Comparative Pharmacokinetics and Metabolisms of Caffeine in Sheep Breeds

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ABSTRACT. The purpose of this study was to investigate the effect of breed on the pharmacokinetics and metabolism of caffeine (CF) and the hepatic metabolic capacity in sheep. CF was administered as a single intravenous dose of 5 mg/kg b.w. in Morkaraman (MK), Akkaraman (AK) and Anatolia Merino (AM) sheep breeds. The plasma levels of CF and its primary metabolites, theobromine (TB), paraxanthine (PX) and theophylline (TP), were measured using high-performance liquid chromatography. Pharmacokinetic parameters of CF and its metabolites were calculated. Plasma TB+PX+TP/CF metabolic ratio was determined as an alternative to CF clearance for the determination of hepatic metabolic capacity. In the three breeds, all kinetic parameters of CF differed significantly (P<0.05) except for volume of distribution. Elimination of CF was slow in the MK (ClT: 0.03 ± 0.01 l/hr/kg, t1/2z: 15.74 ± 7.35 hr) and AM (ClT: 0.05 ± 0.02 l/hr/kg, t1/2z: 9.68 ± 5.21 hr) breeds when compared with the AK breed (ClT: 0.08 ± 0.01 l/hr/kg, t1/2z: 6.84 ± 0.79 hr). There was significant correlation (r²=0.904, P<0.01) between CF clearance and the plasma TB+PX+TP/CF ratio calculated at 7 hr after CF administration. The plasma TB+PX+TP/CF ratios were statistically different (P<0.05) among the breeds (MK, 0.155 ± 0.062; AK, 0.468 ± 0.107; AM, 0.254 ± 0.099). These results suggest that the pattern of drug biotransformation should be consistently tested for all breeds within species. Further studies are needed to determine the biochemical and molecular events underlying such an effect.

KEY WORDS: breed, caffeine, hepatic metabolic capacity, pharmacokinetics, sheep.

The activities of biotransformation enzymes affect either the pharmacological or toxicological impacts of drugs and xenobiotics. During the past decades, much of the detailed knowledge of enzymes responsible for biotransformation has been learned in laboratory animals and humans. In contrast, knowledge of biotransformation enzymes and various factors (breed, age) causing their variability in veterinary food-producing species is still incomplete [10, 12, 15, 19, 26, 35].

Genotyping and phenotyping are the 2 methods that are used today to assess the in vivo activity of biotransformation enzymes. However, the optimal method of describing actual enzyme activity is phenotyping because it is a reflection of the combined effects of genetic, environmental and endogenous factors on enzyme activity [25]. Phenotyping for drug metabolizing enzymes is defined as measuring its actual in vivo activity in an individual. This is performed by administrations of probe substrates for various isoforms of cytochrome P450 (CYP 450) and other enzymes and subsequent determination of appropriate pharmacokinetic parameters or is performed by using metabolism [12].

Caffeine (CF) is now widely used to assess genetic, environmental and race/breed differences for some enzymes and the hepatic metabolic capacity (total activity of enzymes metabolizing CF) because of its pharmacokinetics and almost complete biotransformation by some CYP 450 enzymes in liver [17, 23]. The primary metabolic pathways of CF involve N-3, N-1 and N-7 demethylation to form paraxanthine (PX), theobromine (TB) and theophylline (TP), respectively. These processes account for 95% of caffeine metabolism in humans [17]. CYP1A2 is the enzyme principally responsible for N-3 demethylation of CF, whereas CYP1A2, CYP2E1 [32, 33] and flavin-containing monoxygenases (FMO) [8] are responsible for N-1 and N-7 demethylation of CF. Several metrics to monitor hepatic metabolic capacity by the use of CF as a probe have been proposed. They comprise the elimination half-life (t1/2z), total body clearance (ClT) and area under the plasma concentration-time curve (AUC) parameters of CF [13, 17, 23, 27]. However, uses of these parameters in the determination of the hepatic metabolizing capacity require intensive sampling, and this is cumbersome in clinical practice [23]. Thus, some authors have proposed a simplified method for determination of hepatic metabolizing capacity using plasma metabolic ratios (MR, metabolite/CF) calculated from the concentrations of CF and its metabolites for a single sampling time after CF administration [13, 16, 28, 29].

