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Hepatic and extra-hepatic metabolic pathways involved in flubendazole biotransformation in sheep

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ABSTRACT

Flubendazole (FLBZ) is a broad-spectrum benzimidazole anthelmintic compound used in pigs, poultry and humans. Its potential for parasite control in ruminant species is under investigation. The objective of the work described here was to identify the main enzymatic pathways involved in the hepatic and extra-hepatic biotransformation of FLBZ in sheep. Microsomal and cytosolic fractions obtained from sheep liver and duodenal mucosa metabolised FLBZ into a reduced FLBZ metabolite (red-FLBZ). The keto-reduction of FLBZ led to the prevalent (~98%) stereospecific formation of one enantiomeric form of red-FLBZ. The amounts of red-FLBZ formed in liver subcellular fractions were 3–4-fold higher ($P < 0.05$) compared to those observed in duodenal subcellular fractions. This observation correlates with the higher ($P < 0.05$) carbonyl reductase (CBR) activities measured in the liver compared to the duodenal mucosa. No metabolic conversion was observed following FLBZ or red-FLBZ incubation with sheep ruminal fluid. Sheep liver microsomes failed to convert red-FLBZ into FLBZ. However, this metabolic reaction occurred in liver microsomes prepared from phenobarbital-induced rats, which may indicate a cytochrome P450-mediated oxidation of red-FLBZ. A NADPH-dependent CBR is proposed as the main enzymatic system involved in the keto-reduction of FLBZ in sheep. CBR substrates such as menadione and mebendazole (a non-fluoride analogue of FLBZ), inhibited this liver microsomal enzymatic reaction, which may confirm the involvement of a CBR enzyme in FLBZ metabolism in sheep. This research is a further contribution to the understanding of the metabolic fate of a promissory alternative compound for antiparasitic control in ruminant species.

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Abbreviations: BZD, benzimidazole; FLBZ, flubendazole; red-FLBZ, reduced flubendazole; h-FLBZ, hydrolysed flubendazole; ABZSO, albendazole sulphoxide; MBZ, mebendazole; CBR, carbonyl reductase; P450, cytochrome P450 system; CYP, cytochome P450 subfamily; FMO, flavin-monooxygenase; PB, phenobarbital; MEN, menadione; QRC, quarcetin; WRF, warfarin; PBO, piperonyl butoxide.

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1. Introduction

Livestock animals are continuously exposed to a variety of xenobiotic agents (i.e.: veterinary drugs, feed-additives, pesticides, pollutants, etc.) during their production cycles. These compounds are likely to be metabolised by different enzymatic systems from both hepatic and extra-hepatic tissues. The metabolic activity of different phase 1 and phase 2 xenobiotic metabolising enzymes play a major role in determining the persistence of therapeutically used drugs in target species, which may additionally impose a risk to the consumers as a consequence of the permanence of drug residue levels in edible tissues. Metabolic interactions with such enzymatic systems may drastically affect the disposition kinetics of different drugs used in animal production, which will have a relevant impact on the pattern of drug/metabolites residues in tissues, a major concern for public health and consumer's safety.

Xenobiotic biotransformation takes place predominantly in the liver, although metabolic activity is apparent in extra-hepatic tissues, such as the gastrointestinal tract. In addition to its primary role in the absorption of nutrients and water, the intestine is a major route of entry into the body for many xenobiotics. The intestinal mucosa constitutes an absorptive barrier in the uptake of toxic compounds and/or orally administered therapeutically used drugs. In addition, the gut mucosa has the ability to metabolise a great number of xenobiotic compounds by numerous pathways involving both phase 1 and phase 2 reactions [1].

Ruminants nutritional physiology has been widely studied and a great number of investigations focused on the metabolic activity of the microflora present in the rumen (the largest forestomach cavity in these species). However, most research has mainly examined the fermentation of dietary components and much less attention has been given to the biotransformation of compounds without nutritional relevance [2]. Ruminant animals have a symbiotic relationship with their ruminal microflora (bacteria, protozoa and fungi) which allows them to digest fibrous plant materials [3]. Anaerobic conditions predominate in the ruminal environment and substrates are only partially oxidised. Therefore, while oxidative metabolism predominates in the liver and in the intestinal mucosa, reductive and hydrolytic reactions are of primary importance in the rumen. For instance, ruminal microorganisms are very active in reductive reactions of foreign compounds, particularly those containing -nitro [4,5] and -sulphoxide [6,7] groups. Thus, drug metabolic processes taking place in the rumen are particularly important in ruminant therapeutics.

Flubendazole (FLBZ), methyl ester of [5-(4-fluorobenzoyl)-1*H*-benzimidalzol-2-yl]carbamic acid, is a broad-spectrum benzimidazole (BZD) methylcarbamate anthelmintic available for use in human and veterinary medicine. FLBZ is a fluoride derivative of mebendazole (MBZ) and is widely used for parasite control in pigs, chicken, turkeys and game birds. Unlike other commonly used BZD anthelmintics such as albendazole (aliphatic substitution at position -5) and fenbendazole (aromatic substitution at position -5), MBZ and FLBZ do not contain a sulphur atom at the same position. Instead, a ketone group is present in both anthelmintic molecules, which may have implications in their metabolic pattern within the host. While sulphur-containing BZDs are sequentially oxidised to their sulphoxide and sulphone metabolites by both flavin-monooxygenase (FMO) and cytochrome P450 (P450) systems in the liver [8–12], carbonyl reductases (CBRs) are thought as the main enzymatic system involved in the metabolism of MBZ and FLBZ [13,14].

