1 **Identification of a field isolate of *Fasciola hepatica* resistant to albendazole and**

2 **susceptible to triclabendazole**

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4 [1]Rodrigo Sanabria*, [2]Laura Ceballos*, [2]Laura Moreno, [1]Jorge Romero, [2]Carlos

5 Lanusse and [2]Luis Alvarez[§]

6

7 [1]Centro de Diagnóstico e Investigaciones Veterinarias (CEDIVE), Facultad de

8 Ciencias Veterinarias, Universidad Nacional de la Plata, 7130 Chascomús, Argentina.

9

10 [2]Laboratorio de Farmacología, Centro de Investigación Veterinaria de Tandil

11 (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA. Campus

12 Universitario, 7000 Tandil, Argentina.

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15 Running title: *Fasciola hepatica isolate resistant to albendazole*

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17 *These authors have equally contributed to this work

18

19 [§]Corresponding author: Luis I. Alvarez (lalvarez@vet.unicen.edu.ar), Laboratorio de

20 Farmacología, CIVETAN, CONICET, Facultad de Ciencias Veterinarias, UNCPBA.

21 TE: 00 54 249 443-9850, Argentina.

22

23

1   **Abstract**

2   The experiments described here were designed to characterize the status of

3   susceptibility/resistance to albendazole (ABZ) and triclabendazole (TCBZ) of a

4   *Fasciola hepatica* isolate (named CEDIVE isolate) recovered from infected sheep

5   from in Gualeguay (Argentina) and maintained under laboratory conditions. Two

6   separate clinical efficacy experiments were performed. Experiment 1: Sheep

7   artificially infected with the CEDIVE isolate were randomly distributed into an

8   untreated control and an ABZ (7.5 mg/kg) treated groups (n= 4 each). The systemic

9   exposure of ABZ metabolites was assessed in those ABZ-treated infected animals.

10   Additionally, an untreated control group and a TCBZ (10 mg/kg) treated group was

11   included in Experiment 2 (n=4 each). The fluckicidal efficacy of ABZ and TCBZ was

12   assessed by comparison of the number of flukes recovered from untreated and treated

13   sheep at 15 days post-treatment. The efficacy against the CEDIVE isolate of *F.*

14   *hepatica* was 29% (ABZ) and 100 % (TCBZ). The plasma drug exposure (expressed

15   as AUC and Cmax) observed in the ABZ treated animals (Experiment 1), was in

16   agreement with data obtained in previous studies, which indicate that the low ABZ

17   efficacy was not related to the quality of the pharmaceutical product and/or to a low

18   systemic availability of the active drug/metabolite. The results reported here, clearly

19   show that the CEDIVE isolate of *F. hepatica* behaves as resistant to ABZ and

20   susceptible to TCBZ.

21

22   Key words: *Fasciola hepatica* isolate, resistance, albendazole, triclabendazole.

1 **Introduction**

2 The trematode *Fasciola hepatica* is a cosmopolitan parasite which causes considerable

3 loss in sheep and cattle production systems all over the world (Boray, 1994). As a result

4 of climate change, there has been a dramatic resurgence of sheep and cattle fascioliasis in

5 different regions of the world (Mitchell, 2002). Chemotherapy is the available main tool

6 to control liver flukes. However, the frequent use of effective fluckicidal compounds

7 leads to the development of resistance. Although resistance in flukes has not yet reached

8 the levels roported in nematodes (Wolstenholme et al., 2004), failures of rafoxanide and

9 closantel have been reported (Fairweather and Boray, 1999). Most of the reports

10 involving drug resistance in *F. hepatica* are related to triclabendazole (TCBZ), the most

11 used flukicidal drug in veterinary medicine (Coles and Stafford, 2001). Unlike other

12 benzimidazole (BZD) compounds, the halogenated derivative TCBZ has been shown to

13 have an excellent efficacy against the mature and immature stages of *F. hepatica* (Boray

