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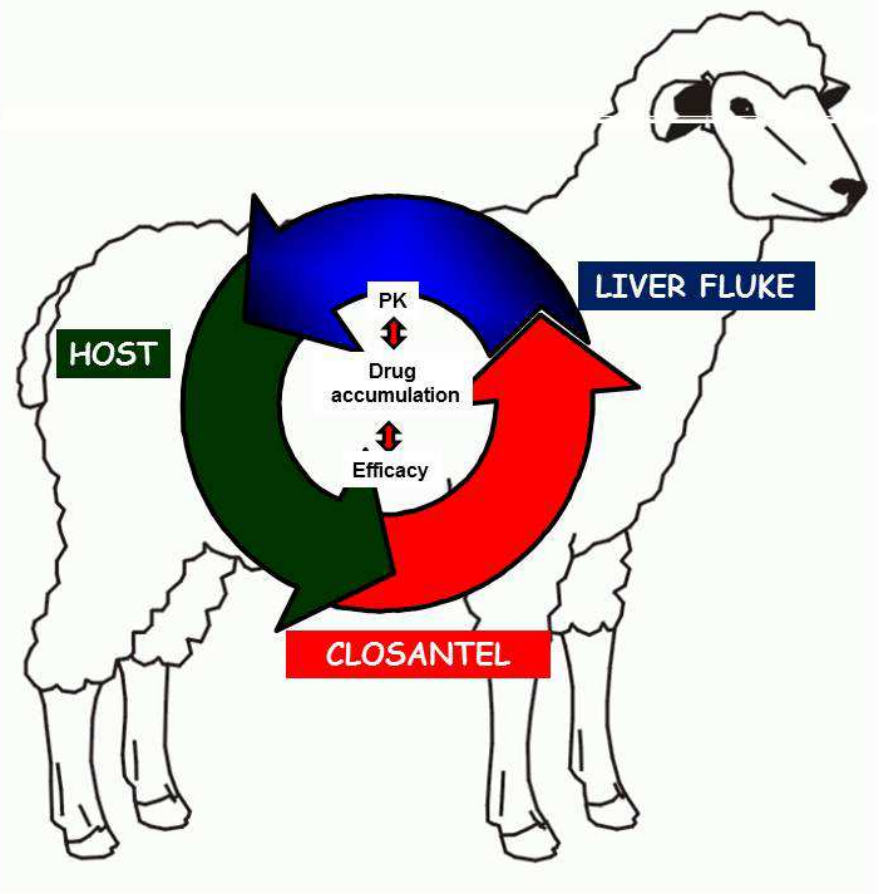

Understanding the main route of drug entry in adult *Fasciola hepatica*: further insights into closantel pharmacological activity

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Running title: In vivo *closantel accumulation in liver flukes*

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Abstract

Closantel (CLS) is highly effective against adult liver flukes after its oral or subcutaneous (sc) administration in ruminants. Trans-tegumental diffusion and oral ingestion are the two potential routes available for the entry of drugs into *Fasciola hepatica*. The work reported here contributes to improve the understanding of CLS pharmacology. The main goals of were: I) to determine the pattern of *in vivo* CLS accumulation into adult *F. hepatica* and relevant tissues in CLS-treated sheep; II) to investigate the influence of the physicochemical composition of the incubation medium on the CLS diffusion process into adult *F. hepatica*; III) to assess the ovicidal activity of CLS against *F. hepatica* eggs; and IV) to investigate the *in vivo* effect of CLS treatment on glutathione S-transferases activity in adult liver flukes exposed to CLS. Fourteen healthy sheep were each orally infected with 75 *F. hepatica* metacercariae. Sixteen (16) weeks after infection, animals were treated with CLS by oral (n= 6, 10 mg/kg) or sub-cutaneous (sc) (n= 6, 5 mg/kg) route. At 12, 24 and 36 h post-treatment, animals were sacrificed (n= 2) and samples of blood, bile and adult *F. hepatica* were collected. In addition, flukes recovered from non-treated sheep (n= 2) were *ex vivo* incubated (60 min) in the presence of CLS in either RPMI or bile as incubation medium. CLS concentration was measured by HPLC. The ovicidal activity of CLS was investigated using eggs obtained from the bile of untreated sheep. Finally, glutathione S-transferase activity in *F. hepatica* recovered from untreated and CLS-treated sheep was assessed. In the *in vivo* studies, the highest CLS concentrations were measured in plasma and adult liver flukes. A positive correlation was observed between CLS concentration in plasma and in *F. hepatica*. Results obtained in the current work indicate that the *in vivo* accumulation of CLS into adult liver flukes occurs mainly by the

oral route. After *ex vivo* incubation, the uptake of CLS by the parasite was markedly diminished in the presence of bile compared with that observed in the presence of RPMI as incubation medium. CLS lacks ovicidal activity at therapeutically relevant concentrations. Lastly, CLS significantly increased glutathione S-transferase activity in flukes recovered at 12 h (oral treatment) and 24 h (sc treatment), compared to the control liver flukes.