Previous work carried out in our laboratory described an integrated pharmacological assessment of the disposition kinetics and the liver microsomal biotransformation of FLBZ in sheep, as well as its ex vivo diffusion through the tegument of the cestode parasite *Moniezia benedeni* [15]. The parent drug and its reduced (red-FLBZ) and hydrolysed (h-FLBZ) metabolites were found in the bloodstream of FLBZ-treated sheep. In addition, sheep liver microsomes were able to metabolise FLBZ into red-FLBZ. The proposed pattern for FLBZ biotransformation is shown in Fig. 1. Considering the potential of FLBZ as a broad-spectrum anthelmintic for use in ruminant species, the work reported here was addressed to gain further insight into the identification of the specific metabolic pathways involved in FLBZ metabolism in the host. The involvement of the ruminal microflora in either the reduction or hydrolysis of FLBZ was also assessed. Overall, this research contributes to characterise the patterns of hepatic and extra-hepatic biotransformation of this anthelmintic drug in sheep, which may be considered critical to optimise its pharmacological activity.

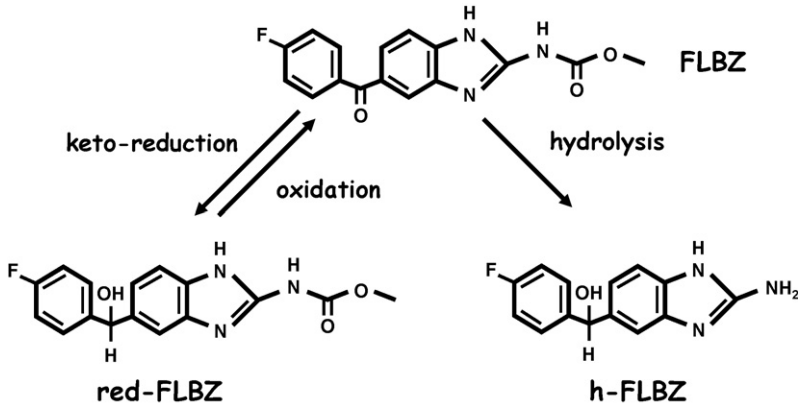

Fig. 1 – Proposed pattern of flubendazole (FLBZ) biotransformation.

2. Material and methods

2.1. Reagents

Reference standards (99.5% pure) of FLBZ and its reduced (red-FLBZ) and hydrolysed (h-FLBZ) metabolites were kindly provided by Janssen Animal Health (Beerse, Belgium). Stock solutions (2.5 mM) of each molecule were prepared in methanol (Baker Inc., Phillipsburg, USA). Albendazole sulphoxide (ABZSO), used as internal standard, was provided by Schering Plough (Kenilworth, New Jersey, USA). Phenobarbital (PB) was purchased from J'anvier (Buenos Aires, Argentina). Mebendazole (MBZ), quercetin (QRC), menadione (MEN), warfarin (WRF), piperonyl butoxide (PBO), chlorpheniramine maleate salt, oleandomycin triacetate, aminopyrine, nicotinamide adenine dinucleotide phosphate (NADP+) and trypsin inhibitor (Type II-S: soybean) were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche Applied Science (Buenos Aires, Argentina). Erythromycin was obtained from the local market (Parafarm, Buenos Aires, Argentina). The solvents used for the chemical extraction and chromatographic analysis were HPLC grade (Baker Inc., Phillipsburg, USA). Buffer salts (KCl, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4 and CH_3COONH_4) were purchased from Baker Inc. (Phillipsburg, USA).

2.2. Animals

Five (5) healthy Corriedale × Merino cross-breed rams were sacrificed to obtain samples of liver parenchyma and duodenal mucosa for preparation of the microsomal and cytosolic fractions. Ruminal fluid was collected from three (3) animals. Sheep were fed with high quality lucerne hay and water ad libitum.

Eight (8) female rats (280–310 g) were randomly allocated in two experimental groups (control and PB-induced) of four (4) animals each other. Both control and PB-treated rats received food and water ad libitum. Drinking water in PB-treated animals was supplied with the drug (1 mg/mL) during 1 week. The average dose of PB administered (68 mg/kg of body weight) was estimated by recording the quantity of water ingested per day. Animal procedures and management protocols were carried out according to the Animal Welfare Policy (Academic Council Resolution 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar). Animals were stunned and exsanguinated immediately in agreement with these institutional and internationally accepted animal welfare guidelines [16].

2.3. Preparation of subcellular fractions

These procedures were adapted from the methodology described by Nebbia et al. [17]. After sacrifice, the abdomen was opened and the liver (sheep and rats) was removed. Samples were rinsed with ice-cold KCl 1.15 % and then stored in aluminium foils, chilled in ice and transported to the laboratory. All subsequent operations were performed between 0 and 4 °C. Liver samples (8 g) from each experimental animal were cut into small pieces with scissors.

Segments (40–50 cm) of sheep duodenum were also obtained. The gut content was discarded and the segment was opened through a longitudinal incision. Then, the mucosa was washed with ice-cold KCl 1.15 %, blotted dry and thereafter scraped using a microscope glass slide. All samples were transported to the laboratory in iced cold homogenisation buffer (0.1 M potassium phosphate, pH 7.4, 0.1 M Tris acetate, 0.1 M KCl, 1 mM EDTA, 18 μM butylated hydroxytoluene, and 1 mg/mL trypsin inhibitor) at 4 °C.

Microsomal and cytosolic fractions from both liver and intestinal tissues were isolated by differential ultracentrifugation. Tissue samples were weighted and homogenised using a Potter-Elvenjem tool (four to six passes) with two volumes of ice-cold homogenisation buffer. Homogenates were filtered through a hydrophilic gauze, centrifuged at 10 000 × *g* for 20 min and the resulting supernatant at 100 000 × *g* for 65 min. Aliquots of supernatants (cytosolic fractions) were frozen in liquid nitrogen and stored at −70 °C. Pellets (microsomal preparations from both tissues) 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 °C until used for incubation assays. An aliquot of each subcellular fraction was used to determine protein content using bovine serum albumin as a control standard [18].