14 et al., 1983). However, TCBZ activity appears to be restricted to the liver fluke and the

15 lung fluke, *Paragonimus* spp. (Weber, et al., 1988; Calvopina et al., 1998), since the drug

16 is inactive against nematodes, cestodes and other trematode parasites (*Dicrocoelium*

17 *dendriticum*, *Paramphistomun* spp. and *Schistosoma mansoni*). The intensive use of

18 TCBZ in endemic areas of fascioliasis has resulted in the development of liver flukes

19 resistant to this compound (Overend and Bowen, 1995; Mitchell, et al., 1998; Moll et al.,

20 2000; Thomas et al., 2000; Olaechea et al., 2011), which is considered a major problem

21 for veterinary therapeutics.

22

23 Albendazole (ABZ) is the only BZD methylcarbamate recommended to control

24 fascioliasis in domestic animals, despite its activity is restricted to flukes older than 12

25 weeks (McKellar and Scott, 1990). A previously reported work (Coles and Stafford,

1   2001) has shown that ABZ was active against a TCBZ-resistant isolate of *F. hepatica*.

2   On the other hand, resistance to both, ABZ and TCBZ in *F. hepatica*, has been reported

3   in Spain (Alvarez-Sanchez et al., 2006). Since no alternative methods for fluke control

4   are available, it is critical to understand how resistance is developed in order to limit its

5   impact on livestock production (Wolstenholme et al., 2004). However, there are

6   relatively few clearly defined isolates of *F. hepatica* available for study, and when new

7   isolates become available they should be subjected to careful scrutiny to establish their

8   response to different anthelmintic drugs (Fairweather, 2011). In order to gain a deeper

9   insight on the mechanism involved on drug resistance in *F. hepatica*, it is relevant to

10 investigate this resistance mechanisms in well characterized fluke isolates. The current

11 work was designed to characterize the status of susceptibility/resistance to ABZ and

12 TCBZ for a field isolate of *F. hepatica*, recovered from an Argentinean farm, which was

13 initially suspected to be susceptible to TCBZ, maintained and artificially produced under

14 laboratory conditions.

15

16

17 **Material and Methods**

18 **Chemicals**

19 Pure (≥99%) standards of ABZ, ABZ-sulphoxide (ABZSO), ABZ-sulphone

20 ($ABZSO_2$), and oxibendazole (OBZ) used as internal standard (IS), were use in the

21 present experiment. The commercial formulation of ABZ (Baxen 3.8%[®], suspension)

22 was from Tecnofarm, Argentina. TCBZ formulation (Fasinex[®] 10%, suspension) was

23 from Novartis, Argentina. All the solvents (acetonitrile and methanol) used during the

24 extraction and drug analysis were HPLC grade and purchased from Baker Inc.

25 (Phillipsburg, NJ, USA). Water was double distilled and deionized using a water

1   purification system (Simplicity®, Millipore, Brazil). Buffer salts ($ClNH_4$) were

2   purchased from Baker Inc. (Phillipsburg, NJ, USA).

3

4   **Experimental design**

5   **Animals**

6   Sixteen (16) healthy male Corriedale sheep (48.6 ± 2.6 kg) aged 24-26 months were

7   involved in this trial. Animals were housed during the experiment and for 15 days

8   before the start of the study. Animals were fed on a commercial balanced concentrate

9   diet. Water was provided *ad libitum*. Animal procedures and management protocols

10   were carried out in accordance with the Animal Welfare Policy (Act 087/02) of the

11   Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de

12   Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar) and

13   internationally accepted animal welfare guidelines (AVMA, 2001).

