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A pharmacology-based comparison of the activity of albendazole and flubendazole against *Echinococcus granulosus* metacestode in sheep

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ABSTRACT

Cyst echinococcosis (CE) is a zoonotic disease caused by the larval stage of the *Echinococcus granulosus* helminth parasite. The work reported here aimed to compare the efficacy of albendazole (ABZ) and flubendazole (FLBZ) against CE in naturally infected sheep. Additionally, their comparative pharmacokinetic behaviour and the assessment of serum liver enzymes activities were studied. Twelve (12) naturally infected sheep were allocated to the following experimental groups: unmedicated control group, FLBZ-treated and ABZ-treated.

Treatments were orally performed every 48 h, over 55 days at dose rate of 10 (FLBZ) and 8.5 (ABZ) mg/kg (equimolar dose rates). The efficacy of the drug treatments was based on protoscoleces' vitality/viability. The kinetic disposition assessment included the Initial and Final Kinetic Studies which implicated the collection of blood samples after both the first and the last drug administration. Blood samples were processed to measure drug concentrations by HPLC. The protoscoleces' vitality observed in the untreated control group (98%) was significantly reduced in the presence of both ABZ and FLBZ. 90% of mice inoculated with protoscoleces in the control group developed hydatid cysts in their peritoneal cavity (viability study). However, only 25% (FLBZ) and 33% (ABZ) of mice inoculated with protoscoleces recovered from treated sheep, developed hydatid cysts in their abdominal cavity. Reduced FLBZ (R-FLBZ) was the main metabolite recovered in the bloodstream after oral administration of FLBZ to sheep. Low plasma concentrations of FLBZ parent drug were measured up to 48 h post-administration. ABZ was not detected in plasma at any time post-treatment, being its metabolites ABZ sulphoxide (ABZSO) and ABZ sulphone ($ABZSO_2$) recovered in plasma. Hepatotoxicity due to the continued treatment with either ABZ or FLBZ was not observed. A 3-fold increase ethoxyresorufin O-deethylase activity, a cytochrome P450 1A (CYP1A)-dependent enzyme reaction, was observed in liver microsomes obtained from sheep receiving ABZ, compared to those of the unmedicated and FLBZ-treated animals. In conclusion, FLBZ is an available anthelmintic which may be developed into an effective and safe drug for the human CE treatment. Despite the low plasma concentrations measured by FLBZ/R-FLBZ, an important reduction in protoscoleces' vitality was observed in cysts located in sheep liver. Modern pharmaceutical technology may help to greatly improve FLBZ systemic exposure improving its efficacy against CE.

1. Introduction

Cystic echinococcosis (CE) is a zoonotic disease caused by the larval stage of *Echinococcus granulosus*. The intermediate hosts include sheep, goats, cattle, horses, pigs, and accidentally, humans. The larval stage frequently infects the liver or lungs (Dueger et al., 1999) and the cysts will expand and contain hundreds or thousands of protoscoleces (PSCs). These PSCs can develop into the adult tapeworm upon ingestion by a definitive host (dogs, wolves, or other wild canids) (Pawlowski et al., 2001; Thompson and McManus, 2001). CE is one of the several parasitic diseases of difficult chemical control, where chemotherapy with currently used drugs is highly variable. This disease is included by the WHO in the list of the neglected tropical diseases, in which the use of

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integrated approaches to cure, prevent and control the disease at the human–animal interface is needed in order to be successful in its prevention and control (WHO, 2012).

Anti-echinoccocal drugs can be employed in patients who are not good candidates for surgery, *e.g.*, due to age, co-morbidities or when the cyst is positioned in such a manner as it compromises resection. Currently, the pharmacological target for the treatment of hydatid cysts is the parasite, mainly across benzimidazole (BZD) compounds, which interferes with mechanisms of glucose absorption leading to glycogen depletion and subsequent degenerative changes in mitochondria and endoplasmic reticulum of germinal cells (Wu et al., 2011). During the past 40 years, various chemotherapeutic agents active against this parasite have been used either as primary or adjunctive treatment. Nowadays, albendazole (ABZ) is the drug of choice for perioperative prophylaxis and treatment of inoperable human cases. However, prolonged and repeated high doses of ABZ are often necessary, and result in curing rates approaching only 30% (Gavidia et al., 2009). Since the development of new molecules for parasite targets is a process that requires a huge investment in time and money (Hennessy, 1997) it is extremely necessary to optimize the use of existing drugs and also search for tools to improve the behaviour of these drugs and their pharmacologic effect. Therefore, it is essential to understand the kinectic and dynamic characteristics of each drug through *in vitro*, *ex vivo* and *in vivo* studies.

Flubendazole (FLBZ) is a broad-spectrum BZD methylcarbamate anthelmintic. It is widely used against gastrointestinal nematode infections in pigs and poultry, as well as against lungworms in pigs. FLBZ is also available for human use to treat nematode infections. Recently, FLBZ has been described as the best macrofilaricide drug tested in animal models when it is given parenterally (Mackenzie and Geary, 2011), and currently it is being reformulated for potential oral treatment of systemic filarial infections. Furthermore, we have previously reported an excellent efficacy of FLBZ against a secondary hidatid disease developed in mice, even superior than that observed for ABZ under the same experimental conditions (Ceballos et al., 2011). The anthelmintic efficacy after a drug treatment may depend not only on the activity of the parent drug, but also on the activity of active metabolite/s. In the case of ABZ, two metabolites are found in the systemic circulation after its administration to different animal species, ABZ-sulphoxide (ABZSO) and ABZ-sulphone ($ABZSO_2$) (Marriner and Bogan, 1980; Prichard et al., 1985; Alvarez et al., 1995; Sánchez et al., 2000). While ABZSO is an active metabolite, $ABZSO_2$ is not used as an anthelmintic and is probably inactive *in vivo* (Lubega and Prichard, 1999). However, most studies on $ABZSO_2$ activity have been carried out in nematodes; its potential activity against other helminths, including cestodes and trematodes, is unknown. On the other hand, the main metabolic pathways for FLBZ include reduction to form reduced-FLBZ (R-FLBZ), and hydrolysis of the methylcarbamate group to form the hydrolysed-FLBZ metabolite (H-FLBZ). R-FLBZ was the main analyte recovered from the bloodstream of sheep (Moreno et al., 2004) and mice (Ceballos et al., 2009) treated with FLBZ, in which only trace amounts of H-FLBZ were detected. Likewise, while H-FLBZ is an inactive metabolite, some biological activity has been described for R-FLBZ including an effect on *Fasciola hepatica* egg hatching (Alvarez et al., 2009) and on PSC of *E. granulosus* under *ex vivo* conditions (Ceballos et al., 2011).