Keywords: closantel; Fasciola hepatica; drug distribution

1. **INTRODUCTION**

Fasciola hepatica is a parasitic flatworm (class Trematoda) responsible for *Fascioliasis,* a foodborne and waterborne zoonotic disease. In mammals, including sheep and cattle, this disease can cause considerable financial losses due to morbidity and mortality (Andrews, 1999; Kaplan, 2001). In Argentina, Mesopotamia and Patagonia are considered endemic areas (Olaechea et al., 2013). As a concern, human fascioliasis is currently recognized by WHO as one of the “neglected tropical diseases”, with an estimated 2.4-17 million people infected and 180 million at risk of infection (WHO, 2010). A significant number of cases were reported in Andean South America, Egypt, and the Bolivian Altiplano, in which a prevalence of up to 72% was reported (Mas Coma et al., 1999; Esteban et al., 1999, 2003).

The main strategy for liver fluke control is still based largely on chemical treatments with five main chemical groups: benzimidazoles (albendazole and triclabendazole), halogenated phenols (niclofolan and nitroxynil), salicylanilides (closantel, rafoxanide) and sulphonamides (clorsulon) (Boray, 1997; Malone, 1984). Of all of them, triclabendazole is considered the drug of choice against *F. hepatica*, due to its high efficacy against both immature and mature stages (Boray et al., 1983). The likely mode of action of the main flukicidal compounds has been described, as well as the stage of liver fluke that they affect (Fairweather and Boray, 1999). However, many elements of the drug-host-parasite relationship are poorly known for most flukicidal compounds, including the salicylanilides. Such information is important, as it will help to improve the understanding of the pharmacokinetic-efficacy relationship.

Closantel (CLS) (N-(5-chloro-4-((4-chlorophenyl)(cyano)methyl)-2-methylphenyl)-2-hydroxy-3,5-diiodobenzamide) is a salicylanilide-derived compound, used in cattle and sheep either for treating liver fluke ≥7-8 weeks old (Mohammed-Ali and Bogan, 1987), or against some nematodes and ectoparasites (Michiels et al.,1987; Lanusse et al., 2009). CLS is a lipophilic drug formulated for oral and sc administration (EMA, 2012). Similarly to other salicylanilides, CLS is extensively bound to plasma proteins (>99%, mainly albumin), which prolongs the therapeutic levels in plasma and limits the tissue distribution (McKellar and Kinabo, 1991). CLS acts as an uncoupler of oxidative phosphorylation in the mitochondria of liver fluke (Kane et al., 1980), leading to an increase of glucose uptake, decrease of glycogen content and decrease in ATP synthesis (Fairweather and Boray., 1999). Moreover, CLS disrupts the mechanisms responsible for maintenance of pH homeostasis and induces spastic paralysis in liver fluke due to an increase in calcium ion concentration in parasite muscle cells (Prichard, 1978). This particular mode of action requires CLS accumulation into cells of the target parasite. Consequently, its therapeutic effect will depend on its ability to reach high and sustained concentrations in the parasite, and to bind its specific receptor for sufficient time to cause the therapeutic effect.

CLS is effective against the adult stage of *F. hepatica*, a blood-consuming parasite located in the bile ducts of mammalian hosts. Plasma protein binding may have an important role in the accumulation of drug into the parasite due to oral ingestion. Therefore, potentially the fluke could be exposed to CLS present in the bile (surrounding medium).There is no data available for either the *in vivo* accumulation of CLS into adult liver flukes or its diffusion capacity through the fluke's tegument. Consequently, the contribution of oral ingestion and/or diffusion through the external surface as potential mechanisms of the

entry of CLS into *F. hepatica* needs to be clarified further. Additionally, flukes that reach the bile ducts become sexually mature and begin laying eggs, which potentially will be exposed to drug concentrations present in the bile. Although some benzimidazole methylcarbamate compounds have demonstrated activity against *F. hepatica* eggs (Coles and Briscoe, 1978; Alvarez et al., 2009), so far no data on the direct ovicidal activity of CLS against *F.hepatica* eggs is available.

Glutathione S-transferases (GSTs) are a family of multifunctional proteins which occur abundantly in most organisms. In fact, GST appears to be one of the major detoxification enzymes in parasitic helminths (Precious and Barrett, 1989). GSTs are essentially involved in the intracellular detoxification of numerous substances, including chemotherapeutic agents, and thus play a major role in the development of drug resistance (Cazenave et al., 1989; Hemingway et al., 2004; Scarcella et al., 2013). GSTs can also serve as non-enzymatic binding proteins involved in intracellular transport (Listowsky et al., 1988) and signalling processes (Cho et al., 2001). Previous work has shown that *F. hepatica* contains high levels of GST (Howell et al., 1988; Brophy et al., 1990a, 1990b). The impact of CLS treatment on GST activity in CLS-susceptible liver flukes may help to understand the physiological role of this important enzyme family in *F. hepatica*.

In spite of the CLS is an “old drug” a further comprehension of its pharmacology properties may be useful to optimize its flukicidal activity. To improve our understanding of the relationship between the plasma disposition kinetics and CLS accumulation into the liver fluke, the concentration profiles of CLS in plasma, adult parasites, bile and liver obtained

from CLS-treated sheep were assessed. In addition, the influence of bile on CLS diffusion under *ex vivo* conditions, the effect of CLS on the *in vitro* hatching of *F. hepatica* eggs, and its *in vivo* effect on GST activity in adult *F. hepatica* were also investigated.

