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Time-course and accumulation of triclabendazole and its metabolites in bile, liver tissues and flukes collected from treated sheep

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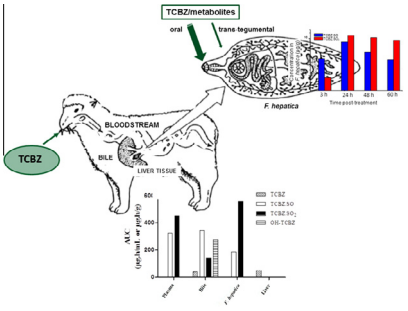
HIGHLIGHTS

- Only the TCBZ sulpho-metabolites were recovered in plasma from TCBZ treated sheep.
- These metabolites were also the main analytes accumulated within adult flukes.
- TCBZ was the main compound accumulated in liver tissue from TCBZ treated sheep.
- The hydroxy-metabolite was recovered in bile from treated sheep.
- Oral ingestion seems to be the main route of drug entry into the flukes.

GRAPHICAL ABSTRACT



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ABSTRACT

The flukicidal compound triclabendazole (TCBZ) has a complex metabolic pattern that includes the systemic presence of its sulphoxide (TCBZ.SO) and sulphone (TCBZ.SO_2) metabolites, usually recovered from the bile of treated animals. The aim of the current work was to evaluate the time-course and pattern of *in vivo* accumulation of TCBZ/metabolites into adult *Fasciola hepatica* specimens recovered from infected sheep. Twelve (12) healthy Corriedale sheep were orally infected with one hundred (100) metacercariae of the TCBZ-susceptible Cullomptom isolate of *F. hepatica*. Sixteen weeks after infection, animals were intraruminally treated with TCBZ (10 mg/kg). At 3, 24, 48 and 60 h post-treatment (pt), animals were sacrificed (n = 3/time period) and samples of blood, bile, liver tissue and adult *F. hepatica* specimens were collected. The concentrations of TCBZ/metabolites were measured by HPLC. TCBZ.SO and TCBZ.SO_2 were the only molecules recovered in the bloodstream, with peak plasma concentrations of 10.8 μg/mL (TCBZ.SO) and 12.6 μg/mL (TCBZ.SO_2). The same metabolites were also the main analytes accumulated within the adult flukes, reaching peak concentrations between 6.35 μg/g (TCBZ.SO) and 13.9 μg/g (TCBZ.SO_2) at 24 h pt, which was coincident with the time when the maximum plasma concentration was attained. Low levels of TCBZ parent drug (0.14 μg/g at 24 h pt) were measured within collected flukes. TCBZ parent drug and its sulpho- and hydroxy-derivatives were recovered in bile collected from treated sheep between 3 and 60 h pt. Although relatively high concentrations of hydroxy-TCBZ (ranging from 0.86 to 10.1 μg/mL) were measured in bile, this metabolite was not recovered within the flukes at any time pt. Finally, TCBZ parent drug was the main compound accumulated in liver tissue over the 60 h pt period. The time-course and drug concentration patterns within the adult liver fluke after TCBZ treatment followed a similar trend to those observed in plasma. Overall, the data reported here confirm that oral ingestion is a main route of drug entry into the trematode *in vivo* exposed to TCBZ/metabolites.

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However, the presence of TCBZ within the adult fluke (despite being absent in the systemic circulation) may be related to some degree of trans-tegumental diffusion from bile or by a direct oral ingestion from portal blood.

1. Introduction

Triclabendazole [6-chloro-5(2-3 dichlorophenoxy)-2-methyl thio-benzimidazole] or TCBZ, an halogenated benzimidazole thiol derivative, shows high efficacy against both the mature and immature stages of *Fasciola hepatica* in sheep and cattle; this is a differential feature compared to other available trematodicidal drugs (Boray et al., 1983). It has been the drug of choice for treating liver fluke infections in livestock for over 20 years and, more recently, has been used successfully to treat human cases of fascioliasis (Fairweather, 2005, 2009). As a consequence of its excellent activity against *F. hepatica*, it has been extensively used and this has led to the selection of TCBZ-resistant populations, which is now a worrying problem in several areas of the world (Fairweather, 2005, 2009, 2011).