A previous work performed in our laboratory demonstrated that improved FLBZ absorption enhanced its clinical efficacy against cystic echinococcosis in mice (Ceballos et al., 2009). Since the cystic echinococcosis in the "mouse model" is broadly different to that occurring in humans, we needed to demonstrate FLBZ activity against hydatid cysts in a different animal model. We choose sheep as an animal model to assess the value of FLBZ in the treatment of the human hydatid disease, as the disease process is similar in both species (Morris et al., 1985). Thus, the goals of the current work were to compare the systemic exposure of ABZ and FLBZ and their activity against CE developed in naturally infected sheep. Complementary work focused on the assessment of liver enzymes activities in response to the extended duration of the dose regimens implemented for both BZD compounds.

2. Materials and methods

2.1. Chemicals

Pure reference standards of FLBZ, R-FLBZ and H-FLBZ were kindly provided by Janssen Animal Health (Beerse, Belgium). Reference standards of oxibendazole (OBZ, used as internal standard), ABZ, ABZSO and $ABZSO_2$ were obtained from Sigma–Aldrich (Dorset, UK). The solvents HPLC grade (acetonitrile and methanol) and ammonium acetate (HPLC grade) were from Sintorgan S.A. (Buenos Aires, Argentina) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Carboxymethylcellulose (CMC) was purchased from Anedra (Buenos Aires, Argentina). The FLBZ suspension (12.5%, w/v) was performed by addition of 12.5 g FLBZ pure standard in a suspension of CMC (0.5%, w/v) prepared with deionized water (100 mL, pH 6.0) under shaking (24 h). The ABZ suspension used was Valbazen® (ABZ 10%, Pfizer, Argentina). Both formulations were vigorously shaken before its administration to sheep.

2.2. Experimental animals

Twelve (12) Corriedale sheep (approximately 8 years old, 50.6 ± 6.0 kg), with hydatid cyst placed in their livers (Type CE1 according to the WHO classification, determined by ultrasonography), were involved in the current trial. Ultrasonography was performed using a Chison 600 Vet equipment (Chison, China) with a 5 MHz transductor. After shaving the anatomical zone close to the last rib on the right side of the animal, a transducing medium was applied to allow a good ultrasound waves transmission and to facilitate the location/identification of hepatic hydatid cysts. Before starting the experiment, approximately 60 sheep from a farm, where the presence of hydatid cyst in adult sheep is common, were scanned by ultrasound. Animals found to have cysts that appeared typical of those *E. granulosus* in the liver were selected for the current trial. The selected animals were kept indoors with commercial balanced food and water *ad libitum* for 15 days prior and during the trial. All sheep were randomly allocated into three experimental groups ($n = 4$, see below). Animal procedures and management protocols were approved by the Ethics Committee according to 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).

2.3. Experimental design

2.3.1. Pharmacokinetic study

The pharmacokinetic study involved two different groups: in FLBZ, animals were treated with the FLBZ formulation and in ABZ, animals were treated with the ABZ formulation. Treatments were performed by the oral route every 48 h for 55 days. An equimolar dose of 0.032 mmol/kg of body weight was used for both, FLBZ and ABZ, which correspond to 10 and 8.5 mg/kg, respectively. The pharmacokinetic study consisted in two time-separated phases named Initial and Final Kinetic Studies, which were carried out after the first (day 1) or last (day 55) treatment, respectively. Details of the treatment schedule are shown in Fig. 1. Blood samples (5 mL) were taken from the jugular vein using 10 mL heparinised Vacutainers®

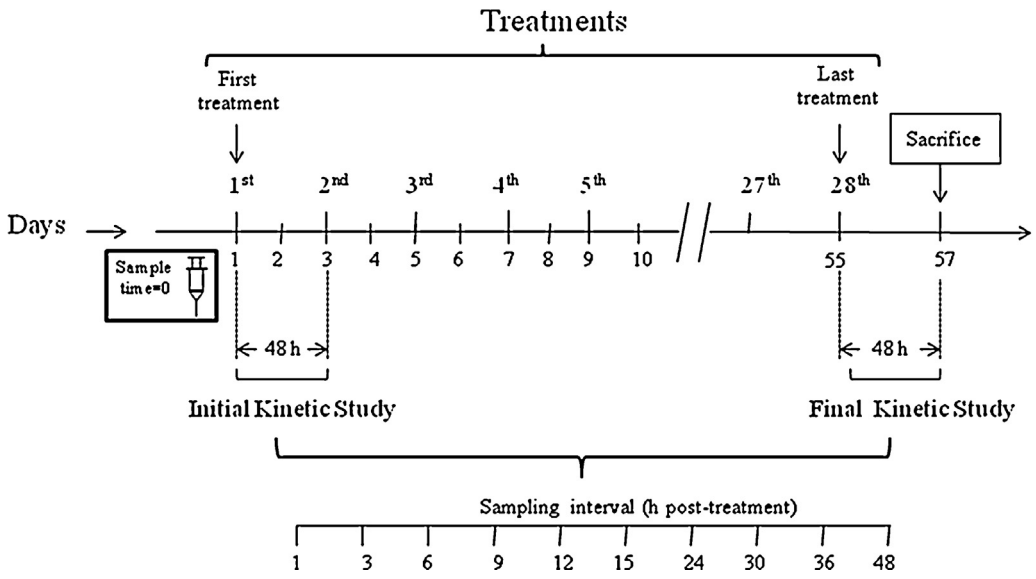

Fig. 1. Schematic representation of the experimental design of the work described here involving both the pharmacokinetic and clinical efficacy trials. Albendazole (ABZ) and flubendazole (FLBZ) treatments were performed every 48 h during 55 days. The pharmacokinetic study was separated in two different phases called Initial and Final Kinetic Studies. In each case, blood samples were taken between 1 and 48 h post-first and -last drug treatment, respectively. Animals were sacrificed 48 h after the last treatment to assess treatment efficacy.

tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation at 2000 × *g* for 15 min, placed into plastic tubes and frozen at −20 °C until analysis by high performance liquid chromatography (HPLC). In order to evaluate the potential toxicity of repeated FLBZ/ABZ treatments, the serum activities of aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine amiotranferase (ALT) were determined prior to and at 1, 2, 3 and 4 weeks after treatment.

2.4. Efficacy study

The efficacy study involved the same FLBZ and ABZ medicated groups plus an unmedicated control group ($n = 4$, control), in which animals were orally treated with water as placebo. Forty-eight (48) hours after the last treatment, animals were sacrificed by captive bolt plus exsanguination. Livers with hydatid cysts were recovered from killed animals. The treatment efficacy was evaluated by the cysts morphological characteristics, PSCs' vitality and viability (Gavidia et al., 2010). PSCs vitality was performed on PSCs recovered from cysts obtained from medicated and unmedicated animals, using the methylene blue exclusion test (Casado et al., 1986). Dead PSCs stain blue and those that are alive exclude the dye and remain clear. The percentage of live PSCs divided by the total number of observed PSCs (live PSCs/total PSCs) was used to calculate the PSCs' vitality, expressed as %. The PSCs' viability was evaluated by intraperitoneal inoculation of Balb/C mice with PSCs (1500/animal) recovered from cysts belonging to ABZ, FLBZ or control group ($n = 10$/group). After 2 months of infection, animals were euthanized and the presence of cysts in the peritoneal cavity was recorded.

