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Species differences in hepatic biotransformation of the anthelmintic drug flubendazole

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Flubendazole (FLBZ) is a broad-spectrum benzimidazole anthelmintic used in pigs, poultry, and humans. It has been proposed as a candidate for development for use in elimination programmes for lymphatic filariasis and onchocerciasis in humans. Moreover, FLBZ has shown promise in cancer chemotherapy, particularly for neuroblastoma. This work investigated the hepatic carbonyl-reducing pathway of FLBZ in different species, including humans. Microsomal and cytosolic fractions were obtained from sheep, cattle, pig, hen, rat, and human liver. Both subcellular fractions of each species converted FLBZ into a reduced metabolite (red-FLBZ). The rate of microsomal red-FLBZ production was highest in sheep (1.92 ± 0.13 nmol/min.mg) and lowest in pigs (0.04 ± 0.02 nmol/min.mg); cytosolic red-FLBZ production ranged from 0.02 ± 0.01 (pig) to 1.86 ± 0.61 nmol/min.mg (sheep). Only subcellular fractions from sheep liver oxidized red-FLBZ to FLBZ in a $NADP^+$-dependent oxidative reaction. Liver microsomes from both pigs and humans transformed FLBZ to red-FLBZ and a hydrolyzed metabolite. Very significant differences in the pattern of FLBZ metabolism were observed among the tested species and humans. These results reinforce the need for caution in extrapolating data on metabolism, efficacy, and safety of drugs derived from studies performed in different species.

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INTRODUCTION

Flubendazole (FLBZ), the methyl ester of [5-(4-fluorobenzoyl)-1*H*-benzimidazol-2-yl] carbamic acid, is a broad-spectrum benzimidazole (BZD) methylcarbamate anthelmintic. FLBZ is marketed for the control of gastrointestinal nematode infections in swine, poultry, and other domestic animals, for control of lungworms in swine, and for human gastrointestinal nematode infections. Flubendazole is also a macrofilaricide, being highly efficacious in a number of animal models, including *Dirofilaria immitis* in dogs and *Brugia pahangi* in cats following parenteral dosing (Mackenzie & Geary, 2011). In this regard, it has been proposed as a candidate compound for development for use in elimination programmes for lymphatic filariasis and onchocerciasis in humans (Mackenzie & Geary, 2011). Moreover, like other BZD derivatives affecting microtubule function, FLBZ has shown promise cancer chemotherapy, particularly for neuroblastoma (Michaelis *et al.*, 2015). A key aspect of development of FLBZ for use in systemic, as opposed to gastrointestinal, diseases is the need to identify an oral formulation that provides much greater bioavailability than currently available preparations (Ceballos *et al.*, 2012, 2014). When considering the suitability of animal models for preclinical development of a systemically available drug, it is essential to determine the consistency of exposure profiles attained in the model vs. target species. As a first step, comparative data on metabolism are important.

Phase 1 and phase 2 xenobiotic metabolizing enzymes (XMEs) play a major role in determining the persistence of drugs in target species. As a class, BZD anthelmintics are extensively metabolized by the host. Unlike other commonly used BZDs, such as albendazole and fenbendazole, FLBZ does not contain a sulfur atom at position -5 of the benzimidazole ring. Instead, a ketone group is present in this molecule, which may have implications for the pattern of biotransformation by the host. In this regard, ketone-containing moieties are likely to be metabolized by carbonyl-reducing enzymes (e.g. aldo-keto reductases, short-chain dehydrogenases/reductases) into their corresponding alcohols (Rosemond & Walsh, 2004; Skarydová & Wsól, 2012). Both aldo-keto reductase and short-chain

dehydrogenase/reductase enzyme superfamilies are ubiquitous in nature and exhibit broad and overlapping substrate specificities (Skarydová & Wsól, 2012). Carbonyl reduction of FLBZ yields a hydroxy metabolite, reduced FLBZ (red-FLBZ). In addition, the parent compound undergoes biotransformation to a hydrolyzed (decarbamoylated) metabolite (h-FLBZ) (Nobilis *et al.*, 2007). These pathways of FLBZ biotransformation are located in cytosolic and microsomal fractions prepared from the liver and the small intestinal mucosa of pigs, pheasants (Nobilis *et al.*, 2007), and sheep (Maté *et al.*, 2008).