After its oral/intraruminal administration to sheep, TCBZ is rapidly metabolized to the sulphoxide (TCBZ.SO) and sulphone ($TCBZ.SO_2$) metabolites (Alvinerie and Galtier, 1986; Mohammed Ali et al., 1986; Hennessy et al., 1987; Ceballos et al., 2010), with plasma peak concentration attained at 18–22 h (TCBZ.SO) and 36–42 h ($TCBZ.SO_2$) post-treatment (pt). Neither TCBZ nor any other metabolites are detected in plasma. Hydroxylation of TCBZ and its two main metabolites also occurs in the liver, but the products are secreted into the bile, mainly in their conjugated form (Hennessy et al., 1987). Maximum levels of the hydroxylated compounds are reached after 8 h (OH-TCBZ), 21 h (OH-TCBZ.SO) and 36 h (OH-$TCBZ.SO_2$) pt (Hennessy et al., 1987). TCBZ.SO and $TCBZ.SO_2$ are the two main (unconjugated) metabolites in both plasma and bile. In plasma, these metabolites bind strongly (>99%) to plasma proteins, mainly albumin (Hennessy et al., 1987), extending the systemic exposure of the drug] in the host. Trans-tegumental diffusion and oral ingestion are the two potential routes available for the entry of drugs into *F. hepatica*. Since adult liver flukes are blood-consuming parasites, plasma protein binding may have an important role in the accumulation of drug into the parasite due to oral ingestion. Therefore, the fluke could be potentially exposed to high drug levels in the bile (surrounding medium). *Ex vivo* studies have demonstrated that TCBZ and all of its metabolic products can diffuse through the tegument of the fluke (Mottier et al., 2004a). Trans-tegumental diffusion appears to be the main route of benzimidazole entry into *F. hepatica*, at least under *ex vivo* conditions (Mottier et al., 2006). Furthermore, the *in vitro* uptake of TCBZ occurs even when the oral route has been closed by ligation (Bennett and Köhler, 1987; Mottier et al., 2006), which suggests that diffusion could play a role in drug uptake *in vivo*. However, it is likely that the drug-parasite interaction could differ *in vivo* because parasites are exposed to a changing drug concentration profile over time in a variable physiological environment. Such a variable environment may be consistent with the different pattern of TCBZ metabolites reported in plasma and bile *in vivo*. Consequently, the contribution of oral ingestion and/or diffusion through the external surface as potential mechanisms of the entry of TCBZ/metabolites into *F. hepatica* needs to be further clarified. The aim of the current work was to evaluate the *in vivo* time-course and patterns of TCBZ/metabolites in adult *F. hepatica* specimens recovered from infected sheep over a 60-h time period.

2. Materials and methods

2.1. Chemicals

Pure reference standards (97–99%) of TCBZ, its sulphoxide (TCBZ.SO) and sulphone ($TCBZ.SO_2$) metabolites, and the hydroxylated compounds (OH-TCBZ, OH-TCBZ.SO, OH-$TCBZ.SO_2$) were kindly provided by Novartis Animal Health (Basel, Switzerland). The different solvents (HPLC grade) and buffer salt used for sample extraction or the chromatographic method were purchased from Baker Ind. (Phillipsburg, USA).

2.2. Animals and experimental design

Twelve (12) healthy male Corriedale sheep (45.8 ± 7.3 kg), aged 14–16 months, were involved in this trial. Animals were housed during the experiment and for 15 days before the start of the study. Animals were fed on a commercial balanced diet. Water was provided *ad libitum*. Animal procedures and management protocols were carried out in accordance with the Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar) and with internationally accepted animal welfare guidelines (AVMA, 2001).