In addition, the following equation expresses the percentage of efficacy (%*E*) of a drug treatment against a given parasite species (*S*) in a treated group (*T*) when compared with an untreated control (*C*):

$$\%E = \frac{\text{mean of}S \text{ in}C - \text{mean of}S \text{ in}T}{\text{mean of}S \text{ in}C} \times 100$$

2.5. Enzyme assays

Four (4) animals of each experimental group (efficacy study) were used as source of liver parenchyma (caudate lobe) for preparation of microsomes. Samples were rinsed with ice-cold KCl 1.15%, and then stored in aluminium foils, frozen in liquid N2, brought to the laboratory and stored at −70 °C. Subsequent procedures were started within one week from sample collection and were carried out between 0 and 4 °C. The preparation of liver microsomes was performed as described previously (Maté et al., 2008). Microsomal suspensions were stored at −70 ° C until being used in the different biotransformation assays. The cytochrome P450 content was determined as the carbon monoxide difference spectrum (450–490 nm) of sodium dithionite-reduced microsomal suspensions (Rutten et al., 1987). The rate of 7-ethoxyresorufin O-deethylation (EROD) was measured for the evaluation of cytochrome P450 1A (CYP1A)-mediated metabolism (Burke and Mayer, 1974). Methimazole (MTZ) S-oxidase was selected as a flavin monooxygenase (FMO)-dependent specific pathway and assayed following the method described by Dixit and Roche (1984) using 1 mM substrate concentration and 0.12 mg of microsomal protein. ABZ S-oxidation was assessed in sheep liver microsomes by the amount of ABZSO formed in the presence of a NADPH generating system following previously reported methods (Maté et al., 2008). The substrate was incubated (8 min at 37 °C) at 50 μM in glass vials in an oscillating water bath under aerobic conditions. Microsomal carbonyl reductase (CBR) activity was measured using menadione (MEN) as substrate and monitoring NADPH oxidation at 37 °C by fluorescence at an excitation wavelength of 380 nm and a maximum of emission wavelength of 460 nm (Maté et al., 2008).

2.6. Chromatographic analysis of FLBZ/ABZ and their metabolites

Chromatography was performed on a Shimadzu HPLC equipment (Shimadzu Corporation, Kyoto, Japan), with two LC-10AS solvent pumps, an automatic sample injector (SIL-10A) with a 50 μL loop, an ultraviolet visible spectophotometric detector (UV) (SPD-10A) reading at 292 nm, a column oven (Eppendorf TC-45, Eppendorf, Madison, WI, USA) set at 30 °C, and a CBM-10A

integrator. The C18 reversed-phase column (5 μm, 250 mm × 4.6 mm) was Kromasil (Kromasil®, Sweden). Elution from the stationary phase for FLBZ/metabolites was carried out with an isocratic elution gradient at a flow rate of 1.2 mL/min using acetonitrile (34%) and ammonium acetate buffer (0.025 M, pH 5.3, 66%). For ABZ/metabolites, the elution from the stationary phase was carried out at a flow rate of 1.2 mL/min using acetonitrile and ammonium acetate buffer (0.025 M, pH 6.6) as a mobile phase. The elution gradient linearly changed from 27:73 (acetonitrile:ammonium acetate buffer) to 50:50 in 5 min, then maintained for 5 min and modified to 27:73 in 3 min, which was then maintained over 3 min.

2.7. Plasma sample extraction

Plasma samples (500 μL) were spiked with OBZ as internal standard (10 μL from stock solution of 50 μg/mL). After 5 min, plasma samples were mixed with 2 mL of acetonitrile for 15 minutes in multivortex and then centrifuged at 2500 × *g* by 15 min. The resulting supernatant was concentrated to dryness in a vacuum concentrator (Speed-Vac®, Savant, CE, USA) and then reconstituted with 150 μL of mobile phase.

Identification of FLBZ, ABZ and its metabolites was undertaken by comparison with the retention time of pure references standards. A complete validation of the analytical procedures for extraction and quantification of each drug and metabolites in plasma was performed before starting the analysis of experimental samples. Retention times for H-FLBZ, R-FLBZ and FLBZ were 5.7, 7.1 and 14.4 min, respectively; and for ABZSO, $ABZSO_2$ and ABZ were 5.4, 6.7 and 11.3 min, respectively. The calibration curves for each analyte constructed by least squares linear regression analysis showed good linearity with correlation coefficients ≥0.998. The limit of quantification was defined as the lowest measured concentration with a CV <20% an accuracy of ±20% and an absolute recovery ≥70%, it was 0.01 μg/mL for FLBZ/metabolites and 0.1 μg/mL for ABZ/metabolites.

2.7.1. Kinetic analysis of the data

The concentration *vs.* time curves for FLBZ and/or its metabolites in plasma for each individual animal were fitted with the PK SolutionsTM computer program (Summit Research Service, OH, USA). The peak concentration (C_{max}) and time to peak concentration (T_{max}) were recorded directly from the measured concentration data. The elimination half-life ($T_{½el}$) was calculated as $\ln 2\lambda_{el}$, where the terminal elimination rate constant (λ_{el}) was calculated by performing regression analysis using data points belonging of the terminal phase concentration–time plot. The area under the plasma concentration–time curve from zero up to the limit of quantification (AUC_{0-LOQ}) was calculated by means of the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity ($AUC_{0-\infty}$) by dividing the last experimental concentration by the terminal elimination rate constant (λ_{el}). Statistical moment theory was applied to calculate the mean residence time (MRT) by using the formula MRT = AUMC/AUC_{0-LOQ} (Perrier and Mayersohn, 1982) where AUMC is the area under the curve of the product of time and the plasma drug concentration *vs.* time from zero to infinity (Gibaldi and Perrier, 1982), and AUC_{0-LOQ} is as defined above.

2.8. Biochemical determinations

Serum AST, ALP and ALT activities were measured using test kits purchased from Kits BioSystem (España), in an autoanalyser (HYCELL LISA 200, Hycell Co., New York, USA).

2.9. Statistical analysis of the data

Data are reported as arithmetic mean (±SD) and statistical analysis was performed using the Instat 3.0 Software (Graph Pad Software, CA, USA). The Mann–Whitney test (non parametric) was used for the statistical comparison of the kinetic data obtained after the initial and the final kinetic trial carried out in both FLBZ- and ABZ-treated groups. CYP contents in liver microsomes are expressed as nmol per mg of microsomal protein. EROD activities are expressed in pmol of resorufin formed per min per nmol of CYP (turnover number: maximal amount of substrate molecules converted per unit of time per catalytic site). All other enzyme activities are expressed in nmol/min per mg of microsomal protein. PSCs vitality, CYP content and enzyme activities (except ABZ S-oxidase) were compared by means of Kruskal–Wallis (non parametric ANOVA) followed by Dunn's multiple comparison test. The Mann Whitney test was employed for the statistical comparison of ABZ S-oxidase activity in control and ABZ-treated animals. For all statistical comparisons, a *P* value less than 0.05 ($P < 0.05$) was considered significant.