Metabolic interactions between drugs can drastically affect their disposition kinetics in livestock and humans. From this point of view, understanding species differences in biotransformation patterns of a given drug is of great importance. In the case of human medicine, these investigations may be useful for searching model species (e.g. rodents) for *in vitro* bioassays to be used in preclinical studies during drug development. This work investigated the hepatic carbonyl-reducing pathway of FLBZ in different species, including humans. Overall, this research contributes to the characterization of the patterns of hepatic biotransformation of this anthelmintic in sheep, cattle, pig, rat, hen, and human. This information is of considerable importance for efforts to optimize and interpret the pharmacological activity of FLBZ in various target species.

MATERIALS AND METHODS

Reagents

Reference standards (99.5% pure) of FLBZ, red-FLBZ, and h-FLBZ were kindly provided by Janssen Animal Health (Beerse, Belgium). Stock solutions (2.5 mM) of each molecule were prepared in methanol (Baker Inc., Phillipsburg, New Jersey, USA). Albendazole sulphoxide (ABZSO), used as internal standard, was provided by Schering Plough (Kenilworth, New Jersey, USA). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche Applied Science (Buenos Aires, Argentina). The solvents used for chemical extraction and chromatographic analysis were HPLC grade (Baker Inc., Phillipsburg, New Jersey, 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, New Jersey, USA).

Animals

Liver samples, free from pathological lesions, were obtained from Texel lambs (n = 6), Aberdeen Angus steers (n = 6), Landrace pigs (n = 4), Wistar male (n = 6) and female (n = 6) rats, and Plymouth Rock Barrada hens (n = 6). Animals were not subjected to any pharmacological treatment prior to slaughter.

Bovine liver samples were obtained from a local slaughterhouse (Mirasur SA, Tandil, Argentina) located 16 km from laboratory facilities. Procedures and management protocols for all animal species 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 (Internal Protocols: 02/2011 and 12/2013; approval dates: March 1, 2012 and October 30, 2013, respectively). Animals were sacrificed in agreement with this institutional animal welfare policy.

Preparation of subcellular fractions

A pool of liver microsomes from adult human beings (mixed age and gender) was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Cattle, sheep, and pig livers were removed, and their caudate processes were immediately excised. The whole liver was used for rats and hens. All liver specimens (10–8 g) were washed with ice-cold 1.15% KCl, covered with aluminum foil, and chilled on ice during transport. Microsomal and cytosolic fractions were isolated by differential ultracentrifugation. Briefly, tissue samples were weighed and homogenized using a Potter-Elvenjem tool (four to six passes) with two volumes of ice-cold homogenization buffer (0.1 M potassium phosphate, pH 7.4, containing 0.15 M Tris acetate, 0.1 M KCl, 1 mM EDTA, and 18 μM butylated hydroxytoluene). Homogenates were filtered through a hydrophilic gauze and centrifuged at 10 000 g for 20 min. The resulting supernatant was centrifuged at 100 000 ***g*** for 65 min. Aliquots of supernatants (cytosolic fractions) were frozen in liquid N_2 and stored at −70 °C. Pellets (microsomal preparations) were suspended in 0.1 M potassium phosphate buffer (pH 7.4, containing 0.1 mM EDTA and 20% glycerol), frozen in liquid N_2, 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 standard (Lowry *et al.*, 1951).

Enzyme assays in liver microsomal and cytosolic fractions

Enzyme assays were carried out in both microsomal and cytosolic hepatic subcellular fractions from sheep, cattle, pigs, rats, and hens, but only in pooled liver microsomes from humans.