Animals were each orally infected with one hundred (100) metacercariae of a TCBZ-susceptible *F. hepatica* isolate, named Cullompton. For details of the history of the Cullompton isolate, see Fairweather (2011). Sixteen weeks after infection, sheep were treated with TCBZ (Fasinex® 5%, Novartis) at 10 mg/kg by the intraruminal (i.r.) route. Three animals were killed at each of 4 time-points after treatment (3, 24, 48 and 60 h) and blood, bile, liver and liver fluke samples were collected. Blood was collected by jugular venepuncture into heparinized tubes and immediately centrifuged at 3000*g* for 15 min to obtain the plasma. After dissection of the animal, samples of liver and bile (from the gall-bladder) were taken. To recover *F. hepatica* adult specimens, the liver, common bile ducts and the gall-bladder of each sheep were removed and opened. The fluke specimens were rinsed extensively with saline solution (NaCl 0.9% w/v) to remove bile and/or adhering materials. All obtained samples were placed into plastic tubes and frozen at −20 °C until analysis by high performance liquid chromatography (HPLC).

2.3. Analytical procedures

2.3.1. Extraction of drug from plasma samples

TCBZ and its metabolites were extracted from plasma as previously described (Virkel et al., 2006). Samples (1 mL) were spiked with 10 μL of oxibendazole (OBZ) (100 μg/mL), which was used as an internal standard (IS). After addition of 2 mL of acetonitrile, samples were shaken for 20 min (multivortex) and then centrifuged at 2500*g* for 15 min. The supernatants were recovered and evaporated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, USA). The dry extracts were reconstituted in 300 μL of mobile phase and an aliquot of 50 μL was injected into the HPLC system.

2.3.2. Extraction of drug from bile samples

TCBZ and its metabolites were extracted from bile samples (0.5 mL). After spiking with 20 μL of the IS (OBZ: 50 μg/mL), the sample was divided into two aliquots for extraction. Aliquots were added to 1.5 mL of acetonitrile and, after shaking for 10 min (multivortex), they were centrifuged at 4000*g* for 10 min (4 °C). The supernatants were recovered and the process repeated. For cleaning, the total supernatant was added to 1.5 mL hexane, shaken, recovered and the process repeated. The total supernatant was evaporated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, USA). The dry extracts were reconstituted in 300 μL of mobile phase and an aliquot of 50 μL was injected into the HPLC system.

2.3.3. Extraction of drug from parasites: F. hepatica

Material (0.1 g) was spiked with 10 μL of an IS (mebendazole: 10 μg/mL). After 10 min, the parasite material was homogenised (15 s, at 4 °C) (Ultraturrax®, T 25, Ika Works Inc., Labortechnik, Wilmington, NC, USA) in preparation for extraction for TCBZ and its metabolites, following the methodology described previously (Mottier et al., 2004a). The liver fluke homogenate was mixed with 1.5 ml of acetonitrile, shaken (multi-tube vortexer, VWR Scientific Products, West Chester, PA, USA) for 5 min and then centrifuged (Jouan®, BR 4i Centrifuge, Saint Herblain, France) to allow phase separation (2000*g* 10 min, 10 °C). This procedure was repeated three times. The final collected acetonitrile phase (4.5 mL) was concentrated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, CA, USA) and then reconstituted with 150 μL of mobile phase. Fifty μL of each solution were injected into the chromatographic system.

2.3.4. Extraction of drug from liver samples

Liver samples were thinly minced and an aliquot of 0.5 g placed into a 5 mL plastic tube. After spiking with 20 μL of the IS (OBZ: 50 μg/mL), samples were added to 0.5 mL of NaOH (1 N). Molecules were extracted by addition of 1.5 mL acetonitrile for 5 min under a high-speed vortexing shaker (Multi-tube Vortexer, VWR Scientific Products, West Chester, PA, US). After mixing, the sample was sonicated (Ultrasound Bath, Lab-Line Instrument, Inc., Melrose Park, OL, US) and centrifuged (BR 4i Centrifuge, Jouan®, Saint Herblain, France) at 2000*g* for 10 min at 4 °C. The clear supernatant was transferred to a tube, and the procedure repeated. The collected supernatant was concentrated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, CA, USA) and then reconstituted with 300 μL of mobile phase. Fifty μL of the reconstituted volume was injected into the chromatographic system.