3. Results

FLBZ and its reduced and hydrolysed metabolites were recovered in plasma after the oral administration of FLBZ formulated as a CMC-suspension to sheep. The mean plasma concentration profiles for FLBZ and its reduced metabolite are shown in Fig. 2. R-FLBZ plasma concentrations were higher than those determined for FLBZ. The C_{max} of R-FLBZ (0.25 ± 0.08 μg/mL) was attained at 15.6 h (T_{max}) post-treatment. FLBZ concentrations were first detected in plasma at 1 h post-treatment and reached the peak value (0.05 ± 0.01 μg/mL) at 18.8 h post-treatment. The H-FLBZ metabolite was the analyte measured at the lowest concentrations, being detectable between 18 and 48 h post-treatment, with concentrations near to the limit of quantification. The trace concentrations detected during a short-time period precluded the development of a complete pharmacokinetic analysis of the data determined for this molecule after the oral administration of FLBZ.

ABZ parent drug was not detected in plasma at any time post-treatment. Fig. 3 shows the mean plasma concentration profiles for ABZSO and $ABZSO_2$ following ABZ administration to sheep. After the first treatment, ABZSO peak plasma concentration (2.41 ± 0.43 μg/mL) was obtained at 10.2 h. The higher plasma concentration achieved for this metabolite compared to $ABZSO_2$ during all sampling times is reflected in higher plasma AUC value obtained for ABZSO (47.07 ± 7.66 μg h/mL) compared to $ABZSO_2$ (11.1 ± 2.22 μg h/mL).

The plasma disposition kinetics of FLBZ/R-FLBZ after the last treatment (Final Kinetic Study) did not show significant differences compared to that observed after the first treatment (Initial Kinetic Study) (Fig. 2a and b). Conversely, following the last ABZ administration to sheep (Final Kinetic Study), the plasma drug exposure of $ABZSO_2$ resulted significantly higher compared to that observed in the Initial Kinetic Study (Fig. 3b). These improved concentrations determine a significant ($P < 0.05$) increment in the $ABZSO_2$ AUC_{0-LOQ} value compared to that obtained after the first treatment (Initial Kinetic Study). The ABZSO plasma concentration profiles were not modified between the first and the last ABZ treatment (Fig. 3a).

A high variability in the serum activities of AST, ALT and ALP was observed in animals from the different groups. Enzyme serum activities were within the normal range in animals from the different groups during the treatment period or slightly above the normal range, specially the ALP activities measured in animals from the control group. Anyway, in medicated animals from both FLBZ and

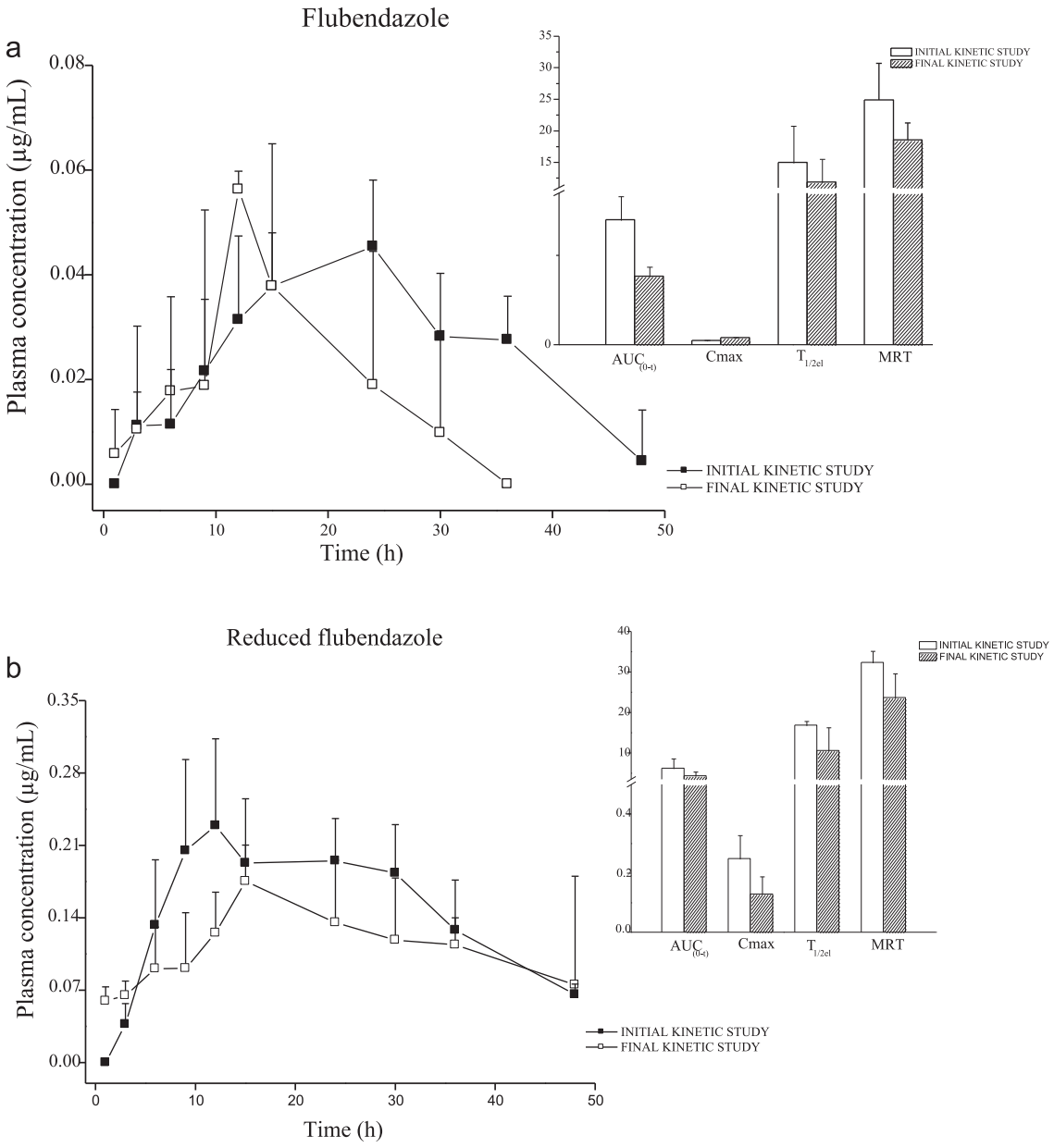

Fig. 2. Plasma concentrations (arithmetic mean ± SD) of flubendazole (FLBZ) (a) and reduced flubendazole (R-FLBZ) (b) at different times after the first and last (Initial and Final Kinetic Studies, respectively) oral administration of FLBZ (10 mg/kg) to sheep. The main pharmacokinetic parameters (arithmetic mean ± SD) obtained for both FLBZ and R-FLBZ after the Initial and Final treatments are shown in the insert of the figure. AUC_{0-t}: area under the plasma concentration *vs.* time curve from 0 to the detection time (μg h/mL); C_{max}: peak plasma concentration (μg/mL); $T_{½el}$: elimination half-life (h). MRT: mean residence time (h).