14

15   *F. hepatica* **isolate**

16   The *F. hepatica* isolate characterized in the current work (named CEDIVE isolate) was

17   recovered from the farm "San Julian", located in the Department of Gualeguay, Entre

18   Ríos, Argentina (32º 52´ S; 59º 25´ O). The farm, with a size of 1200 hectareas, is

19   dedicated to raising cattle (700 heads) and sheep (1000 heads). In this farm, 4 to 5

20   anthelmintic treatments per year have been used by several years in sheep. The

21   treatments were based in the use of BZD compounds (mainly ABZ and oxfendazole),

22   ivermectin and closantel. All anthelmintic treatments used in sheep were directed against

23   gastrointestinal nematodes (*Haemonchus* spp and *Trichostrongylus* spp.). Although the

24   presence of liver flukes has been found in both cattle and sheep, no specific treatments

1   against *F. hepatica* has been implemented, were it not for sporadic drenches in outbreaks,

2   which mainly involved triclabendazole or closantel. Eggs of the *F. hepatica* isolate were

3   recovered from the bile ducts of two sacrificed sheep, and subsequently maintained in

4   donor animals and snails under laboratory conditions at the "Centro de Diagnóstico e

5   Investigaciones Veterinarias" (CEDIVE), Facultad de Ciencias Veterinarias, Universidad

6   Nacional de La Plata, Chascomús, Argentina.

7

8   **Experimental trials**

9   The drug-susceptibility characterization of the CEDIVE isolate of *F. hepatica*

10   involved two separate experiments. ***Experiment 1:*** Each animal was orally infected

11   with two hundred (200) metacercariae of the *F. hepatica* CEDIVE isolate. Sixteen

12   weeks after infection, animals were randomly distributed into two experimental

13   groups (n= 4 each): Control Group, which represented the untreated control and the

14   ABZ Group, where the animals were treated with ABZ (Baxen 3.8%[®], Tecnofarm,

15   Argentina) by the i.r. route at the dose of 7.5 mg/kg. The ABZ reference product

16   (Valbazen, Pfizer) was discontinued in Argentina. Since there were not available data

17   on the plasma exposure of ABZ/metabolites after the use of the Baxen 3.8%

18   formulation and, in order to discard treatment failures derived from the use of a poor

19   quality formulation, a plasma pharmacokinetic study was also performed. For the

20   pharmacokinetic study blood samples were taken by jugular venipunctures into

21   heparinized Vacutainers[®] tubes (Becton Dickinson, USA) before administration (time

22   0) and at 1, 3, 6, 9, 12, 15, 24, 28, 32, 48 and 54 h post-treatment. Plasma was

23   separated by centrifugation at 3000 g for 15 min, placed into plastic tubes and frozen

24   at -20 ºC until analysis by high performance liquid chromatography (HPLC).

25

1 *Experiment 2:* Each experimental animal was orally infected with two hundred (200)

2 metacercariae of the *F. hepatica* CEDIVE isolate. Sixteen weeks after infection,

3 animals were randomly distributed into two experimental groups (n= 4 each): Control

4 Group, which represented the untreated control and TCBZ Group, in which animals

5 were treated with TCBZ (Fasinex[®], Novartis, Argentina) by the i.r. route at the dose

6 of 10 mg/kg.

7

8 **Clinical efficacy study**

9 Fifteen (15) days after treatment all animals were stunned and exsanguinated

10 immediately. Adult *F. hepatica* specimens were recovered from the main bile ducts

11 and the gall bladder of each sheep and counted according to the World Association for

12 the Advancement of Veterinary Parasitology (W.A.A.V.P) guidelines (Wood et al.,

13 1995). The efficacy of each anthelmintic treatment was determined by the comparison

14 of *F. hepatica* burdens in treated versus untreated animals. The following equation

15 expresses the percent efficacy (*% E*) of a drug treatment against *F. hepatica* (*F.h.*) in a

16 single treatment group (*T*) when compared with an untreated control (*C*).

17

18 $$\% E = \frac{\text{Mean of } F.h. \text{ in } C \text{ - mean of } F.h. \text{ in } T}{\text{Mean of } F.h. \text{ in } C} \cdot 100$$

19

20

21 The geometric mean was used as it most accurately represents the distribution of

22 parasite populations within each group (Wood et al., 1995).

