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Naturally infected pig

OFZ-treated

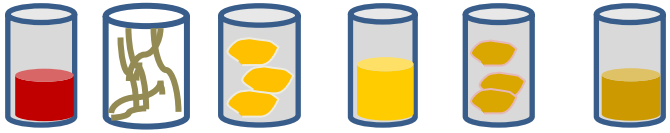
orally administration

(**30 mg/kg**)

Sacrifice and sampling

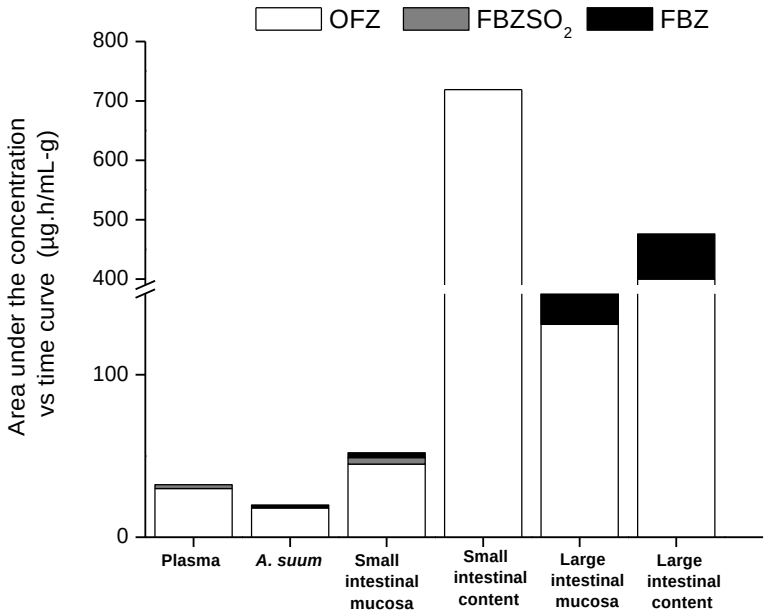
At 3, 6 and 12 h post treatment

Drug distribution Assay



Blood ***A. suum*** **Small intestinal mucosa** **Small intestinal content** **Large intestinal mucosa** **Large intestinal content**

HPLC analysis



Oxfendazole kinetics in pigs: *in vivo* assessment of its pattern of accumulation in *Ascaris suum*

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Abstract

Ascaris suum is a widespread parasitic nematode that causes infection in pigs with high prevalence rates. Oxfendazole (OFZ) is effective against *A. suum* when used at a single high oral dose of 30 mg/kg. The aim of this study was to assess the pattern of distribution/accumulation of OFZ and its metabolites, in bloodstream (plasma), mucosal tissue and contents from small and large intestine and adult specimens of *A. suum* collected from infected and treated pigs. The activity of glutathione-S-transferases (GSTs) in *A. suum* was also investigated. Infected pigs were orally treated with OFZ (30 mg/kg) and sacrificed at 0, 3, 6 and 12 h after treatment. Samples of blood, mucosa and contents from both small and large intestine as well as adult worms were obtained and processed for quantification of OFZ/metabolites by HPLC. OFZ was the main analyte measured in all of the evaluated matrixes. The highest drug concentrations were determined in small (AUC_{0-t} 718.7 ± 283.5 µg.h/g) and large (399.6 ± 110.5 µg.h/g) intestinal content. Concentrations ranging from 1.35 to 2.60 µg/g (OFZ) were measured in adult *A. suum*. GSTs activity was higher after exposure to OFZ both *in vivo* and *ex vivo*. The data obtained here suggest that the pattern of OFZ accumulation in *A. suum* would be more related to the concentration achieved in the fluid and mucosa of the small intestine than in other tissues/fluids. It is expected that increments in the amount of drug attained in the tissues/fluids of parasite location will correlate with increased drug concentration within the target parasite, and therefore with the resultant treatment efficacy. The results are particularly relevant considering the potential of OFZ to be used for soil transmitted helminths (STH) control programs and the advantages of pigs as a model to assess drug treatment to be implemented in humans.

Keywords: *Ascaris suum*, oxfendazole, drug accumulation

1. Introduction

The nematodes *Ascaris lumbricoides* and *Ascaris suum* are widespread parasites of humans and pigs, respectively. Human *ascariosis* is one of the most common soil transmitted helminths infections (STH), and is transmitted through soil contaminated with human faeces containing parasite eggs (WHO, 2018). Current estimations indicate that about a quarter (25%) of the world's population currently has a STH infection (WHO, 2018). Additionally, a high prevalence of gastrointestinal helminthic infections has been found in pig farms (Carstensen et al., 2002), and *A. suum* belongs to the list of the most important species found (Nansen and Roepstorff et al., 1999), causing a chronic illness that varies with geographical region and farm management practices (Dold and Holland, 2010). Porcine ascariasis interferes with the health and performance of pigs, leading to economic losses (Stewart and Hale, 1988).

The control of *Ascaris* spp. is largely based on the use of anthelmintic drugs. The benzimidazole and avermectin compounds are the most used chemical groups. Fortunately, *Ascaris* spp. is extremely susceptible to the aforementioned chemicals. The clinical efficacy of an anthelmintic will depend on its ability to reach high and sustained concentrations within the target parasite (pharmacokinetics), and to bind its specific receptor (pharmacodynamics) for sufficient time to induce the anthelmintic effect (Alvarez et al., 2007; Ceballos et al., 2009). The pharmacokinetics phase involves the time course of drug absorption, distribution, metabolism and elimination from the host, which, in turn, determines the concentration of the active drug reaching the site of parasite location. However, the anthelmintic´s action also depends on the ability of the active

drug/metabolite to reach their specific receptor within the target parasite (Alvarez et al., 2007). Thus, drug entry and the accumulation pattern in target helminths are critical issues to ensure optimal efficacy. The acquired knowledge supports a close relationship between the drug´s pharmacokinetic behaviour in the host and the observed final anthelmintic response (Alvarez et al., 2007).