ABZ treated groups, the AST, ALT and ALP activities resulted similar ($P > 0.05$) to that observed in animals from the control group (Fig. 4).

All cysts (from control and treated groups) appeared turgid with no observable collapse of the germinal layer. The vitality of PSCs obtained from cysts recovered from unmedicated animals (CONTROL) was 97.3 ± 2.72%. Therefore, both treatments provoked a marked protoscolicidal effect, reducing the PSCs vitality at 18.4 ± 5.0% (FLBZ) and 2.6 ± 0.57% (ABZ) (Fig. 5). Furthermore, after 2 months of infection, 90% of mice inoculated with PSCs belonging to control group developed hydatid cysts in their peritoneal cavity, while only 25% (FLBZ) and 33% (ABZ) of mice inoculated with PSCs recovered from treated sheep, presented hydatid cyst in theirs abdominal cavities (viability assay). In agreement to these results, a relevant anthelmintic efficacy was observed in the FLBZ (81%) and ABZ (97%) treated groups.

Table 2 shows CYP contents and enzyme activities measured in liver microsomal fractions obtained from either control, FLBZ- or ABZ-treated sheep. CYP content in liver microsomes from control sheep was similar than those measured in both ABZ- and FLBZ-treated animals. EROD activity in liver microsomes obtained from sheep receiving ABZ resulted between 2.1- and 2.6-fold higher compared to FLBZ-treated ($P < 0.05$) and control ($P < 0.01$) animals, respectively. Conversely, no statistical differences among experimental groups were observed for the microsomal S-oxidation of MTZ and ABZ, and also for MEN reductase activity.

4. Discussion

It has long been considered that FLBZ is probably the 'weakest' of the widely used benzimidazole anthelminthics (especially in humans) and therefore it has often been overlooked.

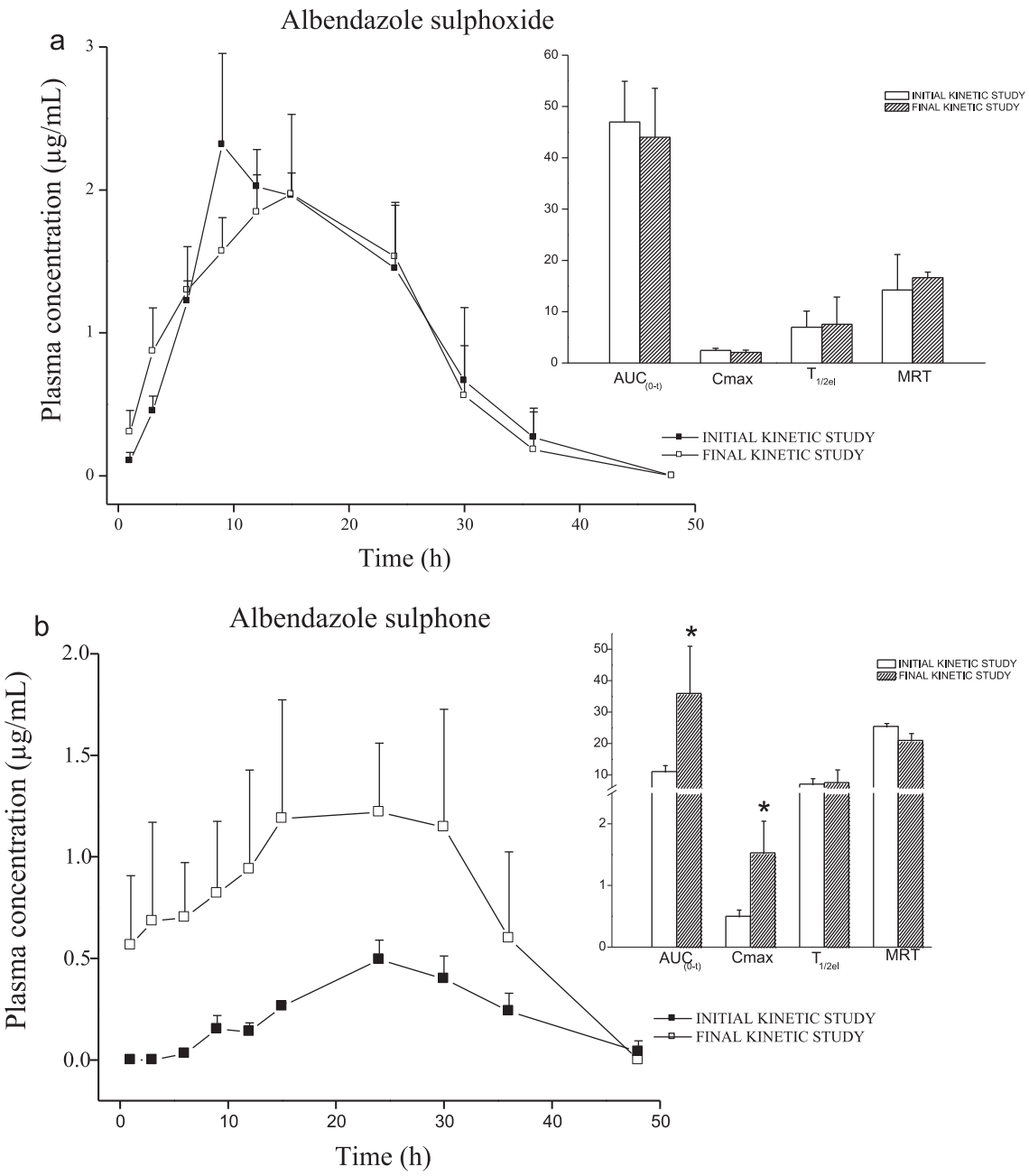

Fig. 3. Plasma concentration profiles (arithmetic mean ± SD) of albendazole sulphoxide (ABZSO) (a) and albendazole sulphone ($ABZSO_2$) (b) at different times after the first and last (Initial and Final Kinetic Studies, respectively) oral administration of albendazole (ABZ) (8.5 mg/kg) to sheep. The main pharmacokinetic parameters (arithmetic mean ± SD) for ABZSO and $ABZSO_2$, obtained after the Initial and Final treatments are shown in the insert of the figure. AUC_{0-t}: area under the plasma concentration *vs.* time curve from 0 to the detection time (g h/mL); C_{max}: peak plasma concentration (μg/mL); $T_{½el}$: elimination half-life (h). MRT: mean residence time (h). *Differences statistically significant ($P < 0.05$) compared to the Initial Kinetic Study for that metabolite.