2.4. Determination of carbonyl reductase (CBR) activities

CBR activities were measured in liver and duodenal subcellular fractions by incubating MEN as a substrate and recording the consumption (oxidation) of NADPH at 37 °C in a spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan, RF-5301PC) at an excitation wavelength of 380 nm and a maximum of emission wavelength of 460 nm. Briefly, each subcellular fraction was pre-incubated at 37 °C over 5 min and then the NADPH disappearance was monitored for 5 min in presence of MEN at an acquisition rate of 1 reading/20 s. Typical reaction mixtures performed for the determination of the kinetic parameters (K_m and V_{max}) contained: 250 μM NADPH, 0.75 mg of liver microsomal protein, 0.1 M potassium phosphate buffer (pH 7.4) and variable concentrations of MEN (62.5–1000 μM) in a final volume of 3 mL. Comparative CBR activities in cytosolic and microsomal fractions from liver and duodenal mucosa were performed using 500 μM MEN. Inhibition studies of CBR activity in liver microsomes were carried out by incubation of 125 μM MEN in presence of quercetin (20 μM). All metabolic reactions started with the addition of the specific substrate.

2.5. Enzyme assays in liver and duodenal microsomal and cytosolic fractions

FLBZ keto-reductase enzymatic biotransformation was assessed in each subcellular fraction by the amount of red-FLBZ formed in the presence of a NADPH generating system. Incubations using red-FLBZ as a substrate were also carried out in liver microsomes. A typical reaction mixture contained (in a final volume of 0.5 mL): phosphate buffer 0.1 M (pH 7.4), 0.5 mg of microsomal or cytosolic protein diluted in 50 μL of

the same buffer, NADPH-generating system ($NADP^+$ 0.32 mM, glucose-6-phosphate 6.4 mM, $MgCl_2$ 5 mM, EDTA 0.8 mM and 1.25 U of glucose-6-phosphate dehydrogenase in phosphate buffer) and 40 μM of FLBZ or red-FLBZ dissolved in 10 μL methanol. All incubation mixtures were allowed to equilibrate (5 min at 37 °C) and the reaction started with the addition of the NADPH-generating system. Incubations (15 or 30 min at 37 °C) were carried out in polypropylene vials in an oscillating water bath under aerobic conditions.

The biotransformation of FLBZ in sheep liver microsomes was also studied in the presence of MEN and WRF (known CBR substrates), mebendazole (MBZ, a non-fluoride analogue of FLBZ) and piperonyl butoxide (PBO, known as a P450 inhibitor). Microsomal preparations containing MEN (40, 80 and 160 μM), WRF (40, 80 and 160 μM), MBZ (40 μM) or PBO (100 and 200 μM) were pre-incubated (5 min at 37 °C) in the presence of FLBZ (40 μM). Then, each reaction was initiated by the addition of the NADPH-generating system. MEN and WRF were dissolved in 0.1 M phosphate buffer; while MBZ and PBO were dissolved in 10 μL of methanol (parallel control tubes contained the same volume of the solvent).

Blank samples, containing all components of the reaction mixture except the NADPH-generating system, were also incubated under the same conditions. These incubations were used as controls for possible non-enzymatic drug conversion. All reactions were stopped by the addition of 0.2 mL of acetonitrile and stored at −20 °C until analysis.

2.6. Cytochrome P450-mediated demethylase activities in rat liver microsomes

The response to PB treatment was assessed by the quantification of P450-dependent *N*-demethylase activities towards several substrates, which in humans and in other laboratory or livestock animal species [17,19] are believed to be markers for the expression of different P450 forms (CYPs), based on molecular models of mammalian enzymes [20]. Thus, oleandomycin triacetate and erythromycin were selected as CYP3A substrates, while aminopyrine and chlorfeniramine were markers of CYP2C-mediated activity. All enzyme activities were determined by aerobic incubations under conditions yielding zero order rates with respect to cofactor and substrate concentrations and ensuring linearity with respect to time and protein concentrations. Oxidative P450-dependent *N*-demethylation activities toward oleandomycin triacetate (0.3 mM), erythromycin (1 mM), aminopyrine (5 mM) and chlorfeniramine (1 mM) were assayed using the NADPH-generating system and 1 mg of microsomal protein. After a suitable incubation time, reactions were quenched with chilled trichloroacetic acid (10%, w/v) and, after centrifugation, the amount of the released formaldehyde was determined fluorometrically on an aliquot of the clear supernatant with Nash's reagent as detailed by Werringloer [21]. Incubations of the same substrates with liver microsomes from untreated rats were used as controls.

2.7. Incubation assays with sheep ruminal fluid

Aliquots (1.96 mL) of sheep ruminal fluid were incubated under anaerobic conditions with 40 μM of FLBZ or red-FLBZ during 15, 30 and 60 min following previously described procedures [12]. Incubations containing 40 μM of ABZSO were used as positive controls since this molecule is extensively reduced by the ruminal fluid under in vitro conditions [22].

2.8. Drug/metabolites extraction

Twenty (20) μL of the internal standard (IS) ABZSO (250 nmol/mL) were added to the inactivated microsomal or cytosolic incubation mixture. Then, samples were mixed with 2 mL acetonitrile, vortexed during 20 min and centrifuged at 4000 × *g* for 15 min at 10 °C. The supernatant was recovered and evaporated to dryness using an Automatic Environmental Speed Vac System (Savant, Holbrook, USA). The dry residue was re-dissolved in 40 % acetonitrile in water and 50 μL were injected into the HPLC system.

Drug/metabolites physico-chemical extraction from ruminal fluid samples was performed following previously reported procedures [12].

2.9. Chromatographic analysis

Samples were analysed for ABZSO (IS), FLBZ, red-FLBZ and h-FLBZ by HPLC. Fifty microlitres (50 μL) of each extracted sample were injected through an autosampler (Shimadzu SIL-10 A Automatic Sample Injector) into a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a Selectosil C_{18} (5 μm, 250 mm × 4.60 mm) reverse-phase column (Phenomenex, CA, USA) and UV detector (Shimadzu, SPD-10A UV detector) reading at 292 nm. The mobile phase was (40/60) acetonitrile/ammonium acetate (0.025 M, pH 5.3). The analytes were identified with the retention times of pure reference standards. Chromatographic peak areas of the analytes were measured using the integrator software (LCsolution, Shimadzu Corporation, Kyoto, Japan) of the HPLC system.