It has been demonstrated that there are marked species differences in the metabolism of CF, as shown in vivo [2] and in vitro [4]. It has also been established that CF is predominantly metabolized to TP in sheep [9], horses, donkeys [20], camels [36], dogs [5] and monkeys [2, 4], while it is predominantly metabolized to PX in humans [2, 4, 17] and cattle [9]. It has also been demonstrated that there are species differences in the pharmacokinetics of CF [4, 9]. However, the effect of breed on the pharmacokinetics and metabolism of CF in food-producing species (e.g., sheep, cattle) has not been reported.

In food-producing species, studies concerning activities of biotransformation enzymes and the determination of biotransformation pathways of veterinary drugs and xenobiotics might be helpful to evaluate the states outlined below.

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(1) The risk of retention of veterinary drugs and xenobiotics in the animal’s body represents a major concern not only for the quality of meat and dairy products [21] but also for humans as a consequence of the accumulation of residues in animal edible tissues [19]. It is therefore evident that better knowledge of the biotransformation enzymes from these species is greatly needed [21, 35]. (2) As laboratory species are poor models for food-producing species on account of marked differences in species of veterinary interest in the activities of biotransformation enzymes, the extrapolation of pharmacotoxicological data from one species to another may not always be feasible. Therefore, a correct mechanistic approach should entail in vivo experiments in target species [19]. These states outlined inter-species may also occur inter-breeds. (3) Enzymes metabolizing CF take part in metabolism of several veterinary drugs (e.g., albendazole, thiabendazole, mebendazole, caffeine and estradiol) [26]. Therefore, differences in enzyme activities may alter the pharmacokinetics of these drugs. In our present study, two kinds of studies were performed in sheep breeds. The first was determination of the breed effect on the pharmacokinetics of CF and its metabolites. The second was estimation and comparison of hepatic metabolic capacity using the plasma (TB+PX+TP/CF)MR ratio and ClT of CF in sheep breeds.

MATERIAL AND METHODS

Chemicals and reagents: TB, PX, TP, CF and β-hydroxyethyl-theophylline were purchased from Sigma (Steinheim, Germany). Acetonitrile and methanol were HPLC-grade and were purchased from Merck (Darmstadt, Germany). Glacial acetic acid (100%) and sodium acetate were of analytical grade and were purchased from Merck (Darmstadt, Germany). The water used in the present study was purified by Milli-Q reverse osmosis (ELGA, Purelab Ultra, Ultra Genetic, England).

Animals, experimental design and sample collection: The Morkaraman (MK, 34.70 ± 1.16 kg, b.w.), Akkaraman (AK, 41.70 ± 2.54 kg, b.w.) and Anatolia Merino (AM, 36.00 ± 1.70 kg, b.w.) sheep breeds, including ten animals of each breed (10–12 months, female), were used in the study. The animals were determined to be healthy based on the results of physical examination and serum biochemical analysis. They were housed in individual pens and were fed barley grains, stalks and dry grass. Drinking water was available ad libitum. They received no pharmacological substances within 2 weeks of beginning the study. The Ethics Committee of the Faculty of Veterinary Medicine (University of Selcuk, Konya, Turkey, report no: 2007/065) approved the study protocol.

Before starting the experiment, a cather was placed into left jugular vein of each animal. A bolus dose of CF was given intravenously (i.v.) at a dosage of 5 mg/kg b.w. Blood samples were collected into tubes with EDTA before and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 16, 24, 32 and 48 hr after CF injection. Blood was centrifuged within one hour after collection, and plasma samples were stored at –20°C until analysis.

Instrument and liquid chromatographic conditions: Chromatographic analysis of CF and its metabolites was performed using HPLC (Finnigan Surveyor, Thermo Electron Corporation, England). The HPLC system consisted of a SRVYR-PDA5 detector system, a SRVYR-AS autosampler and a SRVYR-Lpump LC pump. The ChromQuest software (version 4.1, US) was used for data acquisition and analysis.