2.3.5. Drug quantification by HPLC analysis and validation

Experimental and fortified samples of each matrix (plasma, bile, *F. hepatica* and liver) were analysed by HPLC to determine the concentration of TCBZ and its metabolites. Fifty μL of each previously extracted sample were injected into a Shimadzu 10 A HPLC System (Kyoto, Japan), using a gradient pump, a UV detector set at 300 nm, an autosampler and a controller (Shimadzu Class LC10, Kyoto, Japan). Analytes were identified by the retention times of pure reference standards. Chromatographic retention times were: 8.04 min (OBZ), 11.28 min (OH-TCBZ.SO), 15.30 min (OH-TCBZ.SO_2), 17.95 min (OH-TCBZ), 19.74 min (TCBZ.SO), 20.97 min (TCBZ.SO_2), and 25.67 min (TCBZ). Calibration curves for each analyte in each matrix were prepared by least squares linear regression analysis, which showed good correlation coefficients between 0.995 and 0.998. The absolute recovery of drug analytes from each matrix was calculated by comparison of the peak areas from spiked plasma samples with the peak areas resulting from direct injections of standards in the mobile phase. Mean absolute recoveries within the concentration range 0.1–15 μg/mL (triplicate determinations) were >70% in all cases. The precision (intra- and inter-assay) determined by analysing replicates of fortified matrix samples ($n = 6$) with each compound at three different concentrations had CV (variation coefficient) values <20%. The limit of detection (LOD) was estimated by integrating the baseline threshold at the retention time of each compound for six non-spiked matrix samples. The LOD was defined as the mean 'noise'/internal standard peak area ratio plus 3 standard deviations (SD). The limit of quantification (LOQ) was defined as the lowest measured concentration with a CV < 20% and accuracy of ±20% and an absolute recovery ⩾70%. The LOQ obtained for all molecules assayed was 0.05 μg/mL. Values below LOQ were not included in the pharmacokinetic analysis.

2.4. Analysis of the data

Concentrations of TCBZ/metabolite in plasma, bile, liver and *F. hepatica* are expressed as mean ± standard deviations (SD). The area under the concentration–time curve (AUC) for TCBZ/metabolites in each analysed tissue/fluid with at least four concentration mean determinations, was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982), using the PKSolutions™ computer program (Summit Research Service, Ashland, USA). The AUC value was considered to be an indicator of the total drug availability in each biological matrix assayed. Correlations between individual plasma and *F. hepatica* concentrations for both TCBZ.SO and TCBZ.SO_2 were performed by non-parametric analysis (Spearman r), using the Instat 3.0 Software (Graph Pad Software, San Diego, CA, USA).

3. Results

The mean concentrations of TCBZ and its metabolites measured in plasma, bile, *F. hepatica* and liver (μg/mL or μg/g) after TCBZ administration by the i.r. route to sheep are shown in Table 1. TCBZ.SO and TCBZ.SO_2 were the only molecules recovered in the bloodstream, with peak plasma concentrations of 10.8 ± 2.27 μg/mL (TCBZ.SO) and 12.6 ± 2.45 μg/mL (TCBZ.SO_2). The same metabolites were also the main analytes accumulated within the adult flukes, reaching peak concentrations of 6.35 ± 0.46 μg/g (TCBZ.SO) and 13.9 ± 2.53 μg/g (TCBZ.SO_2) at 24 h pt, which was coincident with the time when the maximum plasma concentration was attained. Plasma and fluke concentrations of TCBZ.SO and TCBZ.SO_2

Table 1
Triclabendazole (TCBZ), triclabendazole sulphoxide (TCBZ.SO), triclabendazole sulphone (TCBZ.SO_2) and hydroxy triclabendazole (OH-TCBZ) concentrations (μg/mL or μg/g, mean ± SD) measured in plasma, bile, *Fasciola hepatica* and liver tissue after the intraruminal administration of TCBZ (10 mg/kg) to *F. hepatica* infected sheep.