The red-FLBZ chromatographic peak fractions were collected into a glass tube by using a fraction collector (FRC-10A Shimadzu Corporation, Kyoto, Japan). The collected fractions were evaporated to dryness and redissolved with 10% 2-propanol in water. Fifty microlitres (50 μL) of each sample were injected into the HPLC system fitted with a chiral stationary phase column (5 μm, 100 mm × 4.0 mm) (Chiral-AGP column, ChromTech, Hägersten, Sweden). This chiral chromatographic method was adapted from a methodology described previously for the chiral separation of ABZSO [23]. Reduced FLBZ enantiomers were identified after the chromatographic analysis of a pure racemic standard of each molecule. The relative proportions (%) of each antipode were obtained using the integrator software of the HPLC system.

2.10. Drugs/metabolites quantification

Validation of the analytical procedures for extraction and quantification of FLBZ and their metabolites was performed before starting the analysis of the experimental samples from the incubation trials. Known amounts of each analyte (2–60 nmol/mL) were added to aliquots of boiled (inactivated) microsomal preparations and ruminal fluid samples. The fortified samples were extracted and analysed by HPLC

Table 1 – Validation parameters determined for the chromatographic analysis of flubendazole (FLBZ) and its reduced metabolite (red-FLBZ) in liver microsomes and ruminal fluid

Validation parameters	Liver microsomes		Ruminal fluid	
	FLBZ	Red-FLBZ	FLBZ	Red-FLBZ
r	0.999	0.998	0.999	0.999
Recovery (%)	80.5–89.7	79.7–84.5	93.6–99.9	92.2–98.2
Precision (CV)	1.58–6.61	2.69–8.11	5.21–8.30	3.47–9.10
Accuracy (%)	0.15–8.10	0.10–9.59	0.39–11.0	0.09–11.8

Calibration curves were prepared by processing inactivated microsomal and ruminal fluid samples fortified with each analyte (3 replicates for each concentration). Recovery, precision and accuracy were evaluated by processing replicates ($n = 4$) of FLBZ and red-FLBZ in each biological sample at 5, 10 and 20 nmol/mL. *r*, correlation coefficient; CV, coefficient of variation.

(triplicate determinations) to obtain calibration curves and percentages of recovery. Calibration curves were prepared using the least squares linear regression analysis (Instat 3.00, Graph Pad Software, Inc., San Diego, USA) of HPLC peak area ratios of analytes/internal standard and nominal concentrations of spiked samples. A lack-of-fit test was also carried out to confirm the linearity of the regression line of each analyte. The concentrations in the experimental samples were determined following interpolation of peak area ratios of analytes/internal standard into the standard curves. Absolute recoveries were established by comparison of the detector responses (peak areas) obtained for spiked microsomal samples (5, 10 and 20 nmol/mL) and those of direct standards prepared in mobile phase. The correlation coefficients, percentages of recovery, precision and accuracy values determined for FLBZ and red-FLBZ in liver microsomes and ruminal fluid are summarised in Table 1.

2.11. *Data and statistical analysis*

The reported data are expressed as mean ± S.D. Metabolic rates are expressed in nmol of metabolic products formed per min per mg of microsomal or cytosolic protein (nmol/min mg). Statistical comparisons were carried out using the Instat 3.00 software (Graph Pad Software, Inc., San Diego, USA). Metabolic rates were compared using Student *t*-test except for inhibition experiments of FLBZ metabolism were ANOVA was the selected statistical test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Characterisation of CBR activities in liver and duodenal subcellular fractions*

The time-dependent oxidation of NADPH was monitored fluorometrically using MEN as a specific CBR substrate. The Eadie-Hofstee plot of CBR activity in sheep liver microsomes and the mean K_m and V_{max} values of the metabolic reaction are shown in Fig. 2. The enzyme contained in the liver microsomal fraction shows typical one-site Michaelis–Menten kinetics. The K_m and V_{max} values ranged from 131.7 to 268 μM and 68.26 to 80.95 nmol/min mg, respectively. Quercetin (20 μM) inhibited (35.7%) the CBR-mediated microsomal keto-reduction of 125 μM MEN (Fig. 2) in sheep liver.

The CBR-mediated activity in microsomal and cytosolic fractions obtained from sheep liver and duodenal mucosa is shown in Table 2. Hepatic metabolic activities were 4.3-fold (microsomes) and 3.4-fold (cytosolic fraction) higher ($P < 0.05$) than those measured in the duodenal mucosa.

3.2. *Hepatic and extra-hepatic biotransformation of FLBZ*

The viability and metabolic capacity of the ruminal microflora was corroborated following the incubation of ABZSO under anaerobic conditions. These metabolic assays were used as positive controls, where ruminal fluid was able to sulphoreduce ABZSO into ABZ. After 60 min incubation, the amount of ABZ formed represented 54.6 ± 6.18% of the total analytes recovered from the incubation mixture. Both FLBZ and red-FLBZ were metabolically stable in ruminal content under the same incubation conditions. No metabolic conversion and/or

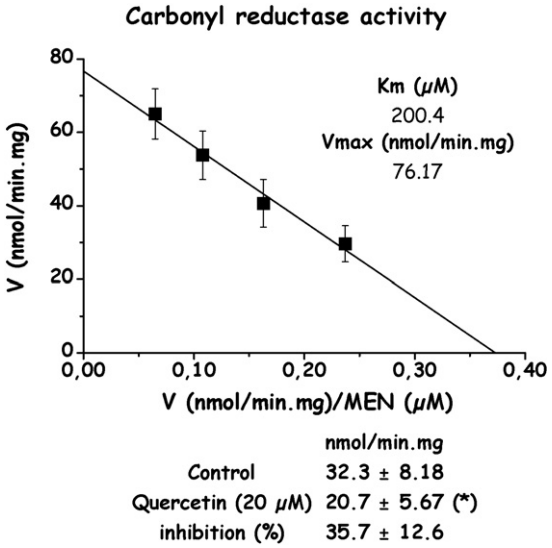

Fig. 2 – Eadie-Hofstee plot and Michaelis–Menten constants of carbonyl reductase (CBR) activity in ovine liver microsomes. The inhibitory effect of quercetin is shown in the inserted table. K_m (μM): substrate concentration giving half-maximal velocity. V_{max} (nmol/min mg): maximal velocity of the metabolic reaction. Data are expressed as mean ± S.D. ($n = 5$ animals). (*): the mean value is significantly different ($P < 0.05$) compared to that obtained in control incubations without quercetin.