The plasma disposition kinetics data obtained for both FLBZ and ABZ in sheep in the current study is well in agreement with the results obtained by Moreno et al. (2004) and Alvarez et al. (1999), respectively. R-FLBZ represented 80% of total analytes recovered from the systemic circulation, followed by the parent drug (20%). The high concentrations of R-FLBZ in plasma, compared to those observed for FLBZ, are given by an efficient presystemic metabolism of FLBZ in both the small intestinal mucosa and liver. In fact, both microsomal and cytosolic fractions obtained from liver and gut mucosa were able to reduce FLBZ into R-FLBZ (Maté et al., 2008). Carbonyl reducing enzymes (CBRs) are a class of oxidoreductase enzymes enclosed within the family of short chain dehydrogenases/reductases. The rate of NADPH oxidation in the presence of the substrate MEN is a good marker of CBR activity (Gonzalez-Covarrubias et al., 2007), whereas this enzyme system was suggested to be involved in the keto-reduction of FLBZ in sheep (Maté et al., 2008). On the other hand, the plasma pharmacokinetic behaviour of ABZ after its oral administration was characterized by the absence of the parent drug and a fast increase in the plasma concentrations of its active ABZSO metabolite, which it also accounted for most of the metabolites recovered from the bloodstream (80%) after ABZ administration. The absence of ABZ in the bloodstream has also been attributed to an extensive first pass metabolism in the liver. In fact, the S-oxidation of ABZ to ABZSO has been shown to be catalysed by the liver microsomal mixed function oxidases in sheep, namely the flavin-monooxygenase (FMO) and the cytochrome P450

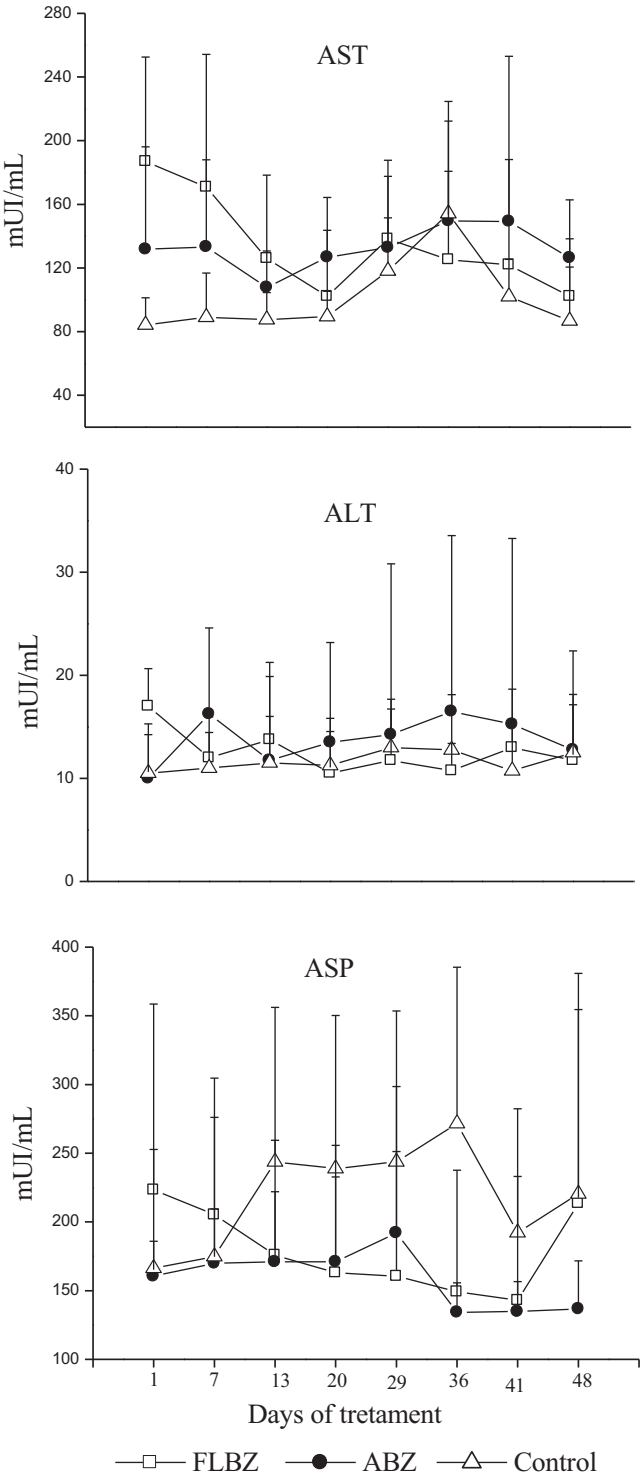

Fig. 4. Enzymatic activities (arithmetic mean ± SD) for the liver enzymes aspartate aminotransferase (AST), alanine amiotranferase (ALT), and alkaline phosphatase (ALP), measured in sheep serum once a week over the 55 days of treatment duration. (□) FLBZ; (●) ABZ and (△) control.

(CYP) enzyme systems (Galtier et al., 1986; Lanusse et al., 1993; Virkel et al., 2004). Furthermore, the active sulphoxide derivative undergoes a second, slower and irreversible oxidative step which forms the inactive sulphone ($ABZSO_2$) in a CYP-dependent mediated reaction (Souhaili-El Amri et al., 1988; Benoit et al., 1992).

Marked differences on the pharmacokinetic behaviour of FLBZ and ABZ following the administration of equimolar doses were

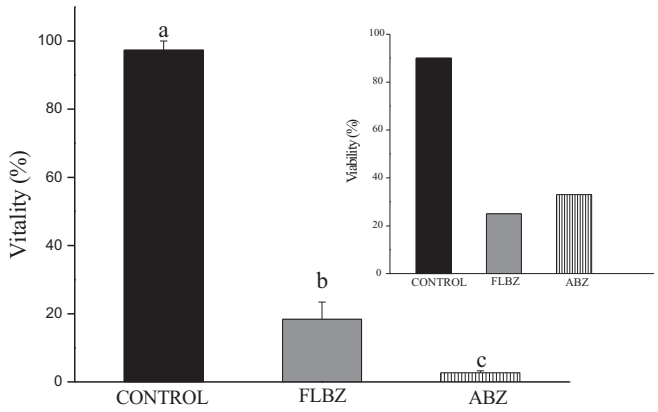

Fig. 5. Vitality (%, means ± SD) of protoscoleces obtained from hydatid cysts collected from the liver of unmedicated (control), flubendazole (FLBZ) and albendazole (ABZ) treated infected sheep. The insert shows the vitality (%) of protoscoleces inoculated into Balb/C mice, estimated by the number of cysts developed 2 months after infection. *Different letters indicate statistically significant differences ($P < 0.05$).