23

1 **Analytical procedures**

2 *Plasma sample extraction:* ABZ and its metabolites were extracted from plasma and

3 quantified by HPLC as previously described (Alvarez et al, 2008). The HPLC was a

4 Shimadzu 10 A System (Kyoto, Japan), which include a gradient pump, a UV detector

5 set at 292 nm, an autosampler and a controller (Shimadzu Class LC10, Kyoto, Japan).

6 Analytes were identified by the retention times of pure reference standards. Retention

7 times for ABZSO, $ABZSO_2$, OBZ, and ABZ were 5.32, 7.24, 9.55, and 11.14 min,

8 respectively. There was no interference of endogenous compounds in the

9 chromatographic determinations. Calibration curves for each analyte were prepared

10 by least squares linear regression analysis, which showed correlation coefficients

11 between 0.996 and 0.999. The absolute recovery of drug analytes from plasma was

12 calculated by comparison of the peak areas from spiked plasma samples with the peak

13 areas resulting from direct injections of standards in mobile phase. Mean absolute

14 recoveries and coefficient of variations (CV) within the concentration range between

15 0.1 and 4 μg/ml (triplicate determinations) were 91.3% (CV: 6.51%) (ABZ), 89.2%

16 (CV: 5.40%) (ABZSO) and 92.2% (CV: 6.72%) ($ABZSSO_2$). Precision (intra- and

17 inter-assay) was determined by analysing replicates of fortified plasma samples (n= 5)

18 with each compound at three different concentrations (0.1, 0.5 and 1 μg/ml). CV

19 ranged from 3.50 to 12.5%. The limit of detection (LOD) was estimated by

20 integrating the baseline threshold at the retention time of each compound in five non-

21 spiked plasma samples. The LOD was defined as the mean 'noise'/internal standard

22 peak area ratio plus 3 standard deviations (SD). The limit of quantification (LOQ) was

23 defined as the lowest measured concentration with a CV <20% and accuracy of ±20%

1   and an absolute recovery ≥70%. The LOQ defined for the three molecules assayed

2   was 0.1 µg/ml. Values below LOQ were not included in the pharmacokinetic analysis.

3

4   **Pharmacokinetic analysis of the data**

5   Noncompartmental pharmacokinetic calculations for the concentration versus

6   time curves for ABZ metabolites in plasma for each individual animal after the

7   different treatments were conducted using the PK Solution 2.0 software (Summit

8   Research Services, CO, USA). The observed peak concentration (C_{max}) and time to

9   peak concentration (T_{max}) were read from the plotted concentration-time curve of each

10   analyte. The elimination ($T\frac{1}{2}el$) half life was calculated as $\ln2/\beta$, where ß represent

11   the terminal slope (h^{-1}). The area under the concentration time-curve (AUC) was

12   calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated

13   to infinity by dividing the last experimental concentration by the terminal slope (β).

14   Statistical moment theory was applied to calculate the mean residence time (MRT) for

15   metabolites in plasma, as follows: MRT= AUMC/AUC, where AUC is as defined

16   previously and AUMC is the area under the curve of the product of time and the

17   plasma drug concentration versus time from zero to infinity (Gibaldi and Perrier,

18   1982).

19

20   **Statistical analysis of the data**

21   Pharmacokinetic parameters are presented as mean ± SD. Fluke counts in each

22   experimental group within each experiment were compared by non parametric test

23   (Mann-Whitney Test). A value of $P<0.05$ was considered statistically significant.

24

25

9

1 **Results**

2 Table 1 shows the parasite counts and the clinical efficacy (%) for ABZ and TCBZ

3 against the CEDIVE isolate of *F. hepatica* in sheep. Efficacy values from 29% (ABZ)

4 and 100 % (TCBZ) were observed. No statistical differences (P>0.05) were observed

5 in fluke counts between ABZ treated and untreated control groups. Furthermore, a

6 significantly (P<0.05) decrease in fluke counts were observed in TCBZ treated

7 animals, in comparison to the counting in untreated animals.