Benzimidazole (BZD) compounds are widely used in veterinary medicine as broad spectrum anthelmintics, showing a high efficacy against most nematode parasites (McKellar and Scott, 1990). Several of them, including fenbendazole (FBZ), have shown efficacy against *A. suum* after their administration at different doses in feed (Campbell, 1990). Oxfendazole (OFZ) is the active sulphoxide metabolite of FBZ which was first marketed to be used in cattle, sheep and horses, for the removal and control of tapeworms (heads and segments), abomasal and intestinal nematodes (adults and 4th stage larvae) and lungworms (adults and larval stages) (Williams and Broussard, 1994). Fenbendazole have also ovicidal activity and is effective for the treatment of *Giardia* infection in calves (O'Handley et al., 1997). OFZ is recommended for the control of the same parasites as its sulfide parent compound (fenbendazole). Besides, its oral administration at a single dose of 30 mg/kg has been reported to be safe (Alvarez et al., 2013) and highly effective for the treatment of cysticercosis (Gonzalez et al., 1996), fasciolosis (Ortiz et al., 2014) and adult stages of *A. suum*, *Oesophagostomum* spp., *Trichuris suis* and *Metastrongylus* spp. (Alvarez et al., 2013).

Glutathione-S-transferases (GSTs) are a family of multifunctional enzymes essentially involved in the detoxification of harmful electrophilic endogenous and exogenous compounds by conjugation of glutathione with target molecules, and also function as non-enzymatic binding proteins involved in intracellular transport (Listowsky et al., 1988) and signaling (Cho et al., 2001) processes. GSTs occur abundantly in most organisms. In

fact, GSTs appears to be one of the major detoxification enzymes in parasitic helminths (Precious and Barrett, 1989), including *A. suum* (Liebau et al., 1994). The impact of OFZ on GSTs activity in *A. suum* recovered from treated pigs has not been investigated, but it could help to understand the drug effect on this important enzyme family.

OFZ could be an alternative anthelmintic to be used in human medicine. Consequently, it is interesting to understand the pharmacological basis supporting its anthelmintic effect and the pattern of drug accumulation inside target parasites. *A. suum* infections in pigs are the very best experimental animal model available to understand the drug-target parasite relationship (Boes and Helwigh, 2000). Information describing the OFZ/metabolites plasma disposition kinetics and tissue residue profiles used at high doses (30 mg/kg), and its efficacy against gastrointestinal nematodes in pigs, is available (Moreno et al., 2012; Alvarez et al., 2013). However, the drug concentration profiles within the different fluid/tissues where target parasites are located; is unknown. To achieve further comprehension of the *in vivo* concentrations required to kill *A. suum* specimens in the pig's gastrointestinal tract, the accumulation of OFZ and its metabolites in blood, mucosal tissue and luminal contents of the small and large intestine and in adults specimens of *A. suum* from OFZ treated pigs. As a complementary indicator of drug exposure to the worm, the *in vivo* and *ex vivo* OFZ effect on GSTs activity was investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

Pure reference standards (99% purity) of OFZ, FBZ and $FBZSO_2$ were from Toronto Chemicals Research Inc. (Toronto, Canada). Oxibendazole (OBZ) and albendazole sulphoxide (ABZSO) were from Sigma–Aldrich (St. Louis, MO, USA). The HPLC grade

119 solvents acetonitrile and methanol were from Baker, Mallinckrodt (Baker, Phillipsburg,
120 USA). Ethyl acetate was from Anedra (BA, Argentina). Water was distilled and deionized
121 using a water purification system (Simplicity®, Millipore, São Paulo, Brazil). The OFZ
122 administered to pigs was Synanthic® 9.06% (Merial, France).

123 **2.2. Animals and experimental design**

124 The study was conducted in eight pigs (15 ± 2.7 kg, 2 months old, local ecotypes breed),
125 naturally infected with *A. suum*. Pigs were fed *ad libitum* with a commercial balanced
126 food and had free access to water. Parasite infection was confirmed by faecal egg counts
127 (FEC) performed by the McMaster technique modified by Roberts and O'sullivan (1950).
128 A 10 days acclimatization period was allowed for the experimental animals to adapt.
129 Animals were housed in pens with concrete floors, protected from rain and prevailing
130 winds, but without temperature control. Animal procedures and management protocols
131 were carried out in accordance with the Animal Welfare Policy (Act 087/02) of the Faculty
132 of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires
133 (UNCPBA), Tandil, Argentina and internationally accepted animal welfare guidelines
134 (AVMA, 2001).

135 Schematic representation of the experimental design mentioned below is shown in Figure
136 1.

137 **Drug tissue distribution trials*:*** Experimental animals (n= 6) naturally infected with *A.*
138 *suum* were orally treated with OFZ at the dose of 30 mg/kg. At 3, 6 and 12 h post-
139 treatment (p.t.), two (2) animals were sacrificed and samples of blood, *A. suum,* mucosal
140 tissue and luminal contents of the GI tract were collected (small and large intestine) (Fig
141 1). *A. suum* specimens were manually collected from pig's intestine and rinsed
142 extensively with physiological saline solution, blotted on coarse filter paper and

immediately processed for drug/metabolites chemical extraction as detailed below. The total number of parasites recovered from each animal was registered. OFZ/metabolites concentration in *A. suum* was quantified only in worms recovered from the small intestine. Untreated animals (n= 2) were also sacrificed in order to obtain blank samples of blood, worms and gastrointestinal tissues/contents. After collection of the intestinal contents, the mucosal tissues of each gastrointestinal section were obtained by scraping. All obtained samples were placed into plastic tubes and frozen at -20 ºC until analysis by high performance liquid chromatography (HPLC).