Assays with FLBZ as substrate. The enzymatic carbonyl reduction of FLBZ toward red-FLBZ and h-FLBZ was assessed in each subcellular fraction (microsomal and cytosolic) by measuring the amount of red-FLBZ and h-FLBZ formed in the presence of a 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 glucose-6-phosphate dehydrogenase in 0.1 M phosphate buffer, pH 7.4). A typical reaction mixture contained (in a final volume of 0.5 mL): phosphate buffer 0.1 M (pH 7.4), 0.5 mg microsomal or cytosolic protein diluted in 50 μL of the same buffer, NADPH-generating system, and 40 μM FLBZ dissolved in 10 μL methanol. Incubation mixtures were allowed to equilibrate (1 min at 37 °C), and the reaction was started by the addition of the NADPH-generating system. Incubations

(15 min at 37 °C) were carried out in glass vials in an oscillating water bath under aerobic conditions. Blank samples, containing all components of the reaction mixture except the NADPH-generating system, were incubated under the same conditions. These incubations were used as controls for nonenzymatic drug conversion. All reactions were stopped by the addition of 0.2 mL acetonitrile and stored at −20 °C until analysis.

Assays with red-FLBZ as substrate. The enzymatic oxidation of red-FLBZ toward FLBZ was assessed in both subcellular fractions by measuring the amount of FLBZ formed in the presence of $NADP^+$ (1 mM), 1 mg/mL of microsomal protein and 40 μM red-FLBZ (dissolved in 10 μL methanol). Incubations mixtures were prepared in a phosphate buffer 0.1 M (pH 7.4), allowed to equilibrate (1 min at 37 °C), and the reaction was started by the addition of the co-factor ($NADP^+$). Incubation conditions and procedures were the same as those described above.

Chromatographic analysis

Twenty microlitre of the internal standard (IS) ABZSO (250 nmol/mL) was added to the inactivated microsomal or cytosolic incubation mixture. Then, samples were shaken for 10 sec and centrifuged at 10 000 *g* for 15 min at 10 °C. Supernatants were directly injected into the HPLC system.

Samples were analyzed for ABZSO, FLBZ, red-FLBZ, and h-FLBZ by HPLC. One hundred microlitre of each sample was injected through an auto sampler (Shimadzu SIL-10 A Automatic Sample Injector) into a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a Kromasil C_{18} (5 μm, 250 mm × 4.60 mm) reverse-phase column (Eka Chemicals, USA) and UV detector (Shimadzu, SPD-10A UV detector) reading at 292 nm. The mobile phase was (32/68) acetonitrile/ammonium acetate (0.025 M, pH 5.3). Analytes were identified by matching their retention times with those 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.

Drug/metabolites quantification

Validation of the analytical procedures for quantification of FLBZ and metabolites was performed before analysis of the experimental samples from incubation assays. Known amounts of each analyte (1.56–100 nmol/mL) were added to aliquots of boiled (inactivated) microsomal preparations. The fortified samples were centrifuged and analyzed by HPLC (triplicate determinations) to validate the analytical method. Calibration curves were prepared using 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 linearity of the regression line of each analyte. Concentrations in the experimental samples were determined following interpolation of peak area ratios of analytes/internal standard into the standard curves.

Complete validation of the analytical procedures for quantification of drug and metabolites in microsomes was performed before starting the analysis of experimental samples. Retention times for red-FLBZ, h-FLBZ, and FLBZ were 8.54, 9.83, and 17.1 min, respectively. The calibration curves for each analyte, constructed by least-squares linear regression analysis, showed good linearity, with correlation coefficients ≥0.998. The limit of quantification for FLBZ and metabolites was 1.56 nmol/mL, defined as the lowest measured concentration with a CV <20%, with an accuracy of ±20%.

Data and statistical analysis

Data are expressed as mean ± SD. Metabolic rates are expressed in nmol of metabolic products formed per min per mg of microsomal or cytosolic protein. Metabolic rates were statistically compared using Kruskal–Wallis test (nonparametric ANOVA). Statistical comparisons were carried out using the Instat 3.00 software (Graph Pad Software, Inc., San Diego, USA). A value of $P < 0.05$ was considered significant. Only one sample ($n = 1$) of pooled human liver microsomes was employed for the incubation assays; therefore, data obtained were not included for the statistical analysis.