Table 2 – Carbonyl reductase (CBR) activities measured in microsomal and cytosolic fractions obtained from sheep liver and duodenal mucosa

	Liver		Duodenal mucosa	
	Microsomal fraction	Cytosolic fraction	Microsomal fraction	Cytosolic fraction
CBR activity (nmol/min mg)	52.8 ± 4.62*	39.9 ± 9.44*	12.1 ± 2.09	11.7 ± 3.94

Metabolic activities (nmol/min mg of protein) were measured using menadione (500 μM) as a substrate and monitoring the oxidation rate of NADPH (see Section 2). Data are expressed as mean ± S.D. ($n = 5$ animals).
* Mean values are significantly different ($P < 0.05$) compared to those measured in the same subcellular fraction from the duodenal mucosa.

chemical degradation was observed following FLBZ or red-FLBZ incubation with sheep ruminal fluid (Fig. 3).

Flubendazole (40 μM) was incubated (30 min at 37 °C) with the cytosolic and microsomal fractions obtained from sheep liver and duodenal mucosa. Both subcellular fractions of each tissue were capable to convert FLBZ into red-FLBZ (Fig. 4). The production of the FLBZ hydrolysed metabolite (h-FLBZ) was not observed under these experimental conditions. The amount of red-FLBZ production ranged from 4 to 12.7 nmol/mg (liver microsomes) and 6.86 to 14.6 nmol/mg (liver cytosolic fraction). The amounts of red-FLBZ formed in liver subcellular fractions were 3-fold (microsomes) and 4-fold (cytosolic fraction) higher ($P < 0.05$) compared to that observed in duodenal subcellular fractions. This observation

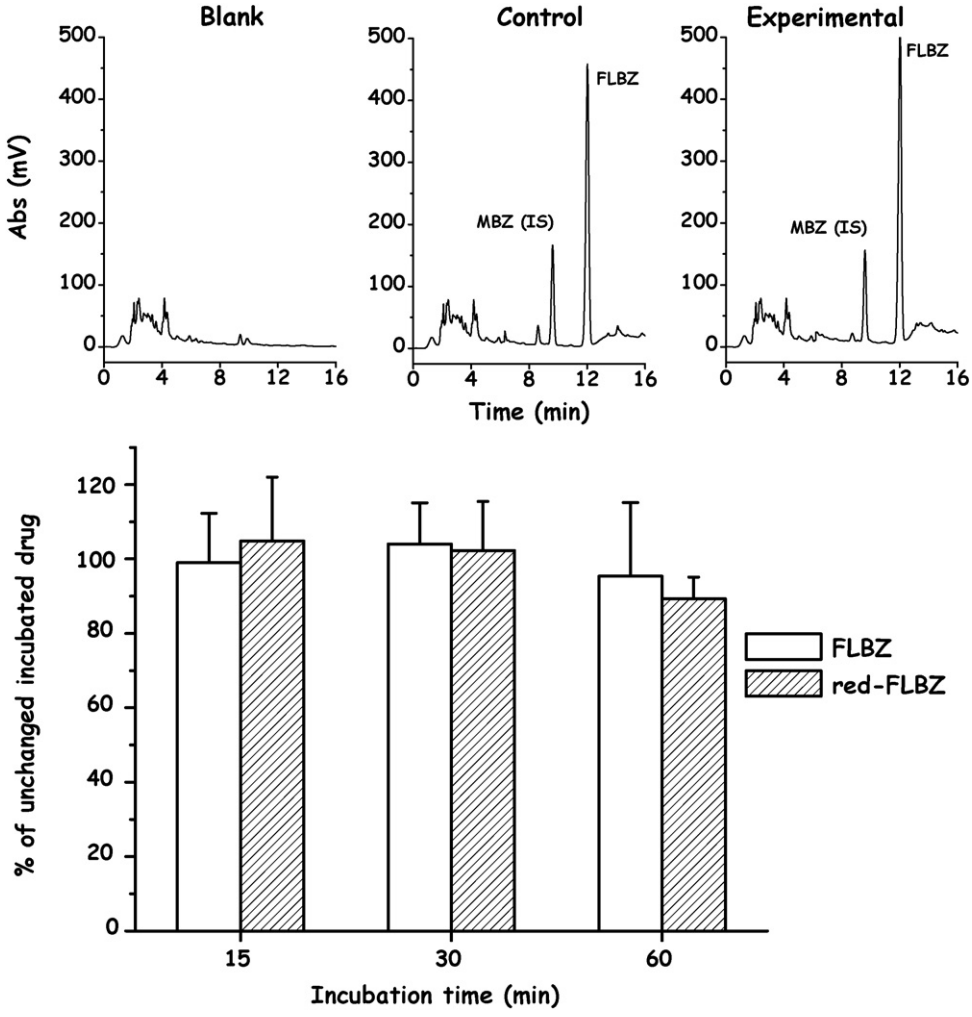

Fig. 3 – Metabolic stability of flubendazole (FLBZ) and reduced flubendazole (red-FLBZ) observed after their incubation (between 15 and 60 min) with sheep ruminal fluid under anaerobic conditions. The results are expressed as the percentage of unchanged parent drug recovered from the incubation medium compared with those determined in metabolically inactive (boiled) ruminal fluid (control incubations). Data (mean ± S.D.) were obtained following FLBZ or red-FLBZ incubations with ruminal fluid obtained from 3 animals. The insert shows typical chromatograms obtained following the analysis of unfortified (blank) ruminal fluid sample (left panel), FLBZ-added (inactivated) ruminal fluid sample (middle panel) and FLBZ incubated in experimental metabolically active ruminal fluid (right panel).