GSTs activity *in vivo* assay*:* The GSTs activity was assayed in *A. suum* specimens either from untreated or treated animals used in the *Drug distribution assay* (sacrificed at 3 and 6 h p.t.) (Fig 1). The low number of worms recovered at 12 h p.t. precluded the assessment of GSTs activity at this sampling point. The parasites were rinsed extensively with saline solution (NaCl, 0.9%, 38 ºC) to remove adhering materials, blotted on coarse filter paper and placed in plastic tubes. From each animal, two samples of 4 *A. suum* specimens each were pooled and processed independently. The samples were kept in a freezer (−80°C). The procedure to obtain microsomal and cytosolic fractions from adult *A. suum* was adapted from the methodology described by Maté et al. (2008). Briefly, parasite samples were weighted and homogenized with two volumes of ice-cold homogenization buffer. Homogenates were filtered through and centrifuged at 10 000 x g for 20 min and the resulting supernatant at 100 000 x g for 65 min. Aliquots of supernatants (cytosolic fractions) were frozen in liquid nitrogen and stored at -70 ºC until used for GSTs activity assay. Pellets (microsomal preparations) were suspended in a 0.1 M potassium phosphate buffer (containing 0.1 mM of EDTA and 20 % of glycerol), frozen in liquid nitrogen and stored at -70 ºC. An aliquot of cytosolic fraction was used to determine protein content using bovine serum albumin as a control standard.

GSTs activity was assayed in the cytosolic fractions using 1-chloro, 2,4-dinitrobenzene (CDNB) as non specific substrate (Habig and Jakoby, 1981). The GSTs activity was determined by a continuous spectrophotometric method (Shimadzu Corporation, Kyoto, Japan).

GSTs activity *ex vivo* assay: Specimens of *A. suum* were collected from the small intestine of untreated control pigs and processed as previously described for the GSTs *in vivo assay*. Worms were incubated for 1, 3 and 12 h at 37 ºC in 5 mL of the RPMI buffer containing OFZ at a final concentration of 5 µM (Fig 1). This is a pharmacologically relevant concentration. There were four replicate assays for each incubation time. Blank samples containing worms and incubation medium without drug were incubated over the same time intervals. Once the incubation time elapsed, worms were rinsed thoroughly with saline solution, blotted on coarse filter paper and processed as described in parasite assays in order to assess GST activity in cytosolic fractions. The parasite material was processed immediately after the incubation assays.

The determination of parasite protein concentration was performed using the Lowry method with bovine serum albumin as standard (Lowry et al., 1951). GSTs activity is expressed as nmol/min/mg protein.

2.3. Analytical procedures

Plasma samples extraction: OFZ, $FBZSO_2$ and FBZ were extracted from plasma by a method adapted from Lanusse et al., (1995). Briefly, plasma samples (1 mL) were spiked with OBZ used as IS and the molecules to be assayed (OFZ, $FBZSO_2$, FBZ) in the validation procedure. Drug molecules were extracted by a solid phase extraction (SPE) procedure using C_{18} cartridges (Strata®, RP-18 100 mg, Phenomenex, CA, USA) previously conditioned. The sample was applied and then sequentially washed with 2 mL of

HPLC water, dried with air for 5 min and eluted with 2 mL of methanol. The elution was evaporated to dryness under a gentle stream of nitrogen at 56 ºC in a water bath (Zymark TurboVap LV evaporator. American Laboratory Trading, Inc. Lyme 06333 CT, USA). The dry residue was dissolved in 250 µL of mobile phase (acetonitrile:water, 27:73). An aliquot (50 µL) of this solution was injected in the chromatographic system.

A. suum* and small/large intestinal mucosa samples extraction*: Two whole parasites (female) from each animal (sampling at 3 and 6 h p.t.), were homogenized independently by means of scissors and scalpel, and 1g of each homogenate was used to quantify OFZ and its metabolites. In the case of the 12 h p.t. sampling time, in which only one parasite was obtained from each treated animal, two determinations were made for each one. As a result, four determinations for each time were obtained. In order to quantify OFZ/metabolites, samples (1 g) of parasite material and mucosa of the small and large intestines were homogenized and spiked with ABZSO as IS. Analytes were extracted by the addition of 1.5 mL of ethyl acetate. After shaking (50 min), the samples were sonicated for 10 min and centrifuged at 3800 rpm for 15 min at 4 ºC, and the clear supernatant (ethyl acetate phase) was transferred to a 5 mL glass tube. This procedure was repeated twice. The total supernatant (4.5 mL approx.) was evaporated to dryness under a gentle stream of nitrogen at 56 ºC in a water bath. For cleaning, the dry residue was dissolved in 2 mL of hexane and 1.5 mL of ACN and vigorously shaken (40 min). The hexane phase was then discarded. The samples were evaporated to dryness under a gentle stream of nitrogen at 56 ºC in a water bath.The dry extracts were reconstituted in 250 µL of mobile phase (acetonitrile:water, 27:73) and an aliquot of 50 µL was injected into the HPLC system.

Small/large intestine luminal contents samples extraction: Samples of small and large intestine luminal contents (1 g) were spiked with ABZSO as IS and sonicated for 40

min. Analytes were extracted by the addition of 1.5 mL of ACN. After shaking (30 min), the samples were sonicated for 10 min and centrifuged at 3800 rpm (15 min, 4 ºC); the supernatant was transferred to a 5 mL glass tube. This procedure was repeated three times. The total supernatant (4.5 mL approx.) was evaporated to dryness under a gentle stream of dry nitrogen at 56 ºC in a water bath. The dry extracts were reconstituted in 250 µL of mobile phase and an aliquot of 50 µL was injected into the HPLC system.

Fluid phase of small intestine luminal content samples extraction: The fluid phase was separated from the particulate phase of small intestine luminal content by centrifugation at 3800 rpm (15 min, 4 ºC). Samples of fluid phase (0.5 mL) were spiked with ABZSO as IS. Analytes were extracted by the addition of 1.5 mL of ACN. After shaking (15 min), and centrifugation (3800 rpm, 15 min, 4 ºC), the supernatant was separated and 1.5 mL of HPLC water were added. Subsequently, the samples were subjected to a solid phase extraction (SPE) identical to that mentioned above for the extraction of plasma samples. The dry extracts were reconstituted in 250 µL of mobile phase (acetonitrile:water, 27:73) and an aliquot of 50 µL was injected into the HPLC system.