RESULTS

Both subcellular fractions of all species converted FLBZ into red-FLBZ (Figs 1 and 2). The rate of microsomal red-FLBZ production ranged from 0.04 ± 0.02 (pigs) to 1.92 ± 0.13 nmol/min.mg (sheep). As shown in Fig. 1, the rate of FLBZ metabolism to red-FLBZ in sheep liver microsomes was higher ($P < 0.001$) than in cattle (4.3-fold), human (21-fold), female

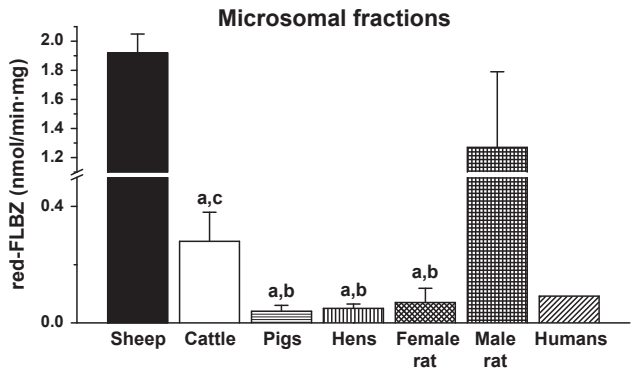

Fig. 1. Comparative biotransformation of flubendazole (FLBZ) by liver microsomal fractions obtained from sheep, cattle, pigs, hens, rats (male and female), and humans. The initial FLBZ concentration was 40 μM. Data (mean ± SD) are nmol of red-FLBZ formed per min.mg of microsomal protein. (a) Statistically different vs. sheep ($P < 0.001$). (b) Statistically different vs. male rats ($P < 0.01$). (c) Statistically different vs. pig ($P < 0.05$).

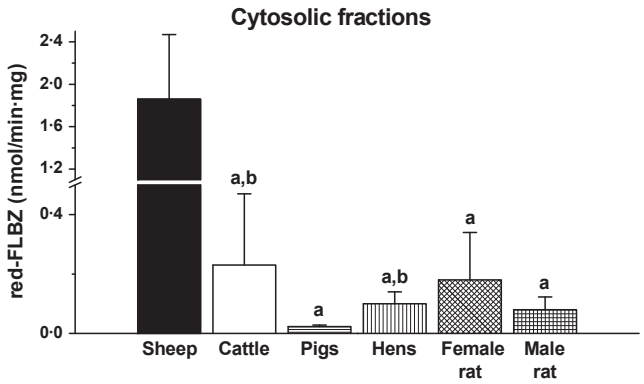

Fig. 2. Comparative biotransformation of flubendazole (FLBZ) by liver cytosolic fractions obtained from sheep, cattle, pigs, hens, and rats (male and female). The initial FLBZ concentration was 40 μM. Data (mean ± SD) are nmol of red-FLBZ formed per min.mg of cytosolic protein. (a) Statistically different vs. sheep ($P < 0.05$). (b) Statistically different vs. pigs ($P < 0.05$).

rat (27-fold), hens (38-fold), and pigs (43-fold). Similarly, cytosolic red-FLBZ production ranged from 0.02 ± 0.01 (pig) to 1.86 ± 0.61 nmol/min.mg (sheep). As shown in Fig. 2, the rate of FLBZ metabolism to red-FLBZ in sheep liver cytosolic fraction was higher ($P < 0.05$) than in cattle (8.1-fold), female rat (10.3-fold), hens (18.5-fold), male rat (23.1-fold), and pigs (80-fold).

The production of h-FLBZ was only observed in pigs (microsomes and cytosols) and humans (microsomes) (Table 1). In pigs, the rate of h-FLBZ production in liver microsomes was 6.9-fold higher ($P < 0.05$) than the rate of generation of red-FLBZ, while in the cytosolic fraction, the rates of production of both metabolites were similar. Human liver microsomes produced similar amounts of each metabolite. Compared to pig liver microsomes, the rate of red-FLBZ production was 2.3-fold higher in human liver microsomes, but the rate of production of h-FLBZ was lower.