8

9 $ABZSO$ and $ABZSO_2$ were the only analytes recovered in plasma after the i.r.

10 administration of ABZ. The active ABZSO was the main metabolite measured in

11 plasma up to 48 h post-treatment. The comparative mean (± SD) plasma concentration

12 profiles of ABZSO and $ABZSO_2$ obtained after the i.r. administration of ABZ are

13 shown in Figure 1. The plasma disposition kinetics data for ABZSO and $ABZSO_2$

14 after i.r. administration of ABZ are summarized in Table 2. The plasma exposure

15 (expressed as AUC and Cmax) of ABZ metabolites observed in the ABZ treated

16 animals (Experiment 1), was in agreement with previous data obtained in our

17 laboratory (Table 3). The ABZSO AUC value obtained after ABZ administration in

18 the current experiment (41.1 ± 13.9 µg.h/ml) was similar to that obtained by Alvarez

19 et al, 1997 (41.8 ± 2.28 µg.h/ml), Alvarez et al, 1999 (46.0 ± 5.72 µg.h/ml) and

20 Moreno et al., 2004 (31.2 ± 5.43 µg.h/ml), in which the reference formulation of ABZ

21 (Valbazen®, ABZ 10%, Pfizer Animals Health, Argentina) was used. A similar

22 pattern was observed for the Cmax parameter.

23

1 **Discussion**

2 The results reported here clearly demonstrate that the CEDIVE isolate of *F. hepatica*

3 behaves as resistant to ABZ and susceptible to TCBZ. Interestingly, in the farm

4 where the CEDIVE isolate of *F. hepatica* was obtained, anthelmintic treatments are

5 only addressed to control GI nematodes. However, it seems clear that the high

6 selection pressure exerted by this practice has contributed to the development of

7 resistance not only in nematodes but even so in liver flukes.

8

9 The manufacturing process, the quality of the active ingredient/excipients and their

10 long standing stability, among other factors involved in the production of generic

11 formulations (suspensions), may substantially affect the drug dissolution process and

12 its consequent GI absorption, which in turns could affect drug effectiveness. In fact,

13 factors related to the quality of the active ingredient have been associated with

14 therapeutic failure of generic rafoxanide formulations against *Haemonchus contortus*

15 in sheep (Van Wyk *et al.*, 1997). Furthermore, it has been recently demonstrated that

16 different generic ABZ formulations commercialized in Uruguay could not be assessed

17 as bioequivalent to the pioneer preparation (Suarez et al., 2011). In the current work,

18 the plasma drug exposure of ABZ metabolites observed after its administration as a

19 "generic" formulation (Baxen 3.8%[®]), resulted similar to that reported after the

20 administration of the reference formulation (Valbazen[®]) in sheep at the same dose.

21 The AUC and Cmax values were in the range of those reported by Alvarez et al,

22 1997; 1999 and Moreno et al., 2004. These results discard the fact that the observed

23 ABZ failure to control liver flues may have given by a poor quality formulation

1   resulting in reduced drug systemic exposure. This was not the case in the work

2   reported here.

3

4   The intrinsic anthelmintic action of BZD compounds relies on a progressive

5   disruption of basic cell functions as a result of their binding to parasite β-tubulin and

6   depolimerization of microtubules (Lacey, 1988). BZD resistance in nematodes has

7   been linked to the loss of high-affinity binding to tubulin (Lubega and Prichard,

8   1991a) and an alteration of the β-tubulin isoform pattern (Lubega and Prichard,

9   1991b), correlated with a conserved mutation at amino acid 200 (phenylalanine to

10   tyrosine: F200Y) in tubulin isotype 1 (Kwa et al., 1994). While experimental data