2.4. Drug quantification by HPLC: analysis and validation

Experimental and fortified samples of each matrix (plasma, *A. suum*, mucosa and content of small and large intestine, and fluid phase of small intestinal content) were analysed by HPLC to determine the concentration of OFZ, $FBZSO_2$ and FBZ. The HPLC system and method to quantify these compounds were as described by Moreno et al. (2012). Calibration curves for OFZ, $FBZSO_2$ and FBZ in each matrix were prepared by least squares linear regression analysis, which showed correlation coefficients >0.994. Mean absolute recoveries for OFZ, $FBZSO_2$ and FBZ in the different biological matrixes,

estimated in the concentration range of 0.1-5 µg/mL (plasma, parasite material and fluid phase of small intestinal content), 0.2-40 µg/mL (mucosa of small and large intestine), and 1-300 µg/mL (total content of small and large intestine), ranged between 72 and 95 % with coefficients of variation (CV) ≤ 15 %. The limit of quantification (LOQ) was defined as the lowest measured concentration with a CV < 20%, an accuracy of ± 20% and an absolute recovery >70%. The limit of quantification was established at 0.1 µg/mL-g for plasma, parasite material and fluid phase of small intestinal content; 0.2 µg/g for mucosa of small and large intestine, and 1 µg/g for total content of small and large intestine. The limit of detection (LOD) was estimated by integrating the baseline threshold at the retention time of each compound for six non-spiked matrix samples. The LOD was defined as the mean 'noise'/internal standard peak area ratio plus 3 standard deviations (SD).

2.5. Analysis of the data

Data are expressed as arithmetic mean ± standard deviations (SD). The area under the concentration-time curve (AUC_{0-t}) for OFZ, $FBZSO_2$ and FBZ in each assayed tissue/fluid was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982), using the PKSolutions™ computer program (Summit Research Service, Ashland, USA). The AUC_{0-t} value was considered to be an indicator of the total drug availability in each biological matrix assayed. Non-parametric (Mann-Whitney) tests were used for statistical comparison of GSTs activity in *A. suum* recovered from *in vivo* and *ex vivo* experiments. Correlation between individual concentrations of OFZ in *A. suum*/small intestinal content, *A. suum*/small intestinal fluid, *A. suum*/small intestinal mucosa and small intestinal content/small intestinal fluid was performed by parametric analysis (Pearson r, r^2). A value of $P<0.05$ was considered statistically significant. Statistical analysis was performed using the Instat 3.0 Software (Graph Pad Software, CA, USA).

3. RESULTS

The presence of eggs in feces before starting the assay demonstrated that all animals involved were parasitized with *A. suum*. Adult *A. suum* were recovered from all sacrificed pigs. A mean of 83 worms were recovered from untreated pigs, 87 from animals sacrificed at 3 h p.t. (4 of them were recovered from the large intestine), 44 from animals sacrificed at 6 h p.t. (13 of them were recovered from the large intestine), and only two (all located in the small intestine) at 12 h p.t..

OFZ/metabolites concentrations (mean ± SD) measured in plasma, *A. suum*, mucosal tissue and contents of the small and large intestine after OFZ administration to pigs (30 mg/kg) are presented in **Table 1**. OFZ was the analyte quantified at highest concentrations in all tissues assayed and at all sampling times, representing about 91% of total analytes (OFZ, $FBZSO_2$ and FBZ). This analyte reached high plasma levels (1.10 ± 0.1 µg/mL) at the first sampling time (3 h p.t.) and achieved its plasma peak concentration (3.70 ± 1.30 µg/mL) within 6 h p.t., while in *A. suum* the maximum OFZ accumulation was observed at 12 h p.t. (2.60 ± 1.60 µg/g). In content of small intestine as well as in content and mucosa of large intestine, the highest OFZ concentrations were measured at the first sampling time (3 h p.t.).

Figure 2 shows the comparative drug availability (expressed as AUC_{0-t}) in each biological matrix, for OFZ, $FBZSO_2$ and FBZ. The highest AUC_{0-t} values for OFZ were observed in the small (718.7 ± 283.5 µg.h/g) and large (399.6 ± 110.5 µg.h/g) intestinal content followed by the large intestinal mucosa (131 ± 25.4 µg.h/g).

Figure 3 shows the comparative concentrations and availability (AUC_{0-t}) observed at 3, 6 and 12 h p.t. in both the content and the fluid phase of the small intestine. OFZ concentrations in fluid phase represented a small proportion of that measured in the

290 content (3.3, 7.6 and 12.3% at 3, 6 and 12 h p.t., respectively). Accordingly, the
291 proportion of OFZ not adsorbed to the particulate material of the small intestinal content
292 which is dissolved in the fluid phase was roughly 4 %. Equivalent OFZ concentrations
293 (ranging between 4.10 and 4.70 µg/g) were observed in samples of small intestinal
294 mucosa of treated pigs at 3, 6 and 12 h p.t..

295 FBZ metabolite was recovered mainly from samples of *A. suum*, mucosa of small and
296 large intestinae and large intestinal content, at all sampling times (Table 1). Its
297 concentrations in plasma, small intestinal content and fluid were below the limit of
298 quantification (LOQ), which preclude any pharmacokinetic analysis (e.g. AUC
299 estimation). Alike, concentrations below LOQ were measured for the inactive $FBZSO_2$
300 metabolite in the most tissues. This analyte could be quantified in samples of small
301 intestinal mucosa and in some sampling times of plasma, *A. suum* and large intestinal
302 content (Table 1).

303 GSTs activities in *A. suum* specimens recovered from both untreated control and OFZ-
304 treated pigs (**GSTs activity *in vivo* assay**) and in worms incubated at different times
305 with OFZ (**GSTs activity *ex vivo* assay**) are shown in **Table 2**. In the *in vivo* experiment,
306 a higher ($P< 0.05$) GSTs activity was observed in *A. suum* recovered from OFZ- treated
307 pigs at 3 h p.t. (183.0 ± 56.7 nmol/min/mg protein) than that obtained in worms from
308 untreated controls (94.3 ± 31.6 nmol/min/mg protein). However, no statistical difference
309 was observed between *A. suum* from untreated and 6 h treated pigs (103.3 ± 15.7
310 nmol/min/mg protein). In the *ex vivo* experiment, OFZ induced an increment in GSTs
311 activity in *A. suum* after 3 and 6 h of incubation (Table 2).