Figure 3 shows the comparative biotransformation of FLBZ and red-FLBZ by both microsomal and cytosolic fractions from sheep liver. Both subcellular fractions oxidized red-FLBZ to FLBZ in a $NADP^+$-dependent oxidative reaction. However, the rates of red-FLBZ production (reductive reaction) were higher ($P < 0.05$) than those measured for the production of FLBZ (oxidative reaction). In both subcellular fractions, the ratios red-FLBZ/FLBZ were similar.

DISCUSSION

BZD anthelmintics require extensive hepatic metabolism to achieve sufficient polarity for excretion. Their metabolic patterns and the resultant pharmacokinetic behavior determine the ability to attain high and sustained concentrations of pharmacologically active drug/metabolites at the site of infection of the target parasites. Carbonyl-reducing enzymes are thought to be the main enzymes involved in the hepatic ketone reduction in FLBZ to red-FLBZ. A great number of ketone-containing moieties are metabolized by the short-chain dehydrogenases/reductases (SDR) superfamily (Skarydová & Wsól, 2012). As a general rule, SDR members catalyze the reversible oxido reduction in a wide variety of endogenous and exogenous compounds. Cytosolic carbonyl reductases (CBRs), like human CBR1, and microsomal hydroxysteroid reductases (HSDs), such as 11 β-HSD, are among the most relevant SDRs. Cytosolic CBRs reduce aldehyde and ketone groups of endogenous compounds and xenobiotics such as prostaglandins, steroids, biogenic amines, and quinones (Forrest & Gonzalez, 2000). Microsomal HSDs specifically catalyze the oxido reduction in steroids, but are able to catalyze ketone reduction in a great variety of nonsteroidal carbonyl-containing compounds (Hoffman & Masser, 2007). Thus, both enzyme types may be involved in FLBZ ketone reduction, and their patterns of expression and function may explain the observed species-related differences in the hepatic biotransformation of this compound (see Figs 1 and 2). Indeed, their metabolic activities may impact on the total amount of metabolites recovered from the bloodstream after FLBZ treatment, which may differ according to the animal species considered. In this regard, the current work shows a much higher rate of FLBZ metabolism to red-FLBZ in sheep liver subcellular fractions than in any of the other animal species under study. An efficient hepatic and intestinal first-pass metabolism of FLBZ after its gastrointestinal absorption may account for the early detection of red-FLBZ, the main metabolite found in the systemic circulation after FLBZ administration to sheep (Moreno *et al.*, 2004; Maté *et al.*, 2008; Krízová *et al.*, 2009; Ceballos *et al.*, 2012). Conversely,

Table 1. Comparative hepatic biotransformation of flubendazole (FLBZ) in pigs and humans

Species	Subcellular fraction					
	Microsomal			Cytosolic		
	red-FLBZ	h-FLBZ	Ratio*	red-FLBZ	h-FLBZ	Ratio*
Pigs	0.04 ± 0.02	0.27 ± 0.09†	6.9 ± 3.0‡	0.02 ± 0.01	0.02 ± 0.00	0.8 ± 0.2
Humans	0.09	0.12	1.30	NA	NA	NA

NA, not applicable. Data (± SD) are nmol of metabolites formed per min.mg of microsomal or cytosolic protein. *Mean ratio h-FLBZ/red-FLBZ. †Statistically different ($P < 0.05$) vs. h-FLBZ production in the cytosolic fraction. ‡Statistically different ($P < 0.05$) vs. the h-FLBZ/red-FLBZ ratio observed in the cytosolic fraction.

