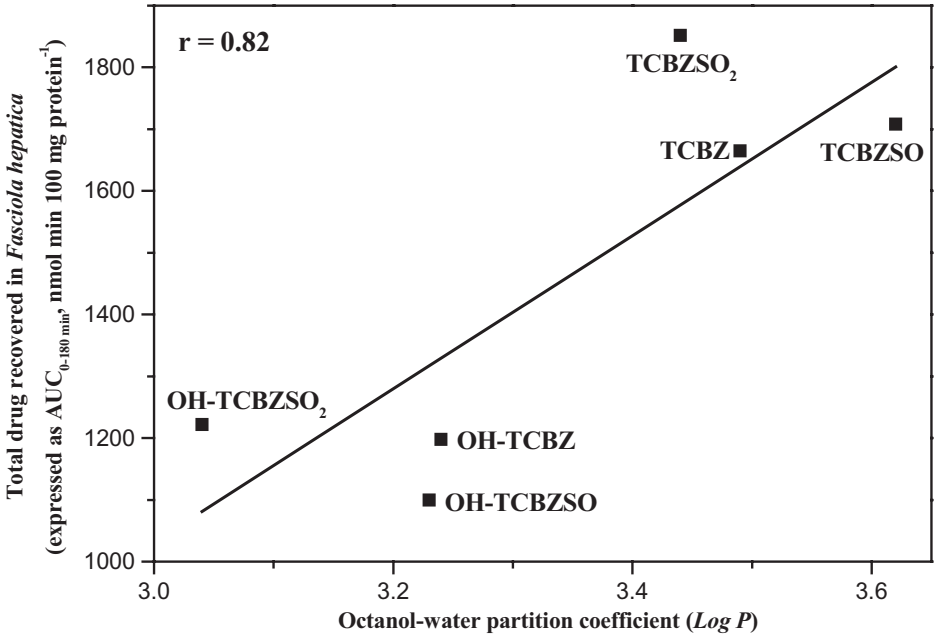

Figure 5. Correlation between total drug recovered in *Fasciola hepatica* ($n = 4$), expressed as area under the concentration versus time curves (AUC) after 180 min of incubation, and the octanol–water partition coefficients (log *P*) estimated for triclabendazole (TCBZ), triclabendazole sulphoxide (TCBZSO), triclabendazole sulphone ($TCBZSO_2$), hydroxy-TCBZ (OH-TCBZ), hydroxy-TCBZSO (OH-TCBZSO) and hydroxy-$TCBZSO_2$ (OH-$TCBZSO_2$).

statistical significance. However, the level of TCBZ measured after 15 min of incubation was significantly higher than that observed for OH-$TCBZSO_2$.

The results obtained from the transtegumental diffusion kinetic studies were correlated with the lipid-to-water PC of the anthelmintic drugs assayed. The PC values obtained were 3.62 (TCBZSO), 3.49 (TCBZ), 3.42 ($TCBZSO_2$), 3.24 (OH-TCBZ), 3.23 (OH-TCBZSO) and 3.03 (OH-$TCBZSO_2$). The correlation between drug diffusion over 180 min of incubation (measured as total drug amount recovered in the liver flukes expressed as AUCs) and drug lipophilicity (measured as octanol–water PC) is shown in figure 5. There was a high correlation ($r = 0.82$) between drug diffusion and drug lipophilicity. Despite the TCBZ, TCBZSO and $TCBZSO_2$ log *P* values tending to be higher than those obtained for the hydroxy-derivatives, these differences did not reach statistical significance.

In spite of the lack of statistical significance between the log *P* values (PC), there was a clear tendency showing an enhanced concentration profile within the parasite for the most lipophilic compounds (TCBZ, TCBZSO, $TCBZSO_2$). Significantly lower $AUC_{0-180\,min}$ values were obtained for the hydroxy-derivatives, OH-TCBZ (1198 nmol min 100 mg protein^{-1}), OH-TCBZSO (1100 nmol min 100 mg protein^{-1}), OH-$TCBZSO_2$ (1222 nmol min 100 mg protein^{-1}), compared with those observed for TCBZ (1665 nmol min 100 mg protein^{-1}), TCBZSO (1708 nmol min 100 mg protein^{-1}) and $TCBZSO_2$ (1852 nmol min 100 mg protein^{-1}). There were no statistically significant differences between the total amount of TCBZ, TCBZSO and $TCBZSO_2$ recovered in

the fluke parasite. Similar concentrations of OH-TCBZ, OH-TCBZSO and OH-TCBZSO$_2$ were recovered in the trematode parasite.