Chromatographic separations were obtained using a Phenomenex Gemini C18 column (5 μm particle size, 250 mm×4.60 mm; Phenomenex Gemini, Torrance, CA, U.S.A.), which was maintained at 25°C. The analytical wavelength was set at 273 nm. The mobile phase consisted of 30% methanol in 25 mM sodium acetate buffer, pH 4.0. The solution was prepared fresh daily. The flow rate was 1.0 ml/min. The mobile phase was filtered through a 0.45-μm filter (Sartorius, Gottingen, Germany; 47 mm) and degassed for 20 min by a degassing system (Sonorex Super RK 100 H, Bandelin Electronic GmbH & Co. KG, Berlin, Germany).

Preparation of stock and working standard solutions: Stock solutions of TB, PX, TP, and CF were prepared in mobile phase at a concentration of 1 g/l. A stock standard mixture (100 mg/l) consisting of TB, PX, TP and CF was prepared from stock solutions. This standard mixture was further diluted with mobile phase to obtain working standard solutions at eight different concentrations (0.01–10 μg/ml) that were used to prepare calibration and quality control samples. β-hydroxyethyl-theophylline was used as an internal standard (IS) and was prepared in mobile phase with a concentration of 1 g/l and further diluted with mobile phase to obtain the working standard solution of 10 μg/ml. All solutions were stored in amber colored bottles at 4°C prior to use.

Sample preparation: The procedure for preparation of plasma samples was as described by Christensen et al. [7], with minor modifications. In brief, 25 μl of 10 μg/ml internal standard β-hydroxyethyl-theophylline was added to 250 μl of plasma. The plasma proteins were precipitated by addition of 750 μl of acetonitrile. The mixture was placed in a vortex blender for 30 sec and then centrifuged at 19,000 g for 5 min. The supernatant was evaporated to dryness at 40°C under a gentle stream of nitrogen gas and reconstituted in 200 μl of mobile phase. The sample (15 μl) was then injected into the HPLC system.

Method validation: Method validation was performed in terms of specificity, linearity, sensitivity, recovery, intraday and interday precision. The specificity of the method was acceptable, since baseline separation and simultaneous quantification was obtained for CF, its primary metabolites (PX, TB, TP), and IS within a single run without any interference from blank plasma samples. Calibration curves were constructed for standards and extracted samples ranging from 0.01 to 10 μg/ml of CF and its metabolites. The correlation coefficients (r²) were greater than 0.9951 for the
extracted samples and were greater than 0.9998 for the standard solutions. The recoveries for CF and its metabolites were determined by comparing the mean peak areas of drug-free samples spiked with CF and its metabolites (0.01–10 μg/ml) with the mean peak areas obtained for standard solutions. The mean percentage recoveries of TB, PX, TP and CF were 99.1 ± 3.1%, 99.7 ± 3.4%, 100.3 ± 2.2% and 97.8±2.8%, respectively. The limit of detection (LOD) was established as a signal to noise ratio (S/N) of 3. The LODs were determined to be 0.020 μg/ml for CF and 0.010 μg/ml for its metabolites. The limit of quantification (LOQ) was established as a signal to noise ratio (S/N) of 6. The LOQs were determined to be 0.025 μg/ml for TP and CF and 0.015 μg/ml for TB and PX. The precision were established by using quality control samples (n=3) at low, medium and high concentrations of CF and metabolites of 25, 100 and 800 ng/ml in plasma. The assay precision [coefficient of variation (CV)] was calculated by assessing the inter- and intraday variability of quality control samples and by expressing the standard deviation of repeated measurements as a percentage of the mean value. The intraday precision ranged from 2.51 to 4.71, 2.37 to 4.30, 2.39 to 3.35 and 2.70 to 3.91%, respectively, for TB, PX, TP and CF based on analysis of three samples analyzed within the same day. The interday variability, as estimated from analysis of three samples on three consecutive days, was low, with the CV ranging from 3.54 to 6.37, 3.66 to 4.27, 5.27 to 6.57 and 4.33 to 7.75%, respectively, for TB, PX, TP and CF.