observed. The peak plasma concentration of ABZSO was 11-fold higher than that obtained for R-FLBZ. Additionally, the AUC of R-FLBZ represented only 13% than that observed for ABZSO (Table 1). Similar differences were reported between ABZSO and oxfendazole (the sulphoxide metabolite of fenbendazole, an aromatic substituted BZD like FLBZ) after the oral administration of their respective parent drugs at the same dose rates to sheep (Lanusse et al., 1995). The low systemic availability (13%) of FLBZ in sheep (Moreno et al., 2004) may account for the observed differences in plasma drug exposure between R-FLBZ and ABZSO. The absence of ABZ in plasma after its oral administration precludes the determination of its absolute bioavailability in sheep (Alvarez et al., 1999). However, when ABZSO AUC were compared after the intravenous and the intraruminal administration of ABZ in sheep, a 90% availability of the parent drug was estimated (Alvarez et al., 1999). Although this approach may overestimate ABZ availability, it may be indicative of a higher rate of ABZ absorption compared to FLBZ. In addition, sheep liver microsomes showed approximately a 2-fold higher ability for ABZ S-oxidation compared to FLBZ keto-reduction when both substrates were incubated at equimolar concentrations (unpublished observations). Altogether these observations may help to explain the marked differences in the systemic exposure between both anthelmintic compounds.

As for other relevant pharmacokinetic difference between FLBZ and ABZ, longer mean residence time and elimination half-life of the R-FLBZ metabolite was observed, compared to those obtained

Table 1
Pharmacokinetic parameters obtained for reduced flubendazole (R-FLBZ) and albendazole sulphoxide (ABZSO) in sheep plasma after the first equimolar (0.032 mmol/kg) administration of Flubendazole (FLBZ) and albendazole (ABZ), respectively.

Pharmacokinetic parameter	ABZSO	R-FLBZ
AUC_{0-t} (μg h/mL)	46.6 ± 7.60	6.26 ± 2.45[a]
AUC_{total} (μg h/mL)	47.07 ± 7.66	8.60 ± 1.43[a]
C_{max} (μg/mL)	2.41 ± 0.43	0.25 ± 0.08[a]
T_{max} (h)	10.2 ± 2.68	15.60 ± 5.63
$T_{½el}$ (h)	6.26 ± 1.23	16.9 ± 1.12[a]
MRT (h)	17.50 ± 0.82	32.37 ± 2.94[a]

Data are expressed as mean ± SD ($n = 4$). AUC_{0-t}: area under the plasma concentration *vs.* time curve from 0 to the detection time; AUC_{total}: area under the concentration *vs.* time curve extrapolated to infinity; C_{max}: peak plasma concentration; T_{max}: time to the C_{max}; $T_{½el}$: elimination half-life; MRT: mean residence time (obtained by non-compartmental analysis of the data).

[a] Statistically significant differences between pharmacokinetic parameters obtained after ABZ and FLBZ administration.

Table 2
Cytochrome P450 (CYP) contents and enzyme activities measured in liver microsomal fractions obtained from either control, FLBZ- or ABZ-treated sheep.

Enzyme activity	Control	ABZ-treated	FLBZ-treated
CYP content (nmol/mg protein)	0.40 ± 0.05	0.44 ± 0.22	0.37 ± 0.05
EROD (pmol/min/nmol CYP)	35.2 ± 9.23	92.4 ± 21.9[a]	43.8 ± 8.20
MTZ S-oxidase (nmol/min mg)	16.8 ± 4.74	13.6 ± 5.04	14.1 ± 3.67
ABZ S-oxidase (nmol/min mg)	1.55 ± 0.67	1.22 ± 0.16	ND
MEN reductase (nmol/min mg)	68.1 ± 12.8	72.5 ± 9.03	62.4 ± 5.17

Data are expressed as mean ± SD ($n = 4$). EROD: 7-ethoxyresorufin O-deethylase; MTZ: methimazole; ABZ: albendazole; MEN: menadione; ND: not determined.
[a] Statistically different compared to control ($P < 0.01$) and FLBZ-treated ($P < 0.05$) animals.

for ABZSO (Table 1). The BZD anthelmintics require extensive hepatic oxidative metabolism to achieve sufficient polarity for excretion (Hennessy, 1993). Differences in the chemical structures of ABZ and FLBZ determine a different biotransformation pattern. Aliphatic substituted BZD at position −5 of the BZD ring, such as ABZ, are sequentially oxidized to their sulphoxide and sulphone metabolites (Lanusse and Prichard, 1993). These metabolites are sufficiently polar when oxidized to be largely excreted in urine (Hennessy et al., 1989). Furthermore, ABZ is extensively metabolized by the host. The efficient "first-pass effect" avoids the detection of the parent compound in the bloodstream. On the other hand, low concentrations of FLBZ are detected in plasma for up to 48 h post-treatment. This aromatic BZD derivative is mainly reduced to R-FLBZ in sheep (Moreno et al., 2004), liver CBRs being the enzymatic system supposed to be involved in its metabolic production (Maté et al., 2008). FLBZ metabolites are largely eliminated by bile. Differences in the metabolic rate/elimination process between FLBZ and ABZ may account for the extended residence in the bloodstream of R-FLBZ compared to that observed for ABZSO.

Usually, pharmacokinetic studies are conducted in healthy individuals/animals. The presence of the parasite itself as well as changes produced by pathogenic action of parasites could induce alterations on pharmacokinetic behaviour, side-effects, distribution to target tissues/parasites and expected efficacy of the chosen anthelmintic for therapy (Alvarez et al., 1997). The results obtained in the current work carried out in naturally *E. granulosus* infected animals, either after FLBZ or ABZ treatments, did not significantly differ with pharmacokinetic data previously reported for these compounds in healthy animals (Ceballos et al., 2012; Alvarez et al., 1997). This outcome was expected since the hydatid cysts do not usually alter the drug pharmacokinetic behaviour in the intermediated hosts (Menezes da Silva, 2003). Changes on drug metabolism could be observed in presence of advanced infections or in the presence of large size cysts which may exert an excessive pressure on nearby organs (Kern, 2003; Eckert and Deplazes, 2004).