2. MATERIALS AND METHODS

The study was conducted on 14 healthy male Corriedale sheep. Their age ranged from 14 to 16 months. The animals were obtained from a farm located in an area free of *F. hepatica* infection. Additionally, the absence of liver fluke infection was checked by analysis of *F. hepatica* eggs in faeces, following routine procedures (MAFF, 1986). During the experiment and for 20 days before, animals were kept indoors and fed with a commercial balanced concentrate diet and supplied with water *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 and internationally accepted animal welfare guidelines (AVMA, 2001).

2.1. In vivo *drug accumulation assay*

Animals (14) were each orally infected with seventy five (75) metacercariae of a TCBZ-susceptible *F. hepatica* isolate, named Cullompton, which behaves as being susceptible to most flukicidal compounds, including CLS and triclabendazole (Fairweather, 2011). The infection was individually checked at twelve (12) weeks after infection by presence of *F. hepatica* egg in feces. Sixteen weeks after infection, animals (two animals were kept without treatment as controls) were randomly allocated into two experimental groups (n= 6) and treated with either CLS at 10 mg/kg orally (Adevet C oral 15%, Vetanco, Argentina)

or at 5 mg/kg sc (Adevet C inyectable/injectable 15%, Vetanco, Argentina). At 12, 24 and 36 h post-treatment (p.t.) two animals from each group were killed and samples of blood, bile, liver and liver fluke specimens were obtained. The collected blood (into heparinized tubes) was immediately centrifuged at 3000 *g* for 15 min to obtain plasma. To recover adult *F. hepatica* 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 and blotted on coarse filter paper. All obtained samples were placed into plastic tubes and frozen at -20ºC until analysis by high performance liquid chromatography (HPLC). Additionally, samples of plasma, bile, liver and *F. hepatica* were obtained from untreated control animals (which were infected two weeks after of the rest of animals). These samples were used in either the ex vivo drug diffusion or ovicidal activity assays described below.

2.2. Ex vivo *assays*

Sixteen weeks after infection, the animals belonging to the control group were killed and adult specimens of *F. hepatica* were recovered as previously mentioned in section 2.1. In addition, parasite eggs were directly recovered from the bile ducts. The *ex vivo* experiments were performed after the completion of the *In vivo* drug accumulation assay, in order to determine the pharmacologically relevant concentration of CLS to be used in the *ex vivo* experiments.

2.2.1. Ex vivo *drug diffusion assay*

Adult liver flukes (approximately 0.1 g in weight) collected from untreated animals were incubated at 37 ºC in either RPMI-1640 buffer (1 mL) or bile (1 mL) and spiked with CLS

(10 µL/tube) dissolved in methanol. The incubation time was 60 min, and the final concentration of CLS in the incubation medium was 3 µg/mL. This is a pharmacologically relevant concentration obtained after the *in vivo* drug accumulation assays described here. For each incubation medium, a single experiment was performed with five (5) replicates. Blank samples containing parasite material and incubation medium (either RPMI-1640 buffer or bile) without drug (spiked with 10 µL of methanol), were incubated for the same time interval. Once the incubation time had elapsed, the flukes were rinsed thoroughly with saline solution, blotted on coarse filter paper and stored at -20 ºC until their preparation for HPLC analysis to measure drug concentrations.

2.2.2. Ex vivo *ovicidal activity of CLS*

F. hepatica eggs were directly recovered from the bile ducts of untreated infected sheep. After several washes with tap water, eggs were suspended in water (approximately 200 eggs/mL, n= 5) and incubated (at 25 ºC) for a 12-h period with CLS at a final concentration of 3 µg/mL. A 12-h period of drug-egg contact represents the approximate time of egg exposure to the drug after an *in vivo* treatment (Alvarez et al., 2009). Untreated eggs were incubated as controls. Untreated and treated eggs were gently washed (x 3) to facilitate drug removal, and kept in darkness at 25 ºC for 15 days. After this period, the trematode eggs were exposed to daylight for 6 h. When this time had elapsed, 0.1 mL of 10% (v/v) buffered formalin was added to each tube in order to stop egg hatching. Hatched and unhatched eggs were evaluated using an optical microscope (x40 magnification) (Leica DM IL LED, Wetzlar, Germany). Approximately 100 eggs were counted to estimate the proportion of hatched eggs in each tube.The “ovicidal activity” expressed as a percentage was estimated using the following formula:

Ovicidal activity % = (% eggs hatched in control - % eggs hatched after drug incubation)/ % eggs hatched in control) x 100

2.3. *GST assay*

The GST activity was assayed in parasites recovered from animals used in either **In vivo *drug accumulation assay*** (n=12; sacrificed at 12, 24 and 36 h p.t.) or **Ex vivo *assays*** (n=2; untreated controls). Adult *F. hepatica* specimens were recovered from the common bile ducts. The parasites were rinsed extensively with saline solution (NaCl, 0.9%, 38 ºC) to remove bile and/or adhering materials, blotted on coarse filter paper and placed in plastic tubes. From each animal, samples of 4 *F. hepatica* specimens were pooled and processed independently. The samples were kept in a freezer (−80°C) until the subcellular fractions were prepared. GST activity was assayed spectrophotometricaly (Shimadzu RF-5301PC; Shimadzu Corporation, Kyoto, Japan) in the cytosol-like fractions obtained from homogenates (two homogenates of 4 adult liver flukes from each animal) prepared from *F. hepatica* specimens recovered from untreated (control) and CLS-treated sheep. Cytosolic-like GST activity was determined using 1-chloro-2,4-dinitrobenzene as a secondary substrate (340 nm) (Habig and Jakoby, 1981). Protein content was determined using the Lowry method with bovine serum albumin as standard (Lowry et al., 1951).