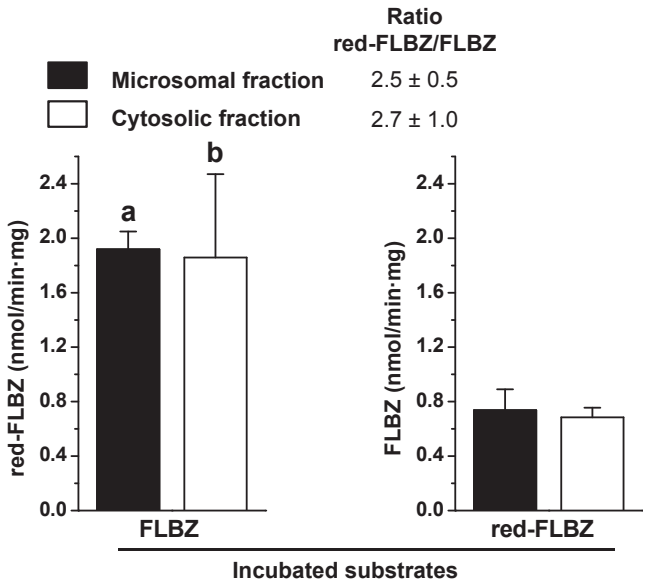

Fig. 3. Comparative biotransformation of flubendazole (FLBZ) (reduction, left panel) and its reduced metabolite (red-FLBZ) (oxidation, right panel) by both microsomal and cytosolic fractions from sheep liver. The reduction in FLBZ toward red-FLBZ was statistically different (a: $P < 0.001$; b: $P < 0.01$) compared to the oxidation of red-FLBZ into FLBZ in the same subcellular fraction. The inserted table compares red-FLBZ/FLBZ ratios found in both hepatic subcellular fractions. Data are expressed as mean ± SD of six livers.

the poor FLBZ biotransformation observed in both cytosolic and microsomal fractions from hens is in agreement with the absence of red-FLBZ in breast and thigh muscle from poultry medicated with the anthelmintic (De Ruyck *et al.*, 2001).

Reduced metabolites formed in a CBR-mediated reaction may be oxidized back to their precursors by the cytochrome P450 (CYP) system. For instance, liver microsomes from phenobarbital-treated female rats oxidized red-FLBZ into FLBZ in a CYP-dependent reaction (Maté *et al.*, 2008). Conversely, sheep liver microsomes failed to conduct this oxidative reaction in the presence of NADPH (Maté *et al.*, 2008). However, in the present work, only sheep liver subcellular fractions oxidized red-FLBZ to FLBZ, but in a $NADP^+$-dependent reaction (see Fig. 3). This observation may indicate that the oxidation of red-FLBZ in sheep is not CYP dependent. As mentioned above, both CBRs and HSDs may contribute to the ketone reduction in FLBZ. These enzymes may also catalyze the dehydrogenation of red-FLBZ back to the parent drug. An interesting feature of the SDR superfamily is that it depends on their predominant function as a dehydrogenase or reductase whether they use either $NADP^+$ or NADPH as co-substrate (Hoffman & Masser, 2007). Other $NAD(P)^+$-dependent cytosolic enzymes, such as aldehyde and alcohol dehydrogenases, may also be involved in red-FLBZ oxidation. In this regard, different members from this group of enzymes are able to oxidize several substrates like retinol to retinaldehyde (Forrest & Gonzalez, 2000; Kumar *et al.*, 2012). Further to these speculations, our work shows that red-FLBZ dehydrogenation into FLBZ may occur only in sheep liver, although the equilibrium favors the ketone reduction in the parent compound.

Cattle liver subcellular fractions reduced FLBZ to red-FLBZ using NADPH as cofactor, but did not oxidize red-FLBZ to FLBZ using $NADP^+$ as cofactor. The rate of FLBZ reduction showed considerable inter-ruminant species variation: FLBZ reduction in red-FLBZ was 4.3- and 8.1-fold higher in sheep liver microsomes and cytosol than in the corresponding cattle subcellular fractions. Moreover, while sheep liver subcellular fractions oxidized red-FLBZ to FLBZ, this reaction was not observed in cattle liver microsomes or cytosols. These results highlight the need for comparative data on drug metabolism in ruminants, because the relative lack of drugs specifically registered for such species commonly results in the extra-label use of veterinary medicinal products already licensed for other species (Gusson *et al.*, 2006).