11   supports a microtubule-based action for TCBZ (reviewed by Fairweather, 2005), it

12   has been shown that the TCBZ-resistant phenotype is not associated with residue

13   changes (specifically, the F200Y mutation) in the primary amino acid sequence of β-

14   tubulin (Robinson et al., 2002). In fact, the accumulated *in vitro* data demonstrate that

15   at least two mechanisms appear to be implicated in TCBZ resistance in *F. hepatica*:

16   increased drug efflux and enhanced oxidative metabolism (Robinson et al., 2004;

17   Alvarez et al., 2005a; Mottier et al., 2006; Devine et al., 2009; Devine et al., 2010).

18   A previous study has shown that ABZ is active against a TCBZ-resistant isolate of *F.*

19   *hepatica* (Coles and Stafford, 2001). The greater trans-tegumental diffusion capability

20   of ABZ compared to TCBZ under *ex vivo* conditions, may account for its efficacy

21   pattern against TCBZ-resistant flukes (Alvarez et al., 2005b). However, the CEDIVE

22   isolate results resistant to ABZ and susceptible to TCBZ. These results may indicate

23   that a different mechanism could be implicated in the development of resistance to each

24   compound, it is likely that TCBZ may target a molecule other than ß-tubulin, which

1 would explain why ABZ continues to act against TCBZ-resistant flukes. To complicate

2 the picture, resistance to ABZ and TCBZ in *F. hepatica* has been reported in Spain

3 (Alvarez-Sanchez et al., 2006). It is, therefore clear, that the mechanism involved on

4 ABZ resistance in *F. hepatica* need to be elucidated.

5

Conclusions

7 The data reported here indicate that the CEDIVE isolate of *F. hepatica* is resistant to

8 ABZ and susceptible to TCBZ. The fact that the CEDIVE isolate is maintained under

9 laboratory conditions, may help to further understand the mechanism(s) of ABZ

10 resistance in *F. hepatica*.

11

15

Figure captions

17 Figure 1: Comparative mean (±SD) plasma concentration profiles (n= 4) for

18 albendazole sulphoxide (ABZSO), after the administration of albendazole

19 (Baxen3.8%®, 7.5 mg/kg) to sheep infected with *Fasciola hepatica*.

20

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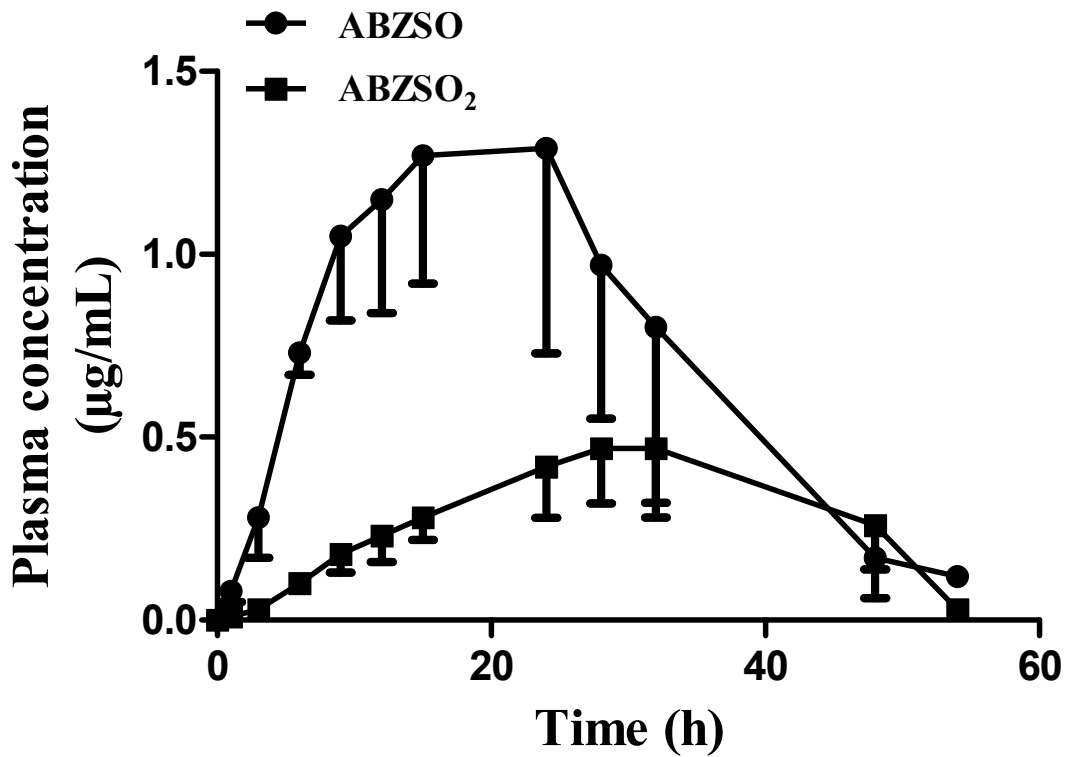21   (bovine, ovine, caprine). Vet. Parasitol. 58, 181-213.