2.4. *Analytical procedures*

Pure reference standards (99% purity) of CLS and demethylated CLS (as internal standard, IS) were gently donated by Janssen Pharmaceutica (Beerse, Belgium), and they were used to validate the HPLC method.

2.4.1. *Extraction of drug from plasma samples*

CLS was extracted from plasma by a method adapted from Iessi et al. (2014). Briefly, plasma samples (1 mL) were spiked with internal standard (IS). After addition of 1 mL of acetonitrile and deionized water (0.25 mL), samples were shaken for 20 min (multi-tube vortexer, VWR Scientific Products, West Chester, PA, USA). The batch of tubes containing the mixtures was placed in an ultrasonic bath (Ultrasound Bath, Lab-Line Instrument, Inc., Melrose Park, OL, US) for 10 min and then centrifuged at 2500 *g* for 15 min (Jouan®, BR 4i Centrifuge, Saint Herblain, France). The supernatants were recovered and the precipitates obtained from the samples were extracted again with 1 mL of acetonitrile as described above. After that, the supernatants were evaporated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, USA). The dry extracts were reconstituted in 250 µL of mobile phase and an aliquot of 50 µL was injected into the HPLC system.

2.4.2. Extraction of drug from bile samples

CLS was extracted from bile samples (0.2 mL). After spiking with the IS, the samples were added to 1 mL of acetonitrile and shaken for 10 min, then centrifuged at 2500 *g* for 15 min (4ºC). The supernatants were recovered and the process repeated. For cleaning, the total supernatant was added to 1 mL hexane, shaken, and recovered. The total supernatant was evaporated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, USA). The dry extracts were reconstituted in 250 µL of mobile phase and an aliquot of 50 µL was injected into the HPLC system.

2.4.3. Extraction of drug from F. hepatica

Parasite material (0.1 g) was homogenized and spiked with IS. The liver fluke homogenate was mixed with1 mL of acetonitrile plus 0.25 mL of deionized water and shaken (multi-tube vortexer) for 5 min and then centrifuged to allow phase separation (3500 *g* 10 min, 10ºC). This procedure was repeated twice. The final collected acetonitrile plus water phase (2.5 mL) was concentrated to dryness in a vacuum concentrator and then reconstituted with 250 µL of mobile phase. An aliquot of 50 µL was injected into the HPLC system.

2.4.4. 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 the IS, samples were added to 0.5 mL of NaOH (1 N). Molecules were extracted by addition of 1.5 mL acetonitrile for 15 min under a high-speed vortexing shaker. After mixing, the samples were sonicated for 10 min and centrifuged at 2500 *g* for 15 min at 4ºC. The clear supernatant was transferred to a tube, and the procedure repeated. The collected supernatant was concentrated to dryness in a vacuum concentrator and then reconstituted with 250 µL of mobile phase. Fifty (50) µL of the reconstituted volume was injected into the chromatographic system.

2.4.5. 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 CLS. Fifty (50) µL of each previously extracted sample was injected by an autosampler (Shimadzu SIL 10AF Automatic Sample Injector) into a Shimadzu LC-20A HPLC system (Shimadzu Corporation, Kyoto, Japan), fitted with a Kromasil C18 (5 mm, 250 x 4.60 mm) reverse-

phase column (Eka Chemicals, Brewster, NY, USA) at 30°C and a fluorescence detector (Shimadzu; RF 10A XL detector) reading at 335 nm excitation and 510 nm emission. The mobile phase consisted of acetonitrile-water (15:85 v/v) containing 0.05% diethylamine at pH 2.5, adjusted with phosphoric acid with a flow rate set at 1.5 mL/min. The total run time for the method was 25 min. The analytes were identified with the retention times of 99% pure reference standards. Chromatographic peak areas of each molecule were measured using the integrator software (Class LC 10; Shimadzu Corporation) of the HPLC system. Calibration curves for CLS 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 0.5-70 µg/mL (triplicate determinations) were >70% in all cases with CV ranging between 5-10 %. The limit of detection (LOD) was defined as the mean "noise"/internal standard peak area ratio plus 3 standard deviations (SD). The LOD obtained was 0.02 µg/mL. The limit of quantification (LOQ) was defined as the lowest measured concentration with a CV < 20% and accuracy of ±20% and an absolute recovery of >70%. The LOQ obtained for CLS in all biological matrices assayed was 0.05 µg/mL. Values below LOQ were not included in the pharmacokinetic analysis.