312 **4. Discussion**

Following the oral treatment of pigs with OFZ, a fast expulsion of the total nematode burden was observed. *A. suum* expulsion started as early as 3 h p.t., recovering worms from the distal section of the large intestine of treated pigs, and *A. suum* elimination was almost complete at 12 h p.t..

OFZ was largely the main analyte quantified in all assayed samples. The high OFZ concentrations achieved after a single oral dose of 30 mg/kg in pigs may account for parasites being exposed to toxic drug concentrations for sufficient time, explaining the early elimination of *A. suum* observed in the current experiment. A fast increment in GSTs activity (3 h p.t.) was observed in worms exposed to OFZ (*in vivo* and *ex vivo* assays), compared to those observed in untreated control worms (Table 2). The increased GSTs activity in *A. suum* could indicate some kind of “response” of the nematode to protect himself from other biochemical alterations (e.g. oxidative stress) induced by "toxic" concentrations of a xenobiotic such as OFZ.

The present study was not performed to estimate worm kinetic expulsion after OFZ treatment, but it is clear that *A. suum* is rapidly affected by high levels of the active compounds measured in the medium surrounding it (small intestinal content/fluid and mucosa), losing its capacity to remain in its specific site of location. The plasma profiles of OFZ/metabolites observed (Table 1) are in agreement with results reported by Moreno et al. (2012) since, after its oral administration, OFZ quickly reaches high plasma levels with a Tmax value as early as 6 h p.t.. The systemic exposure of BZD compounds reflects the amount of drug dissolved at the gastrointestinal level (Alvarez et al., 2013) which would be available for absorption and/or for diffusion through the external surface of parasites located at the gastrointestinal lumen; thus, the higher the drug present at gastrointestinal level, the greater the anthelmintic activity.

The knowledge of drug concentrations achieved within target parasites and the tissues/fluids surrounding them, will contribute to the understanding of the pharmacokinetics-efficacy relationship. OFZ was de most representative analyte measured within the parasites (90 % of total drug). *A. suum* is located in the small intestine, swimming against the flow to maintain its specific location and feeding on with food digested by the host, in contact with the mucosa and surrounded by the intestinal content (Nansen and Roepstorff, 1999). Consequently, anthelmintic drugs can reach their target receptor in *A. suum* by transcuticular diffusion from the intestinal fluid and/or mucosa, and by intestinal absorption from the intestinal content ingested by the worm.

After their administration, BZD compounds are rapidly adsorbed to the digesta particulate material, reaching an equilibrium between drug concentrations in particulate and fluid portions of content (Hennessy, 1993). In fact, we observed a highly positive correlation (r= 0.97) between OFZ concentrations in small intestinal content and in the small intestinal fluid. The amount of OFZ quantified in samples of small intestinal fluid reflects the dissolved drug portion able to diffuse through the external surface of *A. suum*; it represented only 3.45% (3 h p.t.), 12.2% (6 h p.t.) and 15.7% (12 h p.t.) of that observed in small intestinal content samples (ranging between 10.0 and 257 µg/mL) (Table 1). No correlation was observed (P> 0.05) between OFZ concentration in *A. suum* and fluid, content or mucosa of the small intestine; partly explained by the high variability observed in drug concentrations among the different samples. In addition, the OFZ concentration measured in either mucosa or content of the small intestine could partially contribute to the amounts of drug found in the parasite. This metabolite could reach the small intestinal mucosa from the peripheral blood after absorption through the gastrointestinal tract as well as through passive diffusion processes from it, in favour of the concentration gradient.

Hansen et al. (2017) have reported that after treatment of pigs with OFZ (5 mg/kg), OFZ concentrations in the content and the mucosa of the large intestine were far higher than in plasma and inside the parasite *Trichuris suis* (other recognized nematode situated in GI tract with the anterior oesophageal part of the worm is embedded in the mucosa, while the posterior thick part is protruding freely into the lumen). They concluded that OFZ reaches *T. suis* after its gastrointestinal absorption by the host and posterior distribution to the parasites by a systemic circulation-enterocyte pathway. This statement was supported by a high correlation between drug concentrations of OFZ measured in host plasma and worms (Hansen et al., 2017). Besides, FBZ accumulated in the worms originates from the intestinal digesta of the host and some minor part would enter from the systemic circulation (Hansen et al., 2017). A similar *T. suis* drug-accumulation pathway was suggested for OFZ after the oral administration of FBZ to pigs (Hansen et al., 2014). In keeping with these authors' observations, high OFZ concentrations either in mucosa or content of the large intestine were quantified in this study (Table 1). The use of a 30 mg/kg dose of OFZ exposed parasites located at the large intestine such as *Oesophagostomum* spp and *Trichuris suis* (normally "refractory" to anthelmintic treatments) to OFZ concentrations high enough to affect and eliminate them (Alvarez et al., 2013).

Although low FBZ concentrations (range 0.13-0.29 µg/g) were measured in *A. suum*, its contribution to the anthelmintic effect should be taken into account. The greater anthelmintic activity of FBZ compared to OFZ has been demonstrated *in vitro* by assessing binding to parasite tubulin (Lacey and Gill, 1994) and nematode motility (Petersen et al., 1997). The higher anthelmintic potency of FBZ may partially compensate for its lower concentrations achieved inside the parasite, contributing to the final ascaricidal effect. The presence of FBZ after OFZ treatment can be explained by the

OFZ reduction to FBZ, mediated by the microbial activity taking place mainly in the pig´s large intestine (Moreno et al., 2012). In agreement with that, the highest FBZ concentrations were observed in samples of large intestinal content. Reduction by the gastrointestinal microflora plays an important role in the metabolism of a number of drugs, particularly those containing nitro and sulphoxide groups (Lanusse and Prichard, 1993). FBZ may accumulate inside *A. suum* by diffusion through the external parasite surface from the surrounding medium. Since FBZ has a higher "diffusion rate" compared to OFZ due to its higher lipid solubility (Mottier et al., 2003), this metabolite accumulation into the worm could be occurring by passive diffusion from the low (below the limit of detection (0.1 µg/mL) concentrations of the drug present in the small intestinal fluid. Furthermore, FBZ concentrations quantified in small intestinal mucosa would also help to explain FBZ concentrations observed in worms. $FBZSO_2$ metabolite was quantified in *A. suum* only at 12 h post-treatment, likely with a similar accumulation pattern to that described for FBZ. As we mentioned, the greater FBZ accumulation into *A. suum* compared to $FBZSO_2$ could also be explained by differences in drug lipophilicity as a major determinant of the rate of transfer across the nematode cuticle (Thompson et al., 1993, Mottier et al., 2003).