FLBZ metabolism was also studied in hepatic microsomal and cytosolic fractions obtained from male and female rats; subcellular fractions from both sexes converted FLBZ into red-FLBZ. Production of red-FLBZ was significantly higher in male than in female microsomes. Interestingly, the rate of formation of red-FLBZ in liver microsomes from male rats was similar to that observed in sheep liver microsomes (see Fig. 1). The lower red-FLBZ production observed in hepatic subcellular fractions from females may explain the prevalence of FLBZ over red-FLBZ in the systemic circulation of female rats treated with different formulations of the parent drug (Ceballos *et al.*, 2014).

Flubendazole was also incubated with microsomal and cytosolic fractions from pig liver. Both phase 1 FLBZ metabolites (red- and h-FLBZ) were produced by both subcellular fractions. Compared to the cytosolic fraction, the production of h-FLBZ was more efficient and accounted for a higher h-FLBZ/red-FLBZ ratio in pig liver microsomes. These results are in agreement with recent observations prepared in a pharmacokinetic trial conducted in our laboratory (Ceballos *et al.*, 2015). In fact, the hydrolyzed metabolite was the main molecule recovered from pigs treated with different experimental formulations of FLBZ *per os*, being approximately 97% of the total metabolites detected in plasma (Ceballos *et al.*, 2015). In agreement, the *in vitro* production of h-FLBZ observed in the current trial was roughly 80% of the total metabolites formed (see Table 1). It has been suggested that red-FLBZ is an active metabolite, whereas h-FLBZ has no anthelmintic activity (Bártíková *et al.*, 2010; Ceballos *et al.*, 2011). Consequently, in terms of the final anthelmintic activity of FLBZ against systemically located target parasites, the metabolic profile of this anthelmintic may represent a major disadvantage in pigs. In contrast to these *in vivo* and *in vitro* observations, Nobilis *et al.* (2007) reported that the NADPH-dependent ketone reduction in FLBZ led to the prevalent formation of red-FLBZ in the cytosolic compared to the microsomal fraction obtained from liver tissue and intestinal mucosa of pigs, while h-FLBZ was identified only in the cytosolic fraction. Unfortunately, we have no facile explanation for this discrepancy, but the *in vitro* data emerging from the present work corroborated those previous

in vivo observations from the pharmacokinetic study of Ceballos and co-workers (Ceballos *et al.*, 2015).

Elimination or eradication of human filariases will almost certainly require the use of a macrofilaricidal agent. *In vivo* trials in humans and many experimental animal models suggest that FLBZ is a highly efficacious macrofilaricide (Mackenzie & Geary, 2011). However, serious injection site reactions were reported in humans after parenteral FLBZ administration, lending urgency to the search for alternative pharmaceutical strategies to improve the systemic availability of FLBZ and its metabolites in both human and veterinary medicine. Although oral bioavailability of FLBZ has been estimated in humans (EMEA, 1997), no data are available on the plasma pharmacokinetic pattern of FLBZ and its metabolites in humans after parenteral administration. In an earlier research, systemic concentrations of FLBZ were measured in humans by means of a radioimmunoassay (reviewed in Edwards & Breckenridge, 1988). Maximal levels of the anthelmintic in plasma were attained 1–4 h after dosing and the enteral absorption of FLBZ was markedly enhanced when the drug was taken with a meal. Unfortunately, the metabolic profiles of FLBZ metabolites were not reported in the aforementioned investigation. The *in vitro* studies reported here show that human liver microsomes transform FLBZ to red-FLBZ and h-FLBZ, which suggests similarity with the metabolic profile observed in pigs. Species-related differences in plasma drug exposure observed for FLBZ and its metabolites may significantly influence efficacy. As mentioned above, while h-FLBZ is an inactive metabolite, biological activity has been described for red-FLBZ, which may contribute to the overall anthelmintic effect of FLBZ (Ceballos *et al.*, 2014).

In conclusion, remarkable differences in the pattern of FLBZ metabolism were observed among food-producing species and humans. These results illustrate again the need for caution in extrapolating data on metabolism, efficacy, and safety of drugs derived from studies performed in different model and target species.

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