Pharmacokinetic and data analysis: Pharmacokinetic analysis was performed using a specialized computer program (WinNonlin® Professional Version 4.1, Pharsight Corporation, Mountain View, CA, U.S.A.). Plasma concentrations of CF and metabolites were analyzed using noncompartmental analyses to determine model independent parameters such as AUC and volume of distribution (V_{ss}). Plasma elimination rate constant was estimated with least squares regression of the logarithm of the plasma concentration versus the time curve over the terminal elimination phase. AUC from time zero to the last time point with a measurable concentration were calculated by the trapezoidal rule. The maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were determined by direct observation of the plasma concentration-time curve of each metabolite in each animal.

The (TB+PX+TP)/CF\(_{MR}\) ratios of all primary metabolites to CF in blood samples taken at 3-10 hr after CF administration were calculated as a simplified method for determination of hepatic metabolic capacity. Correlations between plasma (TB+PX+TP)/CF\(_{MR}\) at 3–16 hr after CF administration and the Cl⁰ of CF were examined. The ratio of each AUC of the primary metabolites to summed AUCs of the primary metabolites ((metabolite/TP+PX+TP)\(_{AUC}\)) was used for determination of the amounts of TB, PX and TP formed from CF.

Statistical analysis: Data were evaluated for statistical differences in several ways. All data were expressed as means ± SD. The harmonic mean was calculated for τ_{1/2}, The Mann-Whitney U test was used to test for significant differences in this parameter. The other pharmacokinetic data and MRs were analyzed using one way-ANOVA, followed by the Duncan test. Statistical significance was assigned at P<0.05. Correlations between plasma (TB+PX+TP)/CF\(_{MR}\) at 3–16 hr after CF administration and the Cl¹ of CF were assessed using the Pearson correlation test.

RESULTS

The mean plasma concentration-time curves for CF and its primary metabolites after i.v. administration of CF to MK, AK and AM sheep breeds are presented in Fig. 1. The mean pharmacokinetic values of CF and its primary metabolites are summarized in Table 1. These data indicate that all kinetic parameters of CF in the three sheep breeds differed significantly (P<0.05) except for the Vss. Elimination of CF was slow in the MK and AM breeds when compared with the AK breed (Table 1).

The plasma amounts of the primary metabolites of CF in sheep breeds after treatment with CF are indicated in Fig. 1 and Table 1. These data indicate that the main metabolite of CF was TP in the three breeds. The highest correlation coefficient (r²=0.904, P<0.01) between plasma (TB+PX+TP)/CF\(_{MR}\) at 3–16 hr after CF administration and the Cl¹ of CF was determined at 7 hr. The (TB+PX+TP)/CF\(_{MR}\) ratios calculated at 7 hr after CF administration were statistically different (P<0.05) among the breeds (MK, 0.155 ± 0.062; AK, 0.468 ± 0.107; AM, 0.254 ± 0.099). The highest ratio was determined in AK breed.

DISCUSSION

CF was chosen as a test compound for the determination of in vivo hepatic metabolic capacity because its in vivo and in vitro metabolism are well known in other species and its metrics have been validated [2, 4, 6, 9, 13, 33]. Although validation of the assay and method of analysis for simultaneous determination of PX and CF have been reported by Christensen et al. [7], the method of analysis in relation to TB and TP has not been reported. In our study, assay for simultaneous determination of TB, PX, TP and CF was developed and validated. The recoveries of CF and metabolites were nearly complete. The assay is characterized by low inter- and intraday variation; our laboratory aims for both coefficients of variation to be less than 15%, and this target was easily met.