Discussion

The benzimidazole anthelmintics require extensive hepatic oxidative metabolism to achieve sufficient polarity for excretion (Hennessy *et al*. 1993). Despite the plasma pharmacokinetic profile of TCBZ having been described in many species, including cattle, sheep, goats, horses, ponies, donkeys, pigs and humans (Hennessy *et al*. 1987, Bogan *et al*. 1988) the involvement of the cytochrome P450 and FMO systems on the liver biotransformation of TCBZ is still unknown. The implication of these enzymatic systems on the sulphoxidation of albendazole in sheep (Galtier *et al*. 1986), pigs (Souhaili El Amri *et al*. 1987), cattle (Lanusse *et al*. 1993), rats (Moroni *et al*. 1995) and humans (Rawden *et al*. 2000) has been reported. It has been demonstrated that FMO is primarily involved in albendazole hepatic sulphoxidation in sheep (Galtier *et al*. 1986, Lanusse *et al*. 1993) and cattle (Lanusse *et al*. 1993). Recent work from our group reported that FMO-mediated sulphoxidation accounted for 60% of the albendazole sulphoxide production, whilst cytochrome P450 contributed 40% of the albendazole sulphoxide formation in both sheep and cattle liver microsomes (Virkel *et al*. 2004). Similarly, FMO was estimated to be the main enzymatic system involved of total fenbendazole liver sulphoxidation in both species (about 80%). The involvement of both enzyme systems in TCBZ sulphoxidation and TCBZSO sulphonation was shown in the current work after FMO inactivation and/or pre-incubation of microsomes with PB. FMO was inactivated by heat pre-treatment of the microsomal preparation followed by MTZ pre-incubation (Dixit and Roche 1984). Heat treatment inactivates the FMO system without affecting CYP-mediated metabolism (McManus *et al*. 1987), although some loss of activity of CYP2A6 and CYP2C9 isoenzymes has been reported (Grothusen *et al*. 1996). On the other hand, equivalent albendazole sulphoxidation rates were observed in heat-treated compared with MTZ-treated liver microsomes obtained from humans (Rawden *et al*. 2000), sheep and cattle (Virkel *et al*. 2004). Thus, heat treatment of the microsomal preparation followed by MTZ pre-incubation seem to be an adequate strategy to assess the relative contribution of FMO and cytochrome P450 to TCBZ and TCBZSO hepatic oxidation. The relative involvement of both enzymatic systems may be estimated following the assumption that inactivation of FMO leaves the cytochrome P450 system able to metabolize TCBZ or TCBZSO. Thus, the FMO-mediated sulphoxidation accounted for about 62% of TCBZSO and about 48% of TCBZSO$_2$ formation, whilst cytochrome P450 contributed 38% (TCBZSO) and 52% (TCBZSO$_2$) of the oxidized metabolites. Additionally, the greater ability of the sheep liver for TCBZ sulphoxidation rather than TCBZSO sulphonation was demonstrated in the current work. Despite this, the liver capacity to produce the sulphone metabolite from TCBZ parent drug appeared to be greater than that shown for albendazole in a previous work carried out under similar experimental conditions (Virkel *et al*. 2004).

Helminth parasites have an apparent lack of cytochrome P450 as a detoxification route and oxidation pathways in *Ascaris lumbricoides* (nematode) and *Moniezia expansa* (cestode) occur exclusively by the FMO system (Precious and Barret 1989), which requires NADH or NADPH as cofactors (Douch and Buchanan

1979). It has been previously shown that *F. hepatica* has significantly higher oxidative activity than nematode and cestode parasites. Adult liver flukes have the ability to oxidize albendazole to albendazole sulphoxide and albendazole sulphone (Solana *et al.* 2001). On the other hand, the sulphone metabolite has been identified in *F. hepatica* incubated with TCBZSO over 3–12 h (Robinson *et al.* 2004). The work reported here permitted the characterization of the metabolism of TCBZ within the target parasite. The fluke microsomal fraction was able to generate TCBZSO (main product) and $TCBZSO_2$ from TCBZ parent drug. TCBZSO production by the microsomal fraction of *F. hepatica* was about 25% that measured for liver microsomes when the concentration of NADPH was optimal (1 μmol ml^{-1}) (table 1). Although further studies should be conducted to elucidate the metabolic pathways (i.e. enzymatic systems) involved in the oxidation of TCBZ in *F. hepatica*, this set of preliminary results on parasite metabolism are pharmacologically relevant.

F. hepatica infects a variety of hosts such as sheep, cattle, goats, deer, buffaloes, horses, camelids, pigs and humans (Coles 1986). Adult flukes are located in the common bile ducts and the gall-bladder. A large number of experiments have shown that different chemical substances, including anthelmintic drugs, are mainly taken up from the site of parasite location through the external surface, as opposed to oral ingestion. This has been shown in *Haemonchus contortus* (Rothwell and Sangster 1997, Alvarez *et al.* 2000), *Ascaris suum* (Ho *et al.* 1990, Alvarez *et al.* 2001), *Moniezia* spp. (Alvarez *et al.* 1999, Mottier *et al.* 2003), *F. hepatica* (Fetterer and Rew 1984, Alvarez *et al.* 2000, 2001) and *Onchocerca ochengi* (Cross *et al.* 1998) among other helminth parasites. It has been shown that under *in vitro* conditions (Bennett and Köhler 1987), TCBZ can penetrate into liver flukes even when the oral route had been closed off by ligation, which demonstrates the relevance of drug entry through the external surface even in an hematophageous parasite. On the other hand, the large quantities of free (pharmacologically active) TCBZ-related molecules present in bile certainly provide substantial chemical contact with liver-dwelling *F. hepatica* (Hennessy *et al.* 1987).