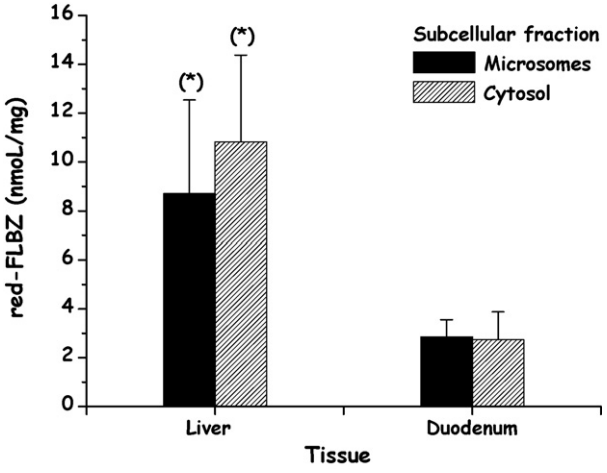

Fig. 4 – Comparative biotransformation of flubendazole (FLBZ) by microsomal and cytosolic fractions obtained from sheep liver and duodenal mucosa. The initial FLBZ concentration was 40 μM. Data (mean ± S.D.) are expressed in nmol of red-FLBZ per mg of microsomal or cytosolic protein ($n = 4$ animals). (*): mean values are significantly different ($P < 0.05$) compared to those obtained in the same subcellular fraction from the duodenal mucosa.

is consistent with the higher CBR activities measured in the liver compared to those observed in the gut mucosal tissue (see Table 2).

Representative chromatograms obtained following the chiral chromatographic analysis of red-FLBZ are shown in Fig. 5. Two enantiomeric forms were detected after the chromatographic analysis of a racemic standard of red-FLBZ (Fig. 5A). The keto-reduction of the pro-chiral FLBZ led to the prevalent (~98%) stereospecific production of one red-FLBZ enantiomeric form (RT = 7.4–7.6 min) in both liver subcellular fractions (Fig. 5B and C).

Sheep liver microsomes failed to oxidise red-FLBZ into FLBZ in the presence of NADPH. However, a NADPH-dependent oxidation produced FLBZ (0.40 ± 0.09 nmol/mg protein) from red-FLBZ in rat liver microsomes. Therefore, characterisation of the enzymatic pathway involved on red-FLBZ oxidation was performed in rat liver microsomes. The effect of PB chronic administration on CYP2C and CYP3A-mediated *N*-demethylase activities measured in rat liver microsomes are shown in Table 3. These metabolic activities were 2.3–3.6-fold higher in PB-induced compared to control rats. Consistently, red-FLBZ oxidation to FLBZ resulted 8.6-fold higher in PB-induced compared to control liver microsomes, while this metabolic reaction was inhibited (84%) in the presence of PBO (Table 4).

3.3. *Modulation of FLBZ keto-reduction in the liver*

The effects of MEN, WRF, MBZ and PBO on the liver microsomal keto-reduction of FLBZ are shown in Fig. 6. The presence of 40 μM MEN in the incubation medium led to a 33.3% inhibition of FLBZ keto-reduction compared to control assays. Higher concentrations of MEN seem to enhance the inhibitory effect. WRF did not inhibit the production of red-FLBZ. A significant inhibition of FLBZ reduction was also observed in the presence of 160 μM MBZ (46.8%) and 100 and 200 μM PBO (56.1–57.7%, respectively).

4. Discussion

The BZD anthelmintics require extensive hepatic oxidative metabolism to achieve sufficient polarity for excretion [24]. Their metabolic pattern and the resultant pharmacokinetic behaviour are relevant in the attainment of high and sustained concentrations of pharmacologically active drug/metabolites at the target parasite [25]. An integrated assessment of the pharmacological properties of FLBZ and its potential development for use in sheep was previously undertaken in our laboratory [15]. A detailed follow up study addressed to

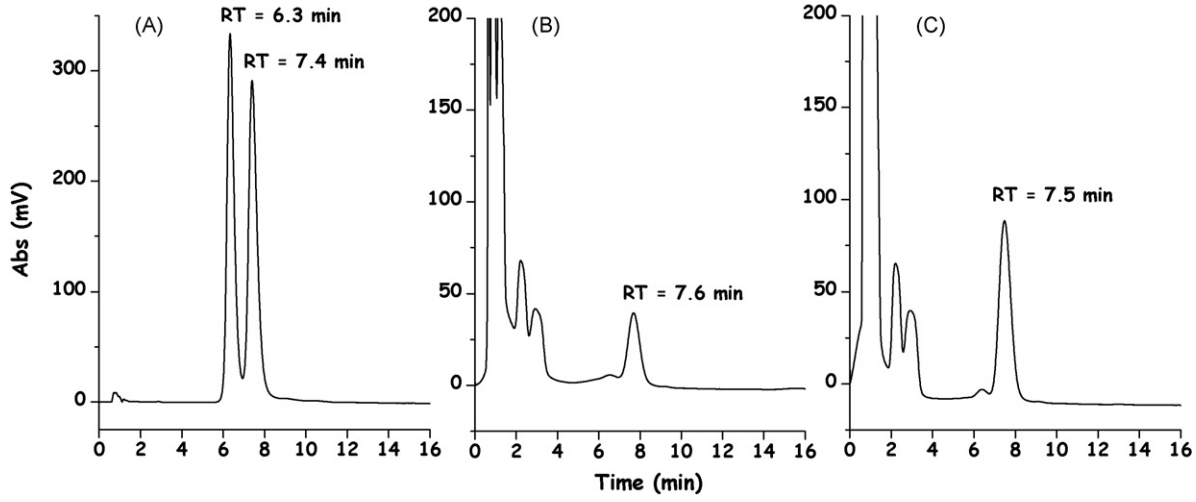

Fig. 5 – Representative chromatograms obtained after the chiral chromatographic analysis of a racemic standard of reduced flubendazole (red-FLBZ) (A) and experimental samples obtained after the incubation of flubendazole (FLBZ) with sheep liver cytosolic (B) and microsomal (C) fractions.