MATRIX	Analyte	Time post-treatment (h)			
		3	24	48	60
PLASMA	TCBZ	ND	ND	ND	ND
	TCBZ.SO	2.08 ± 2.16	10.80 ± 2.27	2.91 ± 0.12	1.50 ± 0.69
	TCBZ.SO_2	0.26 ± 0.29	12.60 ± 2.45	7.09 ± 1.03	6.40 ± 0.54
	OH-TCBZ	ND	ND	ND	ND
BILE	TCBZ	0.71 ± 0.86	1.43 ± 0.37	0.07 ± 0.09	0.16 ± 0.23
	TCBZ.SO	0.09 ± 0.15	15.20 ± 20.7	0.15 ± 0.21	0.16 ± 0.20
	TCBZ.SO_2	0.03 ± 0.05	4.24 ± 3.60	2.30 ± 0.44	0.88 ± 0.88
	OH-TCBZ	0.91 ± 1.01	10.10 ± 1.25	1.25 ± 0.12	0.86 ± 0.65
F. hepatica	TCBZ	ND	0.14 ± 0.13	ND	ND
	TCBZ.SO	0.80 ± 0.66	6.35 ± 0.46	1.73 ± 0.15	0.71 ± 0.20
	TCBZ.SO_2	0.03 ± 0.04	13.90 ± 2.53	11.10 ± 0.14	8.09 ± 0.02
	OH-TCBZ	0.03 ± 0.06	ND	ND	ND
LIVER	TCBZ	0.38 ± 0.44	1.78 ± 0.33	0.31 ± 0.08	0.11 ± 0.15
	TCBZ.SO	ND	0.41 ± 0.15	ND	ND
	TCBZ.SO_2	ND	ND	ND	ND
	OH-TCBZ	ND	0.45 ± 0.14	ND	ND

ND: not detected.

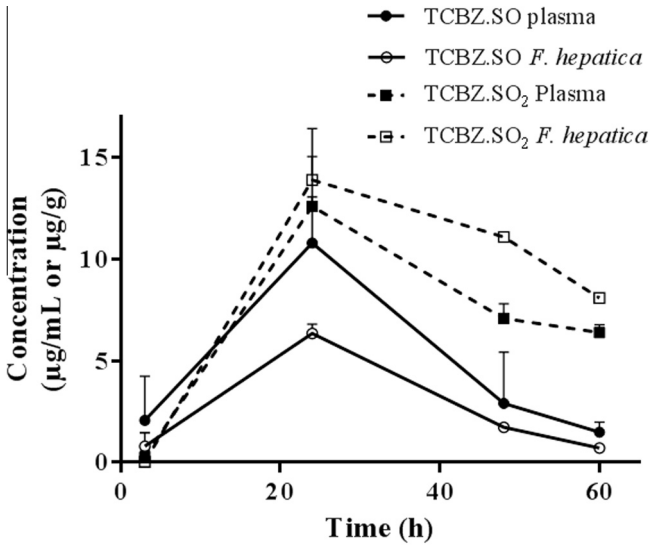

Fig. 1. Plasma and *Fasciola hepatica* concentration profiles (mean ± SD) for triclabendazole sulphoxide (TCBZ.SO) and triclabendazole sulphone ($TCBZ.SO_2$) measured after the intraruminal administration of TCBZ (10 mg/kg) to *F. hepatica* – infected sheep.

obtained following TCBZ administration are shown in Fig. 1. The pattern of drug accumulation in *F. hepatica* matched with the plasma drug exposure of TCBZ.SO and $TCBZ.SO_2$. A significant correlation ($P < 0.05$) between individual plasma and *F. hepatica* concentrations for both TCBZ.SO and $TCBZ.SO_2$ was observed (Fig. 2). Very low TCBZ (0.14 ± 0.13 µg/g) and OH-TCBZ (0.03 ± 0.06 µg/g) concentrations were measured in *F. hepatica* at 24 and 3 h pt, respectively. OH-TCBZ.SO and OH-$TCBZ.SO_2$ were never detected in fluke. Peak levels of TCBZ.SO (15.2 µg/mL), OH-TCBZ (10.6 µg/mL), $TCBZ.SO_2$ (4.24 µg/mL) and TCBZ (1.43 µg/mL) were measured in bile at 24 h pt. The hydroxy metabolites OH-TCBZ.SO and OH-$TCBZ.SO_2$ were not detected in bile at any time pt. The lowest concentrations of TCBZ/metabolites were measured in the liver, the parent drug being the main compound accumulated over the 60 h pt period (Table 1). OH-TCBZ.SO and OH-$TCBZ.SO_2$ were never detected in liver tissue following TCBZ treatment. The AUC values obtained for TCBZ/metabolites in the different biological matrixes assayed is shown in Fig. 3.