In contrast to the patterns of diffusion observed for albendazole and its sulphoxide metabolite (albendazole sulphoxide) into *F. hepatica* (Alvarez *et al.* 2001), the amount of TCBZ and its sulphoxide derivative (TCBZSO) recovered over time did not increase during the incubation period. It could be that 180 min of incubation time was not long enough, since more than 180 min were necessary to demonstrate TCBZ accumulation in flukes under *in vitro* conditions (Bennett and Köhler 1987). Considering the experimental evidence shown here, where the ability of the fluke to oxidize TCBZ into its sulphoxide and sulphone metabolites was demonstrated, it could be hypothesized that the lack of an accumulative process may be due to the capability of the liver fluke to metabolize the drug. Such a finding may agree with the fact that concentrations of the hydroxy-derivatives measured inside the liver flukes decreased after 90 min of incubation. However, the parent drug added to the incubation medium was the predominant analyte recovered within *F. hepatica*. The capability of the parasite to biotransform TCBZ observed in the metabolism assays, was not evident in the drug diffusion assays. It is likely that TCBZ concentrations higher than that used in the current drug diffusion assays (5 nmol ml^{-1}) are necessary to chromatographically detect TCBZ metabolic products. In fact, the presence of $TCBZSO_2$ in flukes incubated with TCBZSO concentrations as high as 140 nmol ml^{-1} has recently been reported

(Robinson *et al*. 2004). Alternatively, other TCBZ-related end products than those identified until now could have been generated by the liver flukes without being detected under the current analytical conditions.

When the transtegumental diffusion of all the molecules assayed was compared at each incubation time, the access of the hydroxylated derivatives to the parasite tended to be lower than that of TCBZ, TCBZSO and $TCBZSO_2$, despite the difference did not reach statistical significance. However, estimation of the total drug amount recovered from the parasite as AUC demonstrated that TCBZ parent drug and its sulpho- metabolites (TCBZSO and $TCBZSO_2$) have a significantly higher ability to penetrate into *F. hepatica* compared with the hydroxylated metabolites. The accumulated data show that the main route of acquisition of broad-spectrum anthelmintics by target parasites appears to be by passive diffusion through their external surface. The rate of penetration of a drug will mainly depend on the intrinsic lipid-to-water partition coefficient of the molecule (Mottier *et al*. 2003), pH/p*K* relationship, molecular size, concentration gradient and the surface area of contact between drug and parasite. Lipid solubility is a major factor determining drug penetration across the nematode cuticle (Alvarez *et al*. 2000, 2001) as well as through the tegument of cestodes (Alvarez *et al*. 1999, Mottier *et al*. 2003) and trematodes (Fetterer and Rew 1984, Alvarez *et al*. 2000, 2001). This correlates with the ability of the drug to reach therapeutically relevant concentrations within the target parasite. TCBZ, TCBZSO and $TCBZSO_2$ are more lipophilic (higher octanol–water PC) and, therefore more soluble in the lipoidal surface membranes of the helminth parasites, than are their respective polar hydroxy-metabolites (lower octanol–water PC). Such a phenomenon accounted for the greater availability of the non-hydroxylated molecules inside the incubated trematode parasite. This was confirmed by the high correlation observed between transtegumental diffusion of TCBZ-related molecules into *F. hepatica* and drug lipophilicity (expressed as log *P*) (figure 5).

In contrast to the differences observed on the diffusion of albendazole and albendazole sulphoxide into *F. hepatica* (Alvarez *et al*. 2001), the results reported here indicated that TCBZ parent drug and its sulpho- metabolites have a similar ability to penetrate through the tegument of the liver flukes. This finding may correlate with the octanol–water PC values obtained for these molecules. While albendazole and albendazole sulphoxide log *P* values were markedly different (3.83 versus 1.24, respectively), the PC values obtained for TCBZ, TCBZSO and $TCBZSO_2$ were similar. The high parasite diffusion capacity observed for the sulphoxide and sulphone metabolites may be pivotal for TCBZ action, since the parent compound is short lived, and both TCBZSO and $TCBZSO_2$ are the main unconjugated analytes recovered in the bloodstream and bile of treated animals (Hennessy *et al*. 1987). Consequently, flukicidal activity has been mainly related to TCBZSO systemic availability, although the sulphone metabolite seems to have some flukicidal activity (41% efficacy on 28-day-old liver flukes) (Büscher *et al*. 1999). On the other hand, the sulphoxide metabolite was found to exert a delayed effect on the motility of the trematode parasite (Bennett and Köhler 1987). In conclusion, the pharmacological activity of TCBZ against liver flukes may depend on the irreversible impairment of essential cellular functions induce by an additive effect of these three molecules.

Knowledge of the patterns of drug/metabolites diffusion into different target parasites, together with the available pharmacokinetic/dynamic information may

substantially contribute to elucidate the mechanism of drug penetration and the pharmacological activity of most anthelmintics. Understanding the relationship among TCBZ metabolism, the relative potency of its metabolic products and their ability to reach the target parasite, may be critical to optimize its flukicidal activity, particularly when TCBZ resistant flukes have already been recognized.

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