2.5. Analysis of the data

Data are expressed as arithmetic mean ± standard deviations (SD). The area under the concentration–time curve (AUC) for CLS in each assayed tissue/fluid (at least four concentrations were included in the determination) 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. Correlation between individual plasma/bile and *F. hepatica* CLS concentrations was performed by parametric analysis (Pearson r, r^2). For the *ex vivo* diffusion assay, the Student's t-test was used to compare drug concentrations obtained at 60 min of incubation in the two different incubation media. For the ovicidal activity assessment, data are presented as eggs hatched (%) ± SD, and the Student's t-test was used to statistically compare both groups. The Mann-Whitney test was used to compare GST activity in adult liver flukes recovered from CLS-treated sheep with the activity in worms recovered from untreated control sheep. In all cases, values were considered different at $P<0.05$.The statistical analysis was performed using the Instat 3.0 Software programme (Graph Pad Software, SanDiego, California).

3. RESULTS

3.1. In vivo *drug accumulation assay*

CLS was recovered in plasma, *F. hepatica*, bile and liver from 12 and up to 36 h pt; their mean concentrations (µg/mL or µg/g) are summarized in **Table 1.** In all matrices evaluated, the peak concentration was reached at 36 h pt (last sampling point). The highest concentrations of CLS were measured in plasma and *F. hepatica*, after both oral and sc administration. No statistical differences ($P>0.05$) in CLS concentration related to the route of administration were observed in the different tissue/fluids assayed. The AUC values obtained for CLS in the different biological matrices is shown in **Fig. 1**. Similar AUC

values were obtained within each matrix after both administration routes. However, the CLS AUC values were higher in plasma and *F. hepatica* than those measured in bile and liver (Table 1, Fig. 1). After both administration routes, the AUC of CLS in bile represented approximately 10 % of that observed in plasma (**Table 1**).

High correlation coefficients between CLS concentrations in *F. hepatica* and plasma were observed, with a Pearson coefficient value (r^2) of 0.705 and a P value of 0.036. Conversely, the correlation between bile and *F. hepatica* CLS concentrations did not show a statistically significant correlation (P value 0.357). Similar results were obtained after the sc administration of CLS with a Pearson coefficient value (r^2) of 0.730 and a P value of 0.030 between CLS concentrations in *F. hepatica* and plasma.

3.2. Ex vivo *drug diffusion assay*

The comparison of CLS concentrations measured in liver flukes incubated in RPMI or bile as incubation medium is shown in **Fig. 2**. In the presence of bile, CLS concentrations recovered from the flukes ranged between 2.20 ± 0.27 µg/g of parasite (wet weight), equivalent to 28% of total drug measured after incubation of adult liver flukes in RPMI (8.10 ± 1.10 µg/g).

3.3. Ex vivo *ovicidal activity of CLS*

The mean egg hatch percentage obtained for untreated eggs ranged between 95 and 97.2% and for CLS treated eggs between 80.8 and 92.1%. The estimated ovicidal activity of CLS was 8.3%.

3.4. GST activity

GST activities in *F. hepatica* recovered from untreated control and CLS-treated sheep are shown in **Table 2**. GST activity was greater after both oral and sc CLS treatments. Significantly higher GST activity in treated than control flukes was observed at 12 and 24 (oral) and 24 (sc) h pt. After oral treatment, the GST activity measured at 36 h pt was significantly lower than that observed in untreated control flukes. A similar trend was observed after sc treatment, but in this case the differences did not reach statistical significance ($P > 0.05$).

4. DISCUSSION

Closantel belongs to the limited number of compounds currently available for the treatment of *F. hepatica* infections (McKellar and Kinabo, 1991). Despite its widespread use, there are still many aspects of the drug-host-parasite relationship that remain unknown. Knowledge of drug concentrations achieved in target parasites and the tissues/fluids surrounding them, will contribute to the understanding of the pharmacokinetic-efficacy relationship.

Mature *F. hepatica* is located in the biliary tract and has a haematophagous habit; therefore, CLS can reach its specific receptor within the parasite by oral ingestion or by diffusion through the external surface of the parasite, or some combination of both routes (Thompson and Geary, 2003). If the oral route pathway is the main route of drug access into the parasite, drug concentrations in blood will be more relevant in terms of flukicidal

activity compared to that present in bile, and *vice versa*. CLS was detected in plasma at the first sampling point (6 h pt) after both routes of administration, reaching concentrations in the bloodstream consistent with those previously reported in other pharmacokinetic studies carried out in sheep (Mohammed-Ali and Bogan, 1987; Hennessy and Ali, 1997). This is the first report of comparing CLS concentrations in *F. hepatica*, plasma, liver tissue and bile, after CLS treatment to infected sheep. The highest CLS concentrations measured up to 36 h pt were obtained in plasma and in *F. hepatica*. In contrast, the relative availability of CLS (expressed as AUC), either in bile or liver tissue, represented less than 20 % of those observed in plasma or *F. hepatica*. This is consistent with its limited tissue distribution due to the high CLS protein binding (greater than 99.9%) (Michiels et al., 1987), which also limits its volume of distribution and extends its terminal plasma half-life.