For BZD compounds, the presence of high drug concentrations for an extended period of time is crucial to achieve optimal anthelmintic efficacy, particularly against parasites located in tissues. In CE human chemotherapy, this is achieved after ABZ administration in two daily doses (10–15 mg/kg) for several months (WHO Informal Working Group, 2001). A similar therapeutic scheme was adopted in the current research in order to compare the efficacy of both FLBZ and ABZ against CE in the ovine model. No accumulative effect was observed for both FLBZ and R-FLBZ after the oral administration of FLBZ parent drug every 48 h during 55 days; their plasma disposition kinetics resulted similar after both the Initial and Final Kinetic Studies (Fig. 2). This fact agrees with similar MEN reductase metabolic activities in control and FLBZ-treated animals (Table 2). A similar finding was observed for the plasma pharmacokinetic behaviour of ABZSO after the Initial and Final Kinetics Studies when ABZ was the administered drug (Fig. 3). This finding is in agreement with a similar ABZ S-oxidase activity measured in liver microsomes obtained from both control and ABZ-treated sheep (Table 2). Unchanged ABZ S-oxidation to ABZSO was also observed in rats treated with ABZ during 10 days (Souhaili-El Amri et al., 1988). On the other hand, the $ABZSO_2$ plasma disposition kinetic drastically changed between the first and last ABZ treatment. The AUC and C_{max} values obtained for $ABZSO_2$ after the last ABZ administration (day 55) were significantly higher than those obtained after the first ABZ treatment (Fig. 3b). Increased $ABZSO_2$ plasma concentrations were observed when ABZ was repetedly administered to sheep (Delatour et al., 1990) and goats (Benoit *et al*., 1992). This fact has been associated with an enzyme-inducing effect of ABZ on the CYP-mediated production of $ABZSO_2$. BZD anthelmintic compounds such as ABZ and oxfendazole may induce the expression and function of CYP1A1 (Gleizes-Escala et al., 1996; Baliharová et al., 2003). The CYP1A subfamily, which comprises CYP1A1 and CYP1A2, is structurally well conserved and constitutively expressed in vertebrates. As far as ruminant species are considered, the expression profiles of constitutive CYP1A1 and CYP1A2 mRNAs have been characterized in cattle liver (Giantin et al., 2010), whereas proteins cross reacting with antibodies raised against rat or rabbit CYP1A have been found in liver microsomal fractions from both cattle (Sivapathasundaram et al., 2001; Nebbia et al., 2003; Giantin et al., 2010) and sheep (Szotáková et al., 2004). The degree of CYP1A1 expression in food producing animals has been found to be related to the rate of *in vitro* O-dealkylation of ethoxyresorufin (EROD) (Ioannides, 2006). Thus, the increased plasma availability of $ABZSO_2$ observed in the current work agrees with the enhanced EROD activity found in liver microsomes from ABZ-treated sheep. In conclusion, the repeated ABZ administration may have induced CYP1A1 and accounted for a higher plasma availability of the inactive $ABZSO_2$ metabolite.

In humans, BZD long term therapy is well tolerated by most patients (El-On, 2003). However, adverse events are not uncommon and include gastrointestinal disturbances, abnormalities in liver functions, abdominal pain, nausea, vomiting and diarrhoea (El-On, 2003). In order to check the potential toxicity by the long term BZD treatment in our animal model, the serum activities of ALT, AST (as indicator of hepatic necrosis) and ALP (as indicator of cholestasis) were determined. Normal values for all animals involved in the study were observed for ALT serum activity. This is expected since in the large domestic species like sheep, the activity of ALT in the liver is low, and in liver injury the serum ALT is not remarkably elevated (Tennant, 1997). Likewise, for all groups there were no clinically significant increases in AST and ALP during the treatment period. However, some animals from the three groups have AST or ALP activities slightly higher than normal on some sampling days, including day 1 (Sample time 0). Likewise, under our experimental conditions it can be concluded that the FLBZ and ABZ formulation administered orally to sheep at 3.2 nmol/kg every 2 days for 55 consecutive days did not significantly affect the health of the animals.

The findings obtained in the current work demonstrated that both FLBZ and ABZ are highly effective against CE. PSC vitality was reduced by 82 (FLBZ) and 98% (ABZ). Similar results were obtained for repeated treatments with albendazole (Morris et al., 1985) and oxfendazole (Blanton et al., 1998; Dueger et al., 1999; Gavidia et al., 2009) in *E. granulosus* naturally infected sheep, where drug treatment resulted in high PSC death rates. Furthermore, a PSC viability of 25 and 33% was observed after FLBZ and ABZ treatments, respectively. The advantage derived from the use of ABZ in CE therapy has been extensively reviewed (Horton, 2003). Furthermore the results described here confirm the anthelmintic effect of FLBZ on hydatid cyst located in the liver of naturally infected sheep. The clear effect on PSC vitality/viability confirms the therapeutic benefit derived from the use of FLBZ in CE, minimizing the risk associated to an accidental planting of PSC in the organics cavities.

Usually, the higher the concentration achieved at the tissue where the parasite is located, the higher the amount of drug reaching the target parasite. In the current work we did not assess drug/active metabolites cyst concentrations. However, findings from different *in vivo* studies (Hennessy et al., 1995; Alvarez et al., 2011) demonstrate the strong correlation between plasma drug exposure of BZD compounds and anthelmintic efficacy. In fact, a high negative correlation between the ABZSO AUC and C_{max} values, and the number of ABZ resistant nematodes recovered from treated lambs has been recently reported (Alvarez et al., 2011; Barrère et al., 2012). ABZ efficacy against CE is based on the capacity of ABZSO to reach the cyst. After FLBZ administration, the ability of both the parent drug and its reduced metabolite to accumulate into the hydatid cyst may account for its *in vivo* activity (Ceballos et al., 2011). Different to that observed in the murine model (Ceballos et al., 2011), in the current work ABZ showed a significantly higher efficacy (measured as PSC vitality) to that observed for FLBZ. In the murine model, the concentrations achieved for FLBZ + R-FLBZ (active molecules) in cysts resulted only 1.3-fold lower than that measured for ABZSO (Ceballos et al., 2011). Although in sheep the cyst concentrations were not assessed, the differences between FLBZ + R-FLBZ and ABZSO plasma concentrations were broader (5.8-fold lower). Thus, the higher efficacy observed for ABZSO in sheep would be directly related with the higher ABZSO systemic exposure and consequently higher cyst exposure to the active drug.

The extrapolation of the results obtained in sheep to humans face the problem of interspecies differences on drug pharmacokinetics mainly due to gastrointestinal anatomy/physiology differences. When a BZD suspension is deposited in the sheep rumen, solid particles mix and distribute through the digesta volume (Hennessy, 1993). The rumen acts as a drug reservoir by slowing the digesta transit time throughout the abomasum (true stomach in ruminants), which results in improved systemic availability of BZD compounds as a consequence of a greater dissolution of drug particles in the acid pH of the abomasum (Lanusse and Prichard, 1993). A different situation may occur in human, where after oral administration the drug directly reaches the stomach. The short gastrointestinal transit time in man may account for a shorter time for dissolution of the administered drug formulation compared to that observed in ruminants, which may limit the absorption of the parent compound. Consequently, drug systemic exposure may result substantially longer in sheep compared to humans. Although drug efficacy results may be difficult to extrapolate between species, the data shown here confirm the potential of FLBZ to be used in the treatment of CE in humans, which generates an interesting therapeutic alternative. Clearly, in spite of the low plasma concentrations (systemic exposure) measured for FLBZ and its reduced metabolite (R-FLBZ), an important reduction in PSC vitality was observed in cysts located in sheep liver. Modern pharmaceutical technology may help to greatly improve FLBZ systemic exposure improving its efficacy against CE in humans.

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