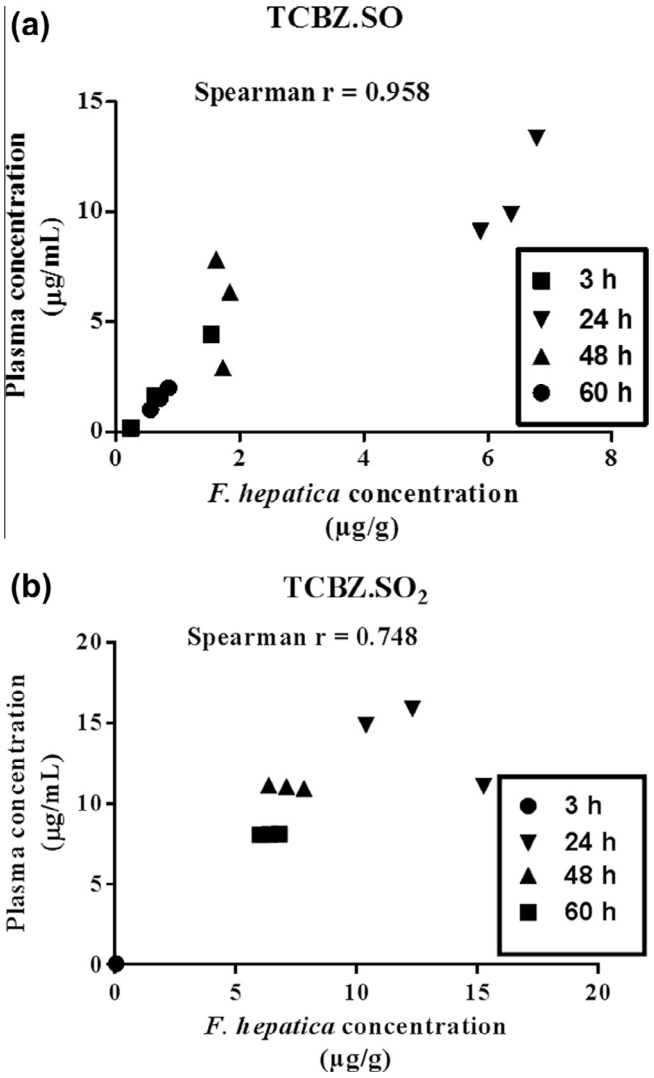

Fig. 2. Spearman correlation coefficient between individual plasma samples and concentrations in *F. hepatica* for both (a) triclabendazole sulphoxide (TCBZ.SO) and (b) triclabendazole sulphone ($TCBZ.SO_2$).

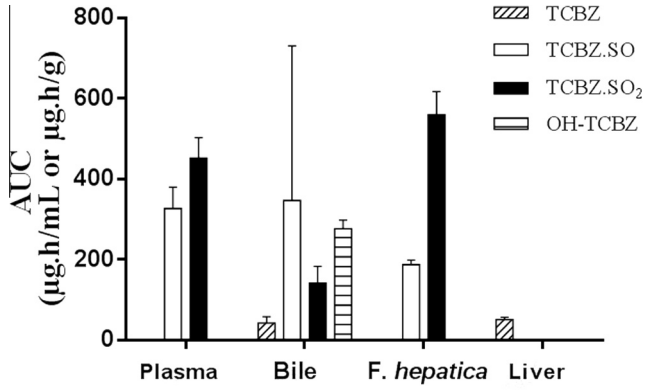

Fig. 3. Mean values of area under the concentration–time curve (AUC) (µg.h/mL or µg.h/g) for triclabendazole (TCBZ), triclabendazole sulphoxide (TCBZ.SO), triclabendazole sulphone ($TCBZ.SO_2$) and hydroxy triclabendazole (OH-TCBZ), measured in plasma, bile, *Fasciola* and liver tissue after the intraruminal administration of TCBZ (10 mg/kg) to *F. hepatica* – infected sheep.