5. Conclusions

The drug concentrations reached in the small intestinal content of pigs, mostly the portion that is dissolved in the fluid phase of it, correlate with the drugs concentration accumulated in *A. suum*, which could have an impact in its pharmacology activity. It is clear that OFZ concentrations around 1-2 µg/g inside the worms are enough to eliminate adult *A. suum*. The characterization of the disposition kinetics of OFZ and its metabolites in the target tissues/fluids with the pattern of drug accumulation into *A.suum* in pigs is a

411 further contribution to the knowledge of the pharmacology of antiparasitic drugs aimed at
412 optimizing parasite control.

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Legends

Figure 1: Schematic representation of the performed experimental design. Drug tissue distribution trials: samples of blood, *A. suum,* mucosal tissue and luminal contents of the

GI tract are taken (small and large intestine) from pigs naturally infected with *Ascaris suum* and either untretated or orally treated with oxfendazole (OFZ) (30 mg/kg), and OFZ/metabolites are analyzed by HPLC. GST *in vivo* and *ex vivo* assay: GST activity is assayed in parasites recovered from animals used in either *In vivo* drug accumulation assay, sacrificed 3 and 6 h post OFZ treatment, or in parasites recovered from untreated control animals, incubated for 1, 6 and 12 h with OFZ (5 nm/mL).

Figure 2: Comparative tissues availabilities of oxfendazole (OFZ), fenbendazole sulphone (FBZSO2) and fenbendazole (FBZ). Area under de concentration vs time curve (AUC_{0-t} , µg.h/mL-g) measured in plasma, *A. suum*, small intestinal mucosa , small intestinal content, small intestinal fluid, large intestinal mucosa and large intestinal content, from naturally infected pigs treated with oxfendazole (30 mg/kg).

Figure 3: Comparative oxfendazole (OFZ) concentrations (arithmetic mean ± SD) detected in small intestinal content and fluid phase of small intestinal content (at 3, 6 and 12 h after OFZ treatment (30 mg/kg) to naturally infected pigs. The insert shows the comparative oxfendazole area under the concentration – time curve from 0 to 12 h (AUC_{0-t}, µg.h/mL-g) measured in small intestinal content and small intestinal fluid recovered from naturally infected pigs treated with oxfendazole (30 mg/kg).

Table 1:

Concentrations (arithmetic mean ± SD) obtained for oxfendazole (OFZ), fenbendazole sulphone ($FBZSO_2$) and fenbendazole (FBZ) in pigs orally treated with oxfendazole (30mg/kg), at 3, 6, and 12 h post treatment (p.t), in small intestinal mucosa, small intestinal content, small intestinal fluid phase, large intestinal mucosa and large intestinal luminal content.

		Tissue concentration (µg/mL-g)					
Time post-treatment (h)		**Plasma**	***A. suum***	**Small intestinal mucosa**	**Small intestinal content**	**Large intestinal mucosa**	**Large intestinal content**
OFZ	3	1.10 ± 0.10	1.80 ± 0.40	4.40 ± 1.40	171 ± 63.7	22.8 ± 0.30	70.5 ± 9.80
	6	3.70 ± 1.30	1.60 ± 0.40	4.10 ± 2.70	31.7 ± 19.1	8.40 ± 6.10	26.8 ± 17.6
	12	3.40 ± 0.70	2.60 ± 1.60	4.70 ± 3.10	21.1 ± 10.1	4.50 ± 1.20	24.5 ± 6.40
$FBZSO_2$	3	<LOQ	<LOQ	0.20 ± 0.10	n.d.	<LOQ	1.30 ± 0.10
	6	0.20 ± 0.10	<LOQ	0.40 ± 0.10	n.d.	<LOQ	<LOQ
	12	0.40 ± 0.10	0.19 ± 0.19	0.60 ± 0.40	n.d.	<LOQ	<LOQ
FBZ	3	<LOQ	0.10 ± 0.10	0.30 ± 0.10	n.d	<LOQ	3.30 ± 2.90
	6	<LOQ	0.20 ± 0.10	0.30 ± 0.10	n.d.	3.60 ± 1.50	5.50 ± 2.90
	12	<LOQ	0.20 ± 0.10	0.70 ± 0.30	n.d.	3.40 ± 0.90	5.10 ± 2.70