Similarly with triclabendazole, after treatment of sheep, the parent drug was detected in minimum quantities in bile and liver tissue, whereas higher concentrations of the main metabolites, triclabendazole sulphoxide and triclabendazole sulphone, were measured either in plasma, *F. hepatica* or bile (Moreno et al., 2014). It should also be pointed out that, conversely to either albendazole or triclabendazole which are rapidly metabolized after their administration to sheep and only their metabolites are detected in the bloodstream (Marriner and Bogan, 1980; Hennessy et al., 1987), CLS is poorly metabolized as 90% of the excreted compound corresponds to the parent drug (Michiels et al., 1987).

As previously mentioned, less than 10% of the CLS concentration detected in plasma was quantified in bile (approx. 3 µg/mL after both routes). The acquisition of broad-spectrum anthelmintics by liver flukes could be by passive diffusion through their tegument (Alvarez et al., 2000, 2001). However, drug diffusion depends on different factors, such as molecule lipophilicity, molecular size, concentration gradient, surface area of contact between drug and parasite (Mottier et al. 2006) and the physico-chemical properties of the surrounding parasite environment (Alvarez et al., 2004). Clearly, under *ex vivo* conditions, the presence of bile modified the diffusion and accumulation of CLS into the parasite, decreasing it by 72 % compared with that observed after incubation in RPMI medium. Only 7.4 % of drug available in ovine bile was able to diffuse into the parasite. Similar results were previously described by Alvarez et al. (2004), when evaluating the diffusion of albendazole, triclabendazole and fenbendazole into *F. hepatica* in the presence of bile or KRT as incubation medium. According to the authors, although all drugs diffuse across the parasite's tegument, the rates of penetration were different in the presence of KRT buffer or bile and the diffusion of the three different molecules was significantly higher in the absence of bile. The same results were obtained in the current work. It is likely that this could be explained by the presence of amphiphilic bile components which induce the micellar solubilization of drug, reducing the proportion of free drug in solution and thus decreasing drug penetration through the parasite tegument.

F. hepatica follows a life cycle which comprises four essential phases, one of which involves the production eggs by the adult fluke which reach the external environment by way of bile and the intestine. Eggs shed with the host's faeces will continue their development in fresh water, via miracidium, sporocyst and redial generations, production

of cercariae and shedding of the latter into water with the potential (in a few hours) to infect the definitive host (Mas Coma and Bargues, 1997). The pharmacological effect of a compound against *F. hepatica* would either be on the inmature and/or mature parasite or on the eggs present in bile. Previous studies have postulated that CLS uncouples oxidative phosphorylation in flukes and it is believed to have a significant neurotoxic effect that causes spastic paralysis, which results in detachment from the food source *in vivo* and starvation (Skuce and Fairweather, 1990). In addition, CLS reduces the availability of ATP, needed (for example) for the energy-demanding processes of gametogenesis and oogenesis, and it has been associated in cattle with a decrease in parasite egg production (Hanna et al., 2006). However, currently no study about the direct ovicidal activity of CLS against fully-formed *F. hepatica* eggs has been reported. The results obtained in the current work show that CLS at 3 µg/mL did not show ovicidal activity, since 92% of eggs *in vitro* exposed to CLS hatched after 12 h incubation. This result differs from the study carried out by Solana et al. (2016) that previously obtained, in which an inhibition of hatching of *F. hepatica* eggs recovered from adult liver flukes obtained from CLS-treated sheep at 36 h pt was reported (Solana et al., 2016).These contrasting results could be explained by the ability of CLS to affect the parasite and, at the same time, the normal development of eggs. However, CLS clearly failed to inhibit the hatching of “normal” *F. hepatica* eggs. The ovicidal action of a compound is dependent on its mode of action and its ability to penetrate the egg shell and accumulate within the egg. It has been suggested that egg hatching inhibition depends on the drug’s hydrophobic nature, where increased activity correlates with higher fat solubility (Lacey, 1988). While highly hydrophilic drugs fail to penetrate the egg shell, highly lipophilic drugs bind to the shell components and would fail to concentrate within the egg, where the target of CLS action is present. Furthermore,

CLS has a high affinity for different proteins, which could also account for the sequestration of CLS within the egg shell.

After oral and sc treatments, GST activity increased, reaching the highest value at 12 (oral) or 24 (sc) h pt, to decrease at 36 h pt to an activity level similar to (oral treatment) or lower than (sc treatment) that observed in adult liver flukes recovered from untreated sheep. GSTs have been detected in a wide range of parasitic helminths (Cvilink et al., 2009). In *F. hepatica*, GSTs represent 4% of the total soluble protein (approx) which appears to indicate an important physiological role for this enzyme system (Howell et al., 1988; Brophy et al., 1990a).The activity of GSTs is based mainly on the conjugation of compounds with electrophilic centers to the tripeptide glutathione (GSH) (Oakley, 2011). However, no conjugates of anthelmintic drugs with GSH have yet been identified in helminths (Matoušková et al., 2016). Helminth GSTs may function as drug-binding proteins, rather than as metabolizing enzymes (Brophy et al., 1990b). In fact, the GST present in *F. hepatica* binds to a number of anthelmintics, including CLS (Brophy et al., 1990b). It has been postulated that the potential passive binding of CLS to helminth GST makes it ineffective and therefore elevated GST activity could confer resistance (Rothwell and Sangster, 1997). As previously mentioned, the Cullompton isolate of *F. hepatica* involved in the current work behaves as CLS susceptible (Fairweather, 2011). Thus, the observed increase in GST activity fails to protect adult liver flukes against the deleterious effect of CLS. In fact, in contrast to most other studies, a strong positive correlation between GST activity and the efficacy of closantel in eliminating flukes has been reported (Miller et al., 1994). A lower GST activity in resistant parasites has been associated with a reduced drug uptake and accumulation into the liver fluke (Miller et al., 1994). The GST activity increase observed in the current study could be related to a “response” of the liver