Table 3 – Comparative N-demethylase activities measured in liver microsomes obtained from control and phenobarbital (PB)-induced rats

CYP subfamily	Enzyme activity	Control	PB-induced
2C	Aminopyrine N-demethylase	11.6 ± 1.92	27.0 ± 4.30 (P = 0.001)
2C	Chlorpheniramine N-demethylase	6.22 ± 2.34	15.7 ± 4.46 (P = 0.009)
3A	Oleandomycin-triacetate N-demethylase	0.98 ± 0.36	3.51 ± 1.09 (P = 0.020)
3A	Erythromycin N-demethylase	0.92 ± 0.45	2.12 ± 1.17 (P = 0.106)

Enzymatic activities (mean ± S.D.) are expressed in nmol of formaldehyde released per min per mg of microsomal protein ($n = 4$ animals). P-values obtained after the statistical comparison (Student t-test) between both experimental groups are shown in parentheses.

Table 4 – In vitro oxidation of reduced flubendazole (red-FLBZ) into flubendazole (FLBZ) by control and phenobarbital (PB)-induced rat liver microsomes

	Control microsomes	PB-induced microsomes	PB-induced microsomes[a]	
			Control incubations	Incubations with PBO 100 μM
FLBZ (%)	100	861 ± 315**	100	15.8 ± 7.41**

[a] Effect of the cytochrome P450 inhibitor, piperonyl butoxide (PBO), on red-FLBZ oxidation by PB-induced rat liver microsomes. Mean ± S.D. values ($n = 4$ animals) are expressed as percentage of FLBZ formed with respect to their respective controls (equal to 100%).
** Significantly different ($P < 0.01$) compared to control.

identify the metabolic pathways implicated on the extensive FLBZ metabolism in sheep is described here.

The metabolic fate of FLBZ was studied in microsomal and cytosolic subcellular fractions obtained from liver and duodenal mucosa of adult male sheep. Both subcellular fractions were able to convert FLBZ into red-FLBZ. Similarly, the keto-reduction of FLBZ led to the production of the reduced metabolite in both cytosolic and microsomal fractions obtained from liver tissue and intestinal mucosa of pigs and pheasants [14]. Following FLBZ administration to sheep, red-FLBZ was the main metabolite found in the systemic circulation. An efficient first-pass metabolism of FLBZ after its gastrointestinal absorption accounted for the early detection of red-FLBZ in the bloodstream [15]. The results presented here confirm that FLBZ reduction both in the duodenal mucosa and in the liver parenchyma contribute to the observed pre-systemic metabolism of FLBZ. Conversely, ruminal fluid failed to reduce FLBZ, which discards the involvement of the rumen microflora as a source of red-FLBZ prior to its gastrointestinal absorption (see Fig. 3). This finding is opposed to that shown for the sulphur-containing BZD anthelmintics, such as albendazole sulphoxide and oxfendazole, which are sulphor-educed to their respective thioethers in the rumen [22,26].

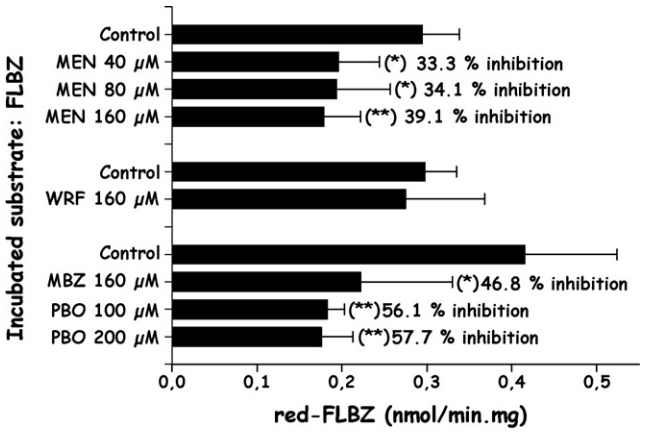

Fig. 6 – Effects of menadione (MEN), warfarin (WRF), mebendazole (MBZ) and piperonyl butoxide (PBO) on the biotransformation of flubendazole (FLBZ) by sheep liver microsomes. The initial FLBZ concentration was 40 μM. Data (mean ± S.D.) are expressed in nmol of red-FLBZ formed per min per mg of microsomal protein ($n = 5$ animals). Mean values are significantly different from control incubations at (*) $P < 0.05$ and (**) $P < 0.01$.

CBRs belong to a class of oxidoreductase proteins enclosed within the family of short chain dehydrogenases/reductases. They are ubiquitous in nature and catalyse the NADPH-mediated reduction of a large number of biologically and pharmacologically active substrates, including a variety of endogenous and xenobiotic carbonyl compounds. Generally, CBRs reduce aldehyde and ketone groups of many endo- and xenobiotics such as prostaglandins, steroids, biogenic amines, and quinones [27]. Considering that CBRs mediate the reduction of many compounds with a ketone group (including FLBZ), this enzymatic activity in both liver and duodenal mucosa from sheep was characterised here. The rate of NADPH oxidation in the presence of the substrate MEN is a good marker of CBR activity [28]. Both cytosolic and microsomal fractions obtained from sheep liver and duodenal mucosa showed CBR-mediated reduction of MEN. A typical Michaelis–Menten kinetics characterised the CBR activity in the hepatic microsomal fraction (see Fig. 2). In addition, metabolic activities measured in liver subcellular fractions were 3.4–4.3-fold higher compared to those observed in duodenal mucosa subcellular fractions (see Table 2). These results are in agreement with a 3–4-fold higher metabolic reduction of FLBZ in the liver compared to the gut mucosal tissue (see Fig. 4). A higher ability for FLBZ reduction was previously observed in the liver compared to the small intestine in both pigs and pheasants [14]. Altogether these observations may indicate that CBR-mediated reduction is the

major pathway involved in FLBZ biotransformation in these animal species.