4. Discussion

A knowledge of anthelmintic drug concentrations achieved in target parasites and tissues/fluids surrounding the parasites will contribute to the understanding of the pharmacokinetic-efficacy relationship. Usually, the higher the concentration achieved in the tissue/fluid where the parasite is located, the higher the amount of drug reaching the parasite. This is strongly supported by the findings from different *in vivo* studies (Hennessy et al., 1995; Barrère et al., 2012; Lloberas et al., 2012). The possible routes of drug entry into the fluke are oral ingestion and/or trans-tegumental diffusion. In the present study, the relationship between plasma, bile, liver and *F. hepatica* drug concentrations have been assessed, in order to gain a better understanding of the mechanism involved in the entry of TCBZ/metabolites into the liver fluke in the *in vivo* situation.

Should drug entry in target parasites occur mainly through the external surface, then drug concentrations achieved at the site (tissue/fluid) of parasite location will influence the amount of drug reaching the site of action inside the worm (Alvarez et al., 2007). For instance, if the trans-tegumental pathway is the main route of drug access into mature *F. hepatica* located in the biliary tract, drug concentrations in bile will be more relevant in terms of flukicidal activity compared to those in the bloodstream. After

TCBZ administration, only 9.7% of the dose has been reported to be secreted as free metabolites in bile, whereas 35.8% is secreted as conjugated metabolites (Hennessy et al., 1987). Since flukes do not ingest/absorb conjugated metabolites from bile (Hennessy et al., 1987), only unconjugated TCBZ metabolites were analysed in bile in the current trial. A fast TCBZ absorption, followed by liver metabolism, explains the early appearance of TCBZ, TCBZ.SO, TCBZ.SO_2 and OH-TCBZ in bile at 3 h pt, and all four compounds were detected at all sampling times. Overall, TCBZ.SO was the analyte present at the highest concentration in bile, followed by OH-TCBZ, TCBZ.SO_2 and TCBZ. Adult liver flukes may be exposed to TCBZ/metabolites passing through their external surface from the bile. Lipophilicity is one of the key factors that determines drug diffusion through the external surface of cestode and trematode parasites (Mottier et al., 2006; Alvarez et al., 2007). A high correlation between molecular lipophilicity (expressed as the octanol–water partition coefficient) and the amount of different benzimidazole (BZD) anthelmintics recovered in *F. hepatica* (Mottier et al., 2004b) and the tapeworm, *Moniezia benedeni* (Mottier et al., 2003), has been reported under *ex vivo* conditions.

The concentration gradient is another important factor that determines drug diffusion. As the concentration gradient increases on one side of the membrane, drug concentrations on the other side will increase in line with the concentration gradient. High correlations have been obtained after linear regression analysis between initial BZD concentration in the incubation medium and drug concentration measured inside a selected number of nematode, cestode and trematode parasites (Mottier et al., 2006). Different *ex vivo* studies have been performed on *F. hepatica* to determine the relative contribution of trans-tegumental versus digestive absorption of TCBZ/metabolites (Mottier et al., 2006). Equivalent TCBZ.SO concentrations were recovered from mouth-ligated (i.e. unable to ingest anything orally) and non-ligated adult *F. hepatica* after 45 min of *ex vivo* incubation, confirming TCBZ entry into the flukes even when the oral route had been closed off by ligation (Mottier et al., 2006). This observation was corroborated later when the relative susceptibilities of the tegument and gut to TCBZ action were evaluated in several *in vitro* experiments, the results pointing to trans-tegumental uptake as the major route of entry of TCBZ into the liver fluke (Toner et al., 2009, 2010). In the present work, after TCBZ administration to infected sheep, the high TCBZ.SO, OH-TCBZ, TCBZ.SO_2 and TCBZ concentrations present in bile (Table 1) certainly provides the potential for substantial chemical contact with the liver-dwelling *F. hepatica*. However, only very low concentrations of OH-TCBZ and TCBZ were detected in the liver fluke at 3 and 24 h pt, respectively, even when their concentrations in bile were relatively high (Table 1). It has been demonstrated that TCBZ parent drug and its sulpho-metabolites have a similar ability to penetrate through the tegument of the liver fluke (Mottier et al., 2004b). Furthermore, accumulation of the hydroxylated derivatives by the fluke is only 33% lower than that observed for TCBZ/TCBZ.SO (Mottier et al., 2004b). Consequently, the results obtained indicate that, under *in vivo* conditions, the trans-tegumental diffusion of TCBZ/metabolites is reduced by the presence of bile as a "solubilizing" medium. Bile is a hepatic aqueous secretion composed of biliary acids and pigments, lipids, amino acids and glucose, amongst others. Biliary secretion has different functions, such as providing an excretory route for metabolic detoxification products, including metabolites and drugs; neutralizing the H^+ in the duodenum; and providing a source of bile acids that are necessary for fat digestion and absorption. In the present work, similar to that previously reported under *ex vivo* experiments in which the incubation medium was bile (Alvarez et al., 2004), the presence of amphiphilic bile components where *F. hepatica* is located may have induced the micellar solubilization of TCBZ and its metabolites, reducing the proportion of free drug in solution and thus avoiding/decreasing drug diffusion through the parasite tegument. The low TCBZ and OH-TCBZ concentrations measured in *F. hepatica* in the present study may indicate some degree of drug diffusion from bile and/or some degree of oral ingestion from portal blood, since these metabolites are not present in the systemic circulation.