fluke to reduce increased "toxic" concentrations of a xenobiotic such as CLS. However, the low metabolic fate of this compound, observed even in sheep (Michiels et al., 1987), avoids any “protective” GST activity in susceptible *F. hepatica*. A similar GST activity increase has been reported previously (Scarcella et al., 2013) in the same adult *F. hepatica* isolate recovered from triclabendazole-treated sheep. Our results show that CLS affects GST activity in adult liver flukes under *in vivo* conditions. However, the physiological implication of this effect needs to be further investigated.

Overall, the results reported here strongly suggest that oral ingestion is the main route of drug entry into *F. hepatica in vivo* exposed to CLS; that low drug quantities present in bile could diffuse into the adult parasite, but have low relevance to the final drug accumulation into the worm; that CLS at pharmacologically relevant concentrations fails to inhibit *F. hepatica* egg hatch; and that susceptible adult liver flukes exposed to CLS show an increase in GST activity. The information provided here is much useful to further understand CLS pharmacology, and could be relevant to optimize its flukicidal activity in ruminants.

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TABLES

Table 1: Closantel (CLS) concentrations (mean ± SD) measured in plasma, *Fasciola hepatica*, bile and liver after its administration by the oral (10 mg/kg) or subcutaneous (sc, 5 mg/kg) route to *F. hepatica*-infected sheep.

	Closantel concentration (µg/mL-g)					
	Time post-treatment					
MATRIX	**12 h**		**24 h**		**36 h**	
	Oral	**sc**	**Oral**	**sc**	**Oral**	**sc**
Plasma	36.2 ± 4.1	24.5 ± 0.9	38.8 ± 14	27.0 ± 2.9	57.2 ± 8 .2	40.7 ± 0.7
F. hepatica	16.1 ± 7.9	16.0 ± 2.3	22.6 ± 4.6	12.3 ± 0.7	33.8 ± 12	22.8 ± 13
Bile	3.30 ± 1.3	1.84 ± 0.6	4.97 ± 2.0	2.34 ± 0.3	4.19 ± 0.5	4.45 ± 3.5
Liver	2.17 ± 0.1	0.66 ± 0.4	2.67 ± 0.1	1.61 ± 1.2	6.40 ± 1.5	3.27 ± 0.8

Table 2: Glutathione S-transferase (GST) activity measured in *Fasciola hepatica* specimens collected from untreated sheep (Time 0, control) and sheep treated with closantel (CLS) by the oral (10 mg/kg) or subcutaneous (sc, 5 mg/kg) route.

Time post-treatment	GST activity (nmol/min/mg prot.)
0 (Control)	133.4 ± 28.0
Oral treatment	
12 h	390.3 ± 29.0*
24 h	162.3 ± 92.0*
36 h	82.0 ± 19.0
sc treatment	
12 h	221.5 ± 103
24 h	265.0 ± 32.2*
36 h	103.3 ± 7.6

Values represent means ± SD from three independent measurements, from a pool of 4–6 adult liver flukes recovered from each animal. *Significantly different from untreated control at P<0.05.

Figure legends

Figure 1

Comparative relative availability of closantel (CLS) in plasma, *F. hepatica,* bile and liver tissue, recovered from CLS-treated sheep by either oral (10 mg/kg) or sub-cutaneous (sc, 5 mg/kg) administration. Values represent mean (± SD) area under the concentration–time curves (AUC_{0-t}, µg.h/mL or µg.h/g).

Figure 2

Diffusion of Closantel (CLS) into *Fasciola hepatica*. Results express drug concentrations (µg/g, mean ± SD) in flukes after 60 min of incubation either in presence of RPMI-1640 medium or bile.*Differences statistically significant at $P<0.05$.