Enantioselectivity of metabolic products occurs when chiral metabolites are generated differentially (in qualitative or quantitative terms) from a single achiral compound through the activity of any xenobiotic metabolising enzyme [29]. The CBR-mediated reduction of haloperidol led to the predominant production of S(−) reduced haloperidol in human liver and brain microsomes, which was also the main enantiomer recovered in urine [30]. Similarly, product enantioselectivity was also described for the CBR-mediated reduction of propiophenone in rats and rabbits, and idarubicin in various animal species and humans [31,32]. The current work showed that the reduction of FLBZ led to the predominant stereospecific production of one enantiomeric form of red-FLBZ in liver subcellular fractions, which had the largest retention time during the chiral chromatographic analysis (see Fig. 5). Unfortunately, the analytical methodology necessary to discriminate between both red-FLBZ enantiomers is not currently available in our laboratory. However, the (+) red-FLBZ was characterised as the main enantiomeric form produced from FLBZ in pigs [14]. Based on these observations made in pigs, a close phylogenetical-related species (artiodactyla order), it is possible to speculate that metabolic activity of liver CBRs may produced the (+) red-FLBZ in sheep.

As it was explained above, red-FLBZ is the main metabolic product formed from FLBZ both in vitro and in vivo. Conversely, the hydrolysis of FLBZ was not observed under these experimental conditions in both liver and intestinal subcellular fractions. In addition, this metabolic reaction did not occur following FLBZ nor red-FLBZ incubation with sheep ruminal fluid. Many other tissues of minor relevance for xenobiotic metabolism, such as blood, lung parenchyma and kidneys, may be involved in the hydrolysis of FLBZ in vivo, which could account for the detection of traces of h-FLBZ in the systemic circulation of FLBZ-treated sheep [15].

Reduced metabolites formed in a CBR-mediated reaction may be oxidised back to their precursor by the P450 enzymatic system. For instance, it has been shown that reduced haloperidol is oxidised to haloperidol by CYP2D6 and other P450 isoforms in human liver microsomes [33]. Based on such observation, it was hypothesised that the P450 system is involved on red-FLBZ oxidation in sheep liver. However, sheep liver microsomes failed to convert red-FLBZ into FLBZ in the presence of NADPH. Conversely, a NADPH-dependent oxidation of red-FLBZ was observed in rat liver microsomes. Thus, complementary assays were carried out to establish whether the P450 system is involved in red-FLBZ oxidation in rat liver. PB is a well-known inducer of several P450 isoforms, including CYP2B, 2C and 3A [34]. Thus, more than 2.3-fold increments on CYP2C and CYP3A-mediated metabolic activities were observed in the liver of PB-induced rats (see Table 3). These findings agree with previous reported data showing that PB induced the expression and the activities of these P450 isoforms [34–36]. In addition to the observed P450 induction, the oxidation of red-FLBZ to FLBZ resulted 8.6-fold higher in PB-induced compared to control rat liver microsomes. Piperonyl butoxide, a well known P450 inhibitor, decreased (84%) red-FLBZ oxidation in liver microsomes from PB-induced rats. Although this oxidative pathway for red-FLBZ is absent in sheep liver, the observations obtained in the rat after both induction and inhibition experiments, may confirm the involvement of the P450 system in this metabolic reaction.

Interference with the liver oxidative metabolism has resulted in pronounced modifications to the pharmacokinetic behaviour of BZD parent drugs or their active metabolites. It has been shown that co-administration of sulphur-containing BZDs with known FMO [37] or P450 [38,39] metabolic inhibitors enhance the plasma availabilities of the parent drug or its metabolites. The higher plasma levels of anthelmintically active molecules may prolong the plasma-tissue recycling process and increase their concentrations at the most important tissues of parasite location [25], which may improve the clinical efficacy of the administered compound as a result of higher levels of active drug/metabolites being presented to the parasite for longer periods of time. It is well known that biotransformation of BZD anthelmintics leads to more polar and less active metabolites. In fact, in terms of parasite uptake [40,41] and mode of action (binding to nematode tubulin) [42], the parent drugs (such as FLBZ) are more efficient than their respective metabolites. Although there is no information available on the anthelmintic activity of red-FLBZ, this metabolite is supposed to be less potent (or may be inactive) compared to the parent drug. Therefore, CBR-mediated FLBZ metabolism may give rise to a considerable reduction on its anthelmintic efficacy. Among the CBR substrates tested, MEN and MBZ inhibited FLBZ keto-reduction in sheep liver microsomes (see Fig. 7), which may also confirm the involvement of this enzymatic system on the production of red-FLBZ in sheep liver. Although PBO is known as a P450 inhibitor, this compound also reduced the formation of red-FLBZ. This finding may indicate that CBRs are also target enzymes for PBO. Interestingly, other enzymes such as non-specific esterases are also inhibited by PBO [43]. It is important to emphasise that this metabolic inhibitor is widely used to improve the efficacy of some ectoparasiticide compounds such as pyrethroids and rotenone [44,45]. Despite the selected inhibitor, the modulation of FLBZ metabolism under in vivo conditions should be considered as a useful tool to improve its systemic availability and clinical anthelmintic efficacy in sheep as well as in other livestock species.

In conclusion, the hepatic and extra-hepatic metabolic fate of FLBZ in sheep was characterised in this work for the first time. CBR activities measured in liver microsomal and cytosolic fractions were higher compared to those observed in both subcellular fractions obtained from the duodenal mucosa. This finding correlates with the higher FLBZ ketone-reduction observed in the liver compared to the small intestinal mucosa. The reduction of FLBZ in sheep liver was enantioselective, presumably to the (+) red-FLBZ. While the P450-mediated oxidation of red-FLBZ into FLBZ was observed in rat liver microsomes, this metabolic pathway is absent in sheep liver. MEN, MBZ and PBO inhibited FLBZ reduction in sheep liver microsomes. These metabolic interferences observed in vitro should be considered as a useful tool to improve FLBZ systemic availability and efficacy. The research reported here is a further contribution to the understanding of the metabolic fate of a promissory anthelmintic drug to be used for parasite control in ruminants. Regardless of the relevance of the specific drug substrate investigated here, the

metabolic approach followed in this work is useful to be applied to assess the unknown pattern of biotransformation for a large number of xenobiotic compounds of pharmaco-toxicological relevance.

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