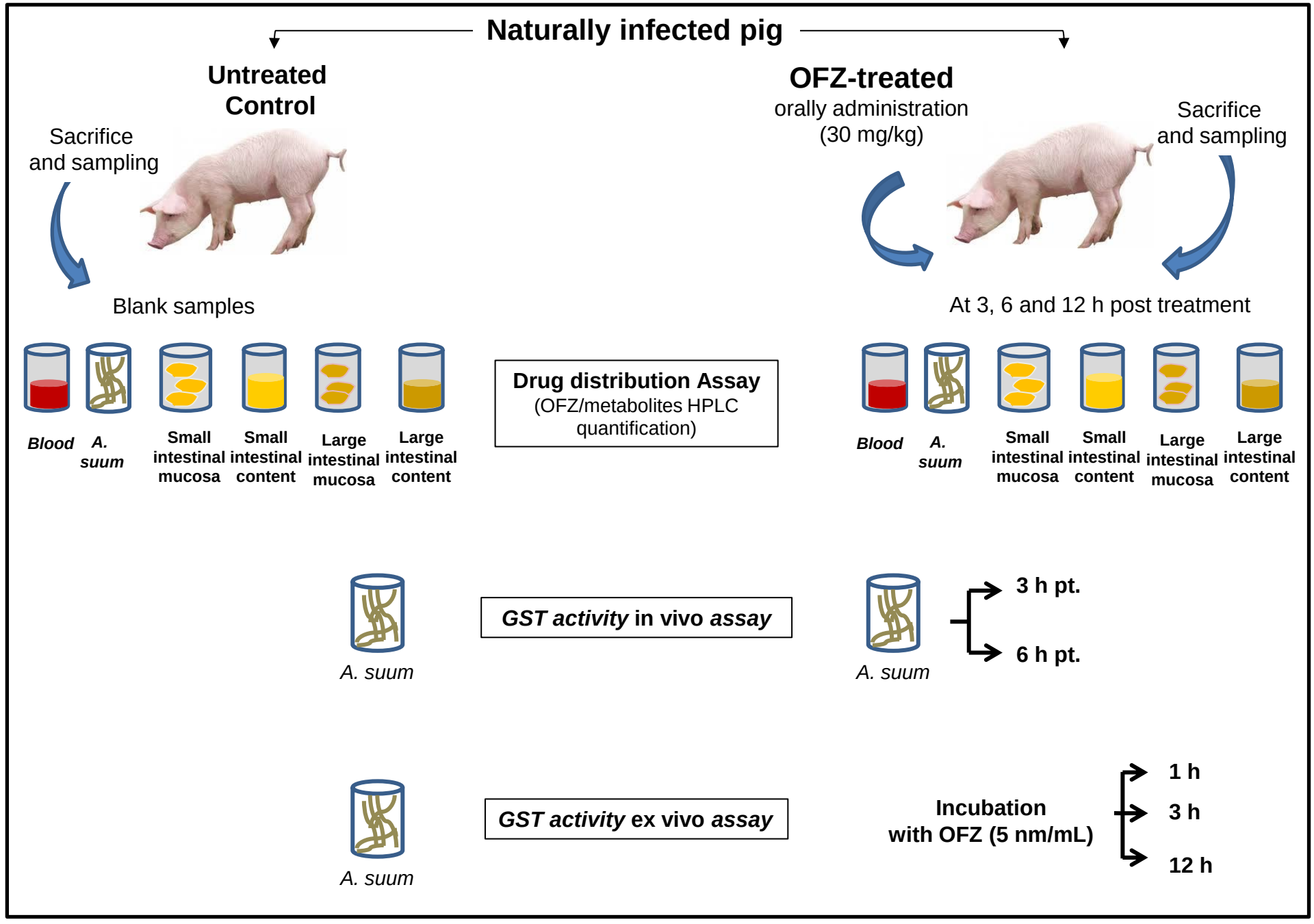
n.d.= not determined; LOQ= limit of quantification.

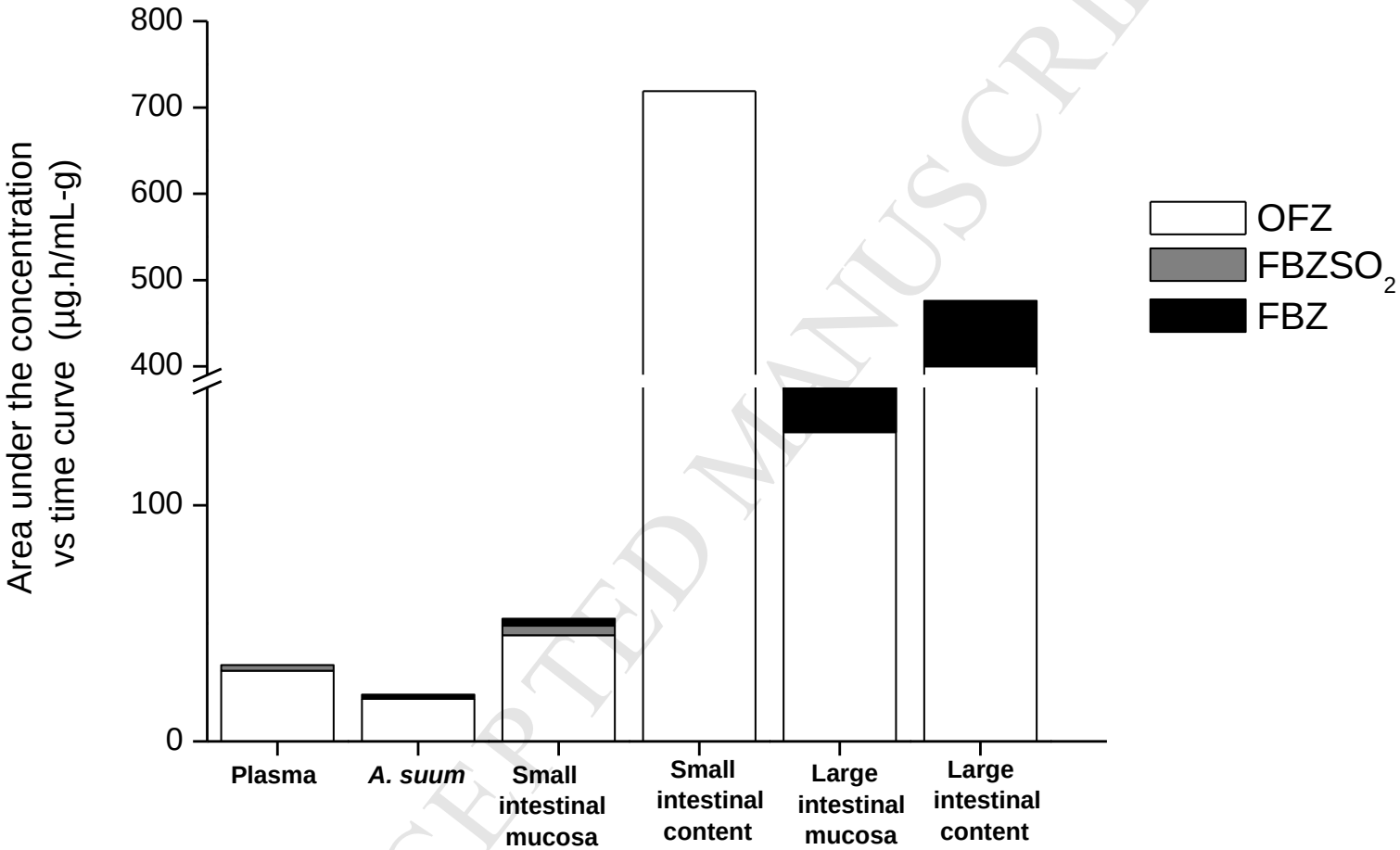
Table 2:

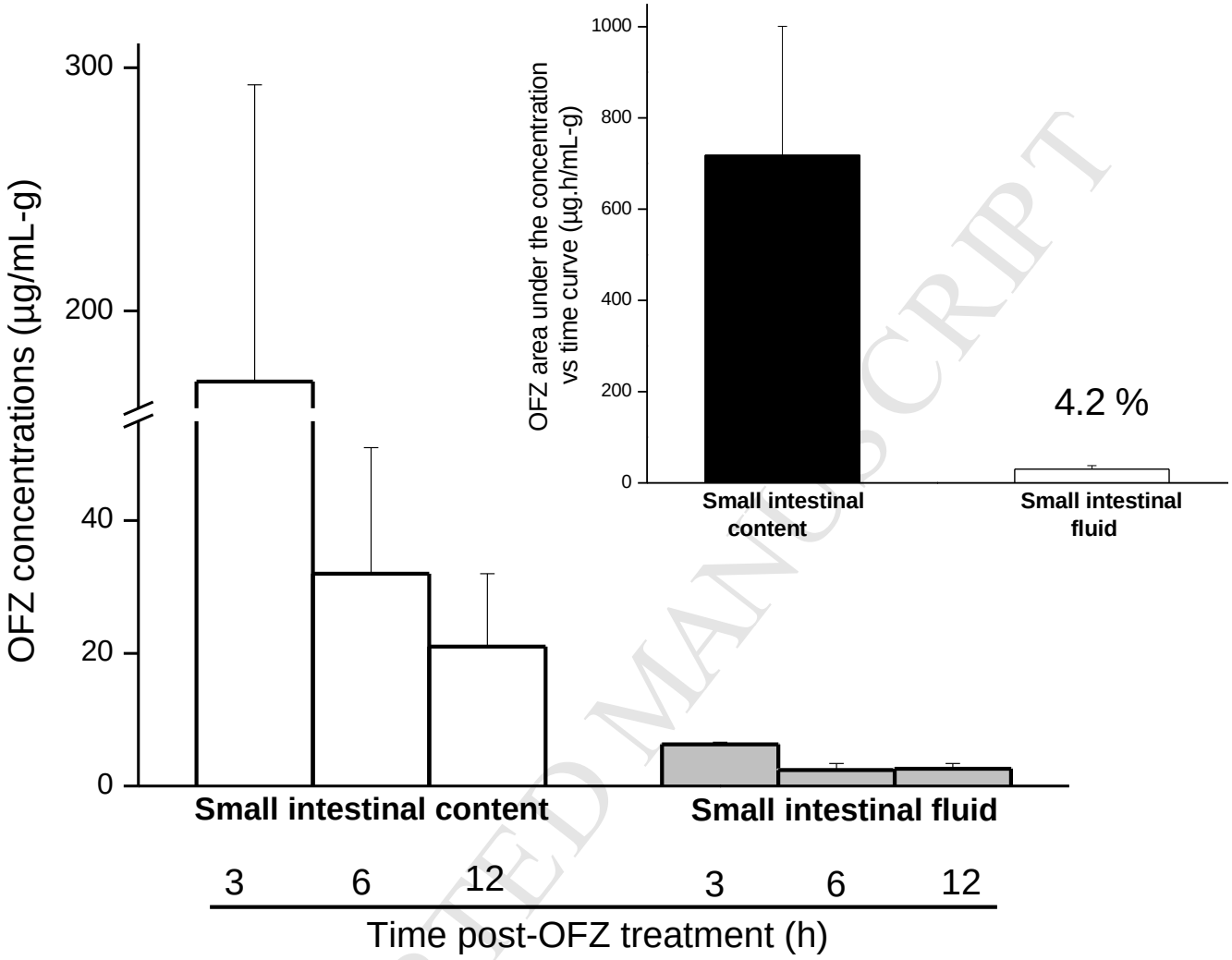
Glutathione S-transferase (GSTS) activity (nmol/min/mg protein) measured in adult *Ascaris suum* obtained at different times from pigs treated with oxfendazole (OFZ, 30 mg/kg) (GSTS activity *in vivo* assay) and in *A. suum ex vivo* incubated with OFZ (5 nmoles/mL) for 1, 3 and 6 h (GSTS activity in vivo assay).

Time post-treatment	***In vivo* assay GST activity**	
0 h	94.3 ± 31.6	
3 h	183.0 ± 56.7*	
6 h	103.3 ± 15.7	
	***Ex vivo* assay[b] GST activity**	
Incubation time	**Control**	**Exposed to OFZ**
0 h	99.5 ± 13.2	-
1 h	123.5 ± 11.0	201.5 ± 73.5
3h	121.0 ± 14.1	209.5 ± 17.1*
6 h	121.5 ± 11.7	214.0 ± 33.6*

Values represent means ± SD of four (4) replicate assays for each time. *Significantly different from untreated control at P<0.05.


Naturally infected pig
Untreated Control
Sacrifice and sampling
Blank samples
OFZ-treated
orally administration (30 mg/kg)
Sacrifice and sampling
At 3, 6 and 12 h post treatment
Blood
A. suum
Small intestinal mucosa
Small intestinal content
Large intestinal mucosa
Large intestinal content
Drug distribution Assay
(OFZ/metabolites HPLC quantification)
A. suum
GST activity in vivo assay
3 h pt.
6 h pt.
A. suum
GST activity ex vivo assay
Incubation with OFZ (5 nm/mL)
1 h
3 h
12 h





- Oxfendazole (OFZ) is effective against *A. suum* when used at a single high oral dose of 30 mg/kg.
- The pattern of distribution/accumulation of OFZ and its metabolites, in adults *A. suum* and related tissues was investigated.
- OFZ was the main analyte measured in all of the evaluated matrixes
- The highest drug concentrations were determined in small and large intestinal content.
- Concentrations ranging from 1.35 to 2.60 µg/g (OFZ) were measured in adult *A. suum*.
- Increments in the amount of drug attained in the tissues/fluids of parasite location correlate with the resultant treatment efficacy.