Figures

Figure 1

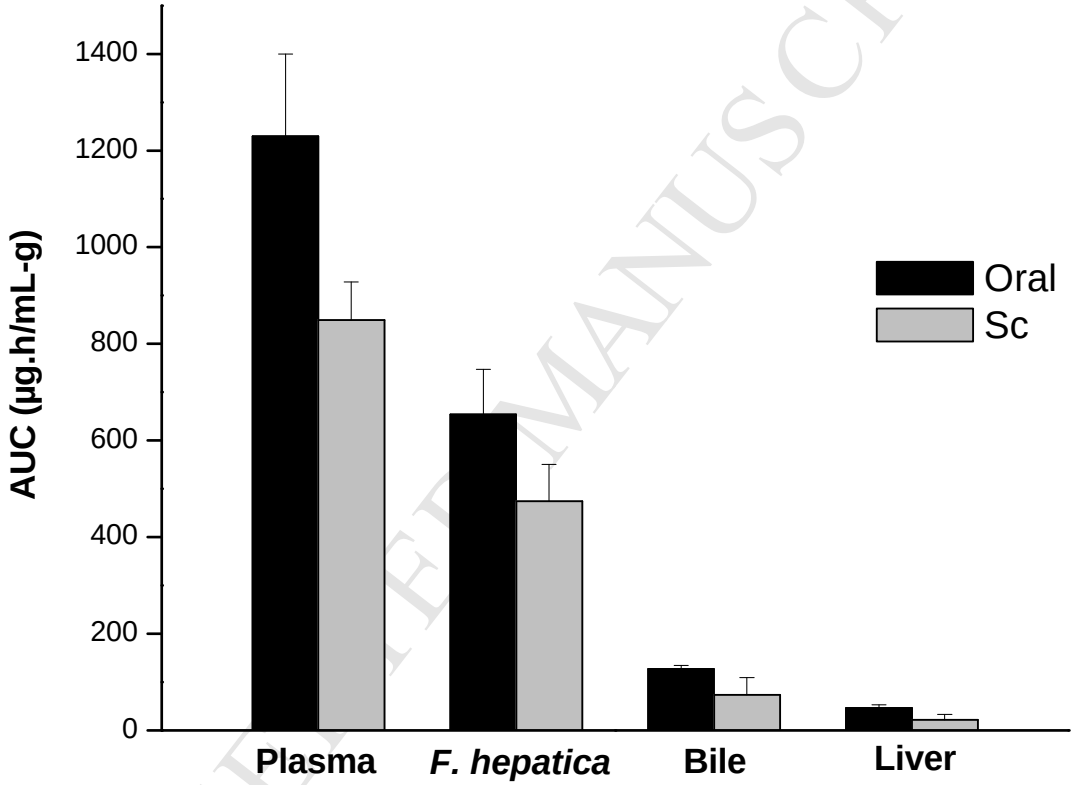
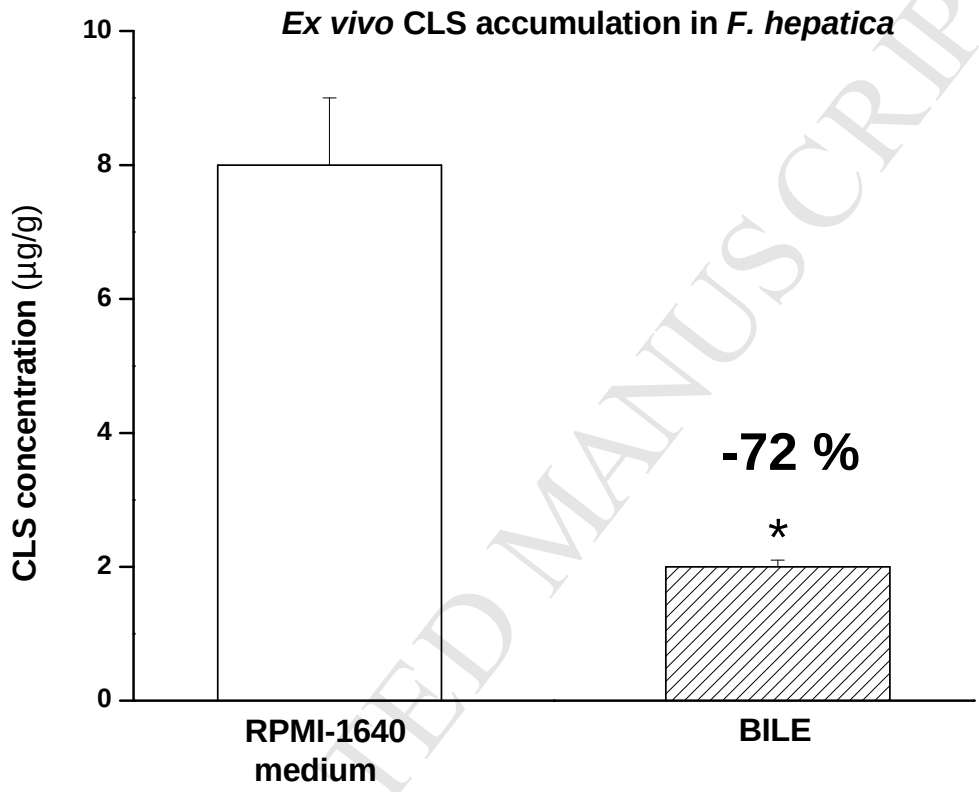


Figure 2



- CLS concentration profile in plasma, adult parasites, bile and liver from CLS-treated sheep were assessed
- Similar CLS concentrations were observed after oral or sc route up to 36.
- The higher concentrations were detected in plasma and parasite samples.
- Oral ingestion is the main route of drug CLS entry into *F. hepatica.*
- The presence of bile decreases the diffusion of drug into the parasite.
- CLS seems not have ovicidal activity at physiological concentrations.