The present study revealed that CF clearance showed significant differences among breeds (Table 1, P<0.05). It was found that CF clearance in the AM breed is similar to that in Suffolk sheep (0.048 l/hr/kg) [9]. The clearance of drugs with limited elimination like CF depends on the intrinsic clearance (total effect capacity of the enzymes metabolizing the drug) and the rate of binding to plasma proteins [1]. CF binds to plasma protein at low rates (10–33%). As the metabolism is mostly performed in the liver (95%), clear-
It was found that the $t_{1/2\lambda z}$ of CF showed statistical differences among the breeds in line with clearance (Table 1, $P<0.05$). The results in AM were similar to those in Suffolk sheep (8.9 hr) [9]. It has also been revealed that there are differences among people of different ethnic origins in elimination half-life depending on metabolism [3]. The terminal half-life ($t_{1/2\lambda z}$) is a variable dependent on the $C_l$ and $V_{ss}$ parameters, and changes in these parameters affect the elimination half-life. The fact that $V_{ss}$ was similar in the breeds (Table 1) indicates that the differences observed in $t_{1/2\lambda z}$ can be attributed to direct clearance; that is, they stem from differences in metabolism.

It was determined that the AUC of CF was statistically different among the breeds ($P<0.05$) and that these differences were in agreement with the $t_{1/2\lambda z}$ and $C_l$ parameters of CF (Table 1). The AUC of CF determined in AM was similar to that determined in Suffolk sheep (106 $\mu g\ hr/\text{ml}$) [9].

The fact that the $V_{ss}$ of CF is similar among the breeds indicates that the AUC value in sheep can also be used as a reliable parameter for hepatic metabolic capacity.

The results showed that the $V_{ss}$ of CF is similar among sheep breeds. However, the $V_{ss}$ values determined in the three breeds (Table 1) were relatively higher than that determined in Suffolk sheep (0.56 $l/kg$) [9]. It has previously been determined that the $V_{ss}$ of CF is generally similar in different species and that the pharmacokinetic differences among species are related to clearance more than to distribution [4]. It has also been reported that $V_{ss}$ of CF does not show differences in various types of liver diseases [5, 16].

It was established that there were statistically significant differences among the breeds in the peak concentrations of TB and TP (Table 1, $P<0.05$). No difference was found in PX. In three breeds, the peak concentrations in TB and PX were similar to those determined in Suffolk sheep (maximum 0.2 $\mu g/\text{ml}$). The peak concentrations of TP in the AK and AM breeds were determined to be in compliance with the results in Suffolk sheep (approximately 1 $\mu g/\text{ml}$) [9].

In the present study, CF was found to be metabolized basically into three different dimethylxanthines (TB, PX, TP). These results were found to be in agreement with the results of the studies on Suffolk sheep by Danielsson and Golsteyn [9]. Furthermore, it has also been revealed that CF is primarily metabolized to these three dimethylxanthines in in vivo studies on mammals such as cattle [9], camels [36], horses, donkeys [20], dogs [5] and humans [23], whose metabolism pathways are similar.

In the present study, TP was found to be the primary metabolite of CF in sheep. In the MK, AK and AM breeds, based on the AUCs of TP and CF, the transformation rates were calculated as 61, 78 and 67%, respectively. TP was also found to be the primary metabolite after CF administration in Suffolk sheep (72%) [9]. In a similar way, although
CF is the primary metabolite in camels [36], horses, donkeys [20], dogs [5] and cynomolgus monkeys, in humans [2] and cattle [9], the primary metabolite is PX. Berthou et al. [2] determined that PX made up 81% of total dimethylxanthines resulting from CF metabolism in human liver microsomes and that TP made up 99% of total dimethylxanthines in liver microsomes of cynomolgus monkeys.

It was determined that there were breed differences in the rates of CF's transformation into main metabolites (theobromine/TB+PX+TP)AUC. The highest rates of metabolic transformation for TB were found in the MK and AM breeds, that for PX was found in the MK breed and that for TP was found in the AK breed. It was determined that TB/PX/TP formations were 18/20/61%, 14/8/78% and 18/15/67% in the MK, AK and AM breeds, respectively. Danielson and Golsteyn [9] determined that the rates of metabolic transformation from CF to TB/PX/TP in plasma samples at 5 hr were 15/7.35/72%, respectively, and that metabolic formation followed the PX<TB<TP order. In the present study, although the results obtained from the AK and AM breeds were similar to the values reported by Danielson and Golsteyn [9], the results were different in the MK breed (TB<PX<TP).