As reported before (Alvinerie and Galtier, 1986; Mohammed Ali et al., 1986; Hennessy et al., 1987), TCBZ parent drug was not detected in plasma after its i.r. administration, TCBZ.SO and TCBZ.SO_2 being the only metabolites measured in the bloodstream of TCBZ-treated sheep. Since TCBZ.SO and TCBZ.SO_2 bind strongly to plasma proteins, the slow appearance of TCBZ metabolites in plasma could be related to the rate of release of albumin from the liver. Similar to that observed in plasma samples, TCBZ.SO and TCBZ.SO_2 were the main molecules quantified in liver flukes. However, the most interesting result was that the concentration versus time curve for TCBZ metabolites observed in *F. hepatica* copies very well their concentrations in plasma. In fact, a high correlation between plasma and *F. hepatica* concentrations was observed (Fig. 2). This high correlation can only be explained by the oral ingestion of the compounds due to the haematophagous behaviour of *F. hepatica*. Therefore, oral ingestion is confirmed as the main mechanism of uptake of TCBZ/metabolites in liver flukes *in vivo*. Similar results have been reported previously in *in vitro* experiments for the flukicidal drug clorsulon, due to the binding of the drug to the red blood cells (Meaney et al., 2005a,b). Interestingly, the AUC plasma/*F. hepatica* ratios for TCBZ.SO (1.74) and TCBZ.SO_2 (0.81) were substantially different. These results could be related to the capacity of the trematode parasite to oxidise TCBZ.SO into its sulphone metabolite (Robinson et al., 2004). Finally, accumulation of TCBZ in the liver was clearly higher than that observed for its metabolites, as TCBZ.SO and OH-TCBZ were the only metabolites detected at 24 h pt. Since it is unlikely that bile could be ingested by immature flukes migrating through the liver parenchyma, the parent compound appears to be the main molecule implicated in the efficacy against the immature stage of *F. hepatica*.

In line with previous studies (Alvarez et al., 2004), the present work reinforces the idea that the physicochemical features of the environment where the target parasite is located play a pivotal role in the drug accumulation process into the parasite. Overall, the data reported here confirm that oral ingestion is the main route of drug entry into the fluke *in vivo* exposed to TCBZ/metabolites. However, the presence of TCBZ within the adult fluke (despite it being absent in the systemic circulation) may be related to some degree of trans-tegumental diffusion from bile or by direct oral ingestion from portal blood. The absence of OH-TCBZ in liver flukes suggests that the trans-tegumental route of entry for this compound has low significance under *in vivo* conditions. The flukicidal activity of TCBZ could be related mainly to the systemic exposure of both TCBZ.SO and TCBZ.SO_2, since the latter seems to have at least some flukicidal activity (Büscher et al., 1999; Halferty et al., 2009).

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