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23

24

1   **Figures**

2   **Figure 1. ABZSO and ABZSO2 plasma concentrations** in *Fasciola hepatica*-

3   infected sheep (mean and standard deviations).

4

5

6   <u>**Table 1.**</u> Individual and mean fluke counts and clinical efficacy (%) against the

7   CEDIVE *Fasciola hepatica* isolate, obtained after the administration of albendazole

8   (ABZ, 7.5 mg/kg, i.r.) or triclabendazole (TCBZ, 10 mg/kg, i.r.) in sheep.

9

Animal	Control Group	ABZ Group	Control Group	TCBZ Group

# 1	112	60	27	0
# 2	67	25	23	0
# 3	43	69	15	0
# 4	67	55	29	0
Arithmetic mean	72.3	52.3	23.5	0
Efficacy*	-	**29 %**		**100%**

1   * The efficacy was calculated using geometric means.

2

3

4   **Table 2.** Plasma pharmacokinetic parameters (mean ± SD) for albendazole

5   sulphoxide (ABZSO) and albendazole sulphone (ABZSO$_2$) obtained after the

6   intraruminal (i.r.) administration of albendazole (ABZ, 7.5 mg/kg) to *Fasciola*

7   *hepatica* infected sheep.

8

PHARMACOKINETIC PARAMETERS	ABZSO	ABZSO$_2$
Cmax (µg/mL)	1.40 ± 0.47	0.50 ± 0.10
Tmax (h)	16.5 ± 5.20	30.0 ± 2.30
AUC$_{0-t}$ (µg.h/mL)	41.1 ± 13.9	15.9 ± 5.30
T½el (h)	8.70 ± 0.80	7.30 ± 0.60
MRT (h)	23.1 ± 2.14	29.6 ± 1.80

9

1   **Table 3.** Comparative peak plasma concentration (Cmax)and area under the

2   concentration vs time curve (AUC) for albendazole sulphoxide (ABZSO), obtained

3   after the i.r. administration of albendazole (7.5 mg/kg) as a 3.8% formulation (Baxen

4   3.8%[®], Tecnofarm, Argentina, current work) or as a 10% formulation (Valbazen,

5   Pfizer Animal Health, Argentina, reference formulation)(works from Alvarez et al.,

6   1997; 1999 and Moreno et al., 2004) to sheep.

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ABZSO PK Parameter	Current work	*Alvarez et al., 1997*	*Alvarez et al., 1999*	*Moreno et al., 2004*
Cmax (µg/ml)	1.40 ± 0.47	1.76 ± 0.16	1.92 ± 0.26	1.23 ± 0.24
AUC (µg.h/ml)	41.1 ± 13.9	41.8 ± 2.28	46.0 ± 5.72	31.2 ± 5.43

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