In determination of hepatic metabolic capacity, the AUC, C1 and t1/2 parameters of CF and MRs can be used in mammal species. However, uses of CF parameters (AUC, C1, t1/2) in determination of CYP1A2 activity vary across species. This difference depends on the contributions of enzymes to CF metabolism. AUC, C1 and t1/2 parameters can be used in determination of CYP1A2 activity in humans because the significant metabolic pathway in humans is transformation to PX with CYP1A2 and CYP1A2 also plays role in the formation of other metabolites [23]. However, in species like camels [36], horses, donkeys [20], dogs [5] and sheep [9], in which TP is an important metabolic pathway in CF metabolism, these parameters may be used for determination of hepatic metabolic capacity rather than CYP1A2 activity.

In the present study, it was determined that there are statistically significant differences in the (TB+PX+TP/CF)MR ratios calculated at 7 hr after CF administration among the breeds (P<0.05). A number of enzymes play roles in the transformation of CF to its main metabolites [8, 18, 23], and it has been revealed that CYP1A2 is the most significant enzyme responsible for N-1, N-3 and N-7 demethylation of CF in humans [11, 33]. CYP2E1 enzyme plays roles in N-1 and N-7 demethylation of CF, and it has been reported that its coefficient is smaller in these reactions compared with that of CYP1A2 enzyme [32]. However, Chung and Cha [8] reported that FMOs are more efficient enzymes in N-1 and N-7 demethylation of CF. Rettie and Lang [22] reported that FMOs are of no significance in N-1 and N-7 demethylation of CF. The differences in (TB+PX+TP/CF)MR ratios in the sheep breeds can be attributed to differences in the synthesis profiles and the activities of CYP1A2, CYP2E1 and FMO enzymes.

Different sampling times in development of the use of MRs as a practical test in determination of hepatic metabolic capacity have been suggested, and their relation with other tests that indicate their validity have been revealed in many studies [14, 23, 28-31]. It has been determined that there are high correlations between plasma MRs determined at specific sampling times (2 and 12 hr) after CF administration.

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Table 1. Mean ± SD pharmacokinetic parameters for caffeine and its primary metabolites (theophylline, theobromine and paraxanthine) in Morkaraman, Akkaraman and Anatolia Merino sheep breeds (n=10) after i.v. administration of caffeine at a dosage of 5 mg/kg of body weight

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<tr>
<th>Parameters</th>
<th>MK 149 ± 38&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AK 60 ± 6&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AM 103 ± 30&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>AUC&lt;sub&gt;0-48&lt;/sub&gt; (µg·hr/ml)</td>
<td>15.74 ± 7.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.84 ± 0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.68 ± 5.21&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>t&lt;sub&gt;1/2&lt;/sub&gt; (HM) (hr)</td>
<td>0.73 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>V&lt;sub&gt;m&lt;/sub&gt; (l/kg)</td>
<td>0.03 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Cl&lt;sub&gt;T&lt;/sub&gt; (l/hr/kg)</td>
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a, b, c Different letters in the same column are statistically significant (P<0.05). MK, Morkaraman; AK, Akkaraman; AM, Anatolia Merino; AUC<sub>0–48</sub>, area under the curve from time 0 to the last detectable concentration; t<sub>1/2</sub>, terminal half-life; V<sub>m</sub>, volume of distribution at steady state; Cl<sub>T</sub>, total body clearance; C<sub>max</sub>, peak concentration; T<sub>max</sub>, time to peak concentration; HM, harmonic mean.
and CF clearance [16, 24, 28-31, 34]. In the present study, as there were high correlation between plasma (TB+PX+TP/CF)MR ratios calculated at 7 hr after CF administration and the Clh of CF, it was concluded that this ratio determined at a single sampling time point can be used instead of CF clearance, which requires multiple samples.

In conclusion, marked breed differences in hepatic metabolic capacity and pharmacokinetics of CF and its metabolites were established in three sheep breeds (MK, AK and AM). From these results, it appears that the pattern of drug biotransformation should be consistently tested for all breeds within species. Further studies are needed to determine the biochemical and molecular events underlying such an effect. Uses to predict enzyme activities of phenotyping drugs could also be developed in animals like in humans.

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REFERENCES


