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SYSTEMIC CLEARANCE AND DEMETHYLATION OF CAFFEINE IN SHEEP AND CATTLE

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ABSTRACT:

Pharmacokinetics of caffeine have been studied in sheep and cattle treated with caffeine (5 mg/kg) by intravenous injection. Terminal-phase elimination half-lives were 8.9 hr in sheep and 8.1 hr in cattle. Noncompartmental analyses of data collected from individual animals indicate that neither terminal-phase rate constants (β) nor mean residence times of caffeine in plasma differed between species. Each of the three possible *N*-demethylated primary metabolites of caffeine was detected in plasma from each species, with theophylline predominating in sheep and paraxanthine predominating in cattle. These data indicate that hepatic capacity to clear caffeine from the systemic circulation is similar between sheep and cattle, but that the preferred routes of metabolism differ. Expression of cytochromes P4501A (CYP1A subfamily) may differ between these species.

Hepatic metabolism of many drugs and xenobiotics critically involves the CYP[1] superfamily of enzymes, and dealkylation of various 7-alkoxyresorufin homologs has been proposed to reflect activities of several CYP subtypes (1, 2). For example, deethylation of 7-ethoxyresorufin has been related to CYP1A enzymes, although specific association with CYP1A1 or CYP1A2 isoenzymes may be species-dependent (3–5). Using this substrate, and noninduced hepatocytes, Van't Klooster *et al.* (6) have studied this reaction and have determined a less active deethylation process in hepatocytes from sheep than from cattle or goats. To determine whether this observation reflects a species difference between sheep and cattle, we have studied the *in vivo* pharmacokinetics and demethylation of caffeine, an alternate CYP1A substrate.

Caffeine (1,3,7-trimethylxanthine) is metabolized by hepatic CYP enzymes to four primary metabolites: 1,3-dimethylxanthine (theophylline), 1,7-dimethylxanthine (paraxanthine), 3,7-dimethylxanthine (theobromine), and 1,3,7-trimethyluric acid. Its clearance from plasma is thought to reflect hepatic metabolic capacity (7). *N*-Demethylation of caffeine also seems to involve enzymes of the CYP1A subfamily (8–10) and *N*-3 demethylation, to paraxanthine, has been applied as a specific index of CYP1A2 activity (11–13).

Materials and Methods

Chemicals. Authentic samples of caffeine, its demethyl metabolites and 7-(β-hydroxyethyl)-theophylline were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Solvents were HPLC grade and were purchased from BDH Chemicals (Vancover, British Columbia, Canada). Solutions of caffeine or each metabolite were prepared in water containing an equimolar amount of sodium benzoate.

Animals. Three Suffolk ewes (body weights, 74–107 kg) and three Hereford heifers (body weights, 264–290 kg) were provided by the Canada Alberta Livestock Research Trust, Inc. (Lethbridge, Alberta, Canada). Sheep were maintained on alfalfa/grass hay and cattle were maintained on cubed alfalfa.

Animal Treatments. On treatment mornings, cannulae were placed into the left and right external jugular veins of sheep or cattle. Filter-sterilized solutions of caffeine (sheep, 20 mg/ml; cattle, 50 mg/ml) were injected rapidly through the left cannula to a final dose of 5 mg/kg. Samples of blood were drawn into heparinized vacutainers (Becton Dickinson Vacutainer Systems, Rutherford, NJ) through the right cannula at 30, 60, 90, 120, 150, 180, 240, 300, 360, 420, and 1440 min after treatment. Blood was centrifuged, and plasma was stored at −40°C until analysis.

Preparation of Internal Standard: 7-(β-Hydroyethyl)-theophylline Acetate. Under the chromatographic conditions adopted, an endogenous component of cattle plasma eluted simultaneous to 7-(β-hydroxyethyl)-theophylline. To achieve separation, an internal standard was prepared by acetylation of 7-(β-hydroxyethyl)-theophylline at room temperature with acetic anhydride/pyridine in acetonitrile solvent. Evaporation of solvent and recrystallization from methanol afforded 7-(β-hydroxyethyl)-theophylline acetate [m.p. 104°C, lit. m.p. 105°C (14)]. This product chromatographed as a single peak by HPLC and capillary column GC. Mass spectral analysis was consistent with 7-(β-hydroxyethyl)-theophylline acetate.

Standard Solutions and Standard Curves. Stock solutions of caffeine and metabolites (1 mg/ml) were prepared in distilled water containing sodium benzoate (1 mg/ml). Working solutions were prepared by dilution of stock solutions into acetonitrile (0.1 or 0.01 mg/ml). Standard curve (0.1, 0.5, 1.0, 5.0, 10.0, and 12.5 μg/ml) and quality control (0.1 and 0.5 μg/ml) samples were prepared in duplicate, on each analysis day, by addition of exact volumes of working solutions to plasma (1.0 ml) previously drawn from a representative animal of the species being studied. These samples were assayed each day in parallel to unknown samples.

Analysis of Caffeine and Its *N*-demethylated Metabolites. Caffeine and its *N*-demethylated metabolites were extracted from plasma, and analyzed by HPLC as described by Haughey *et al.* (15), except that a C_8 rather than a C_{18} column was used, and the volume of plasma employed was 1 ml. Briefly, samples of plasma (1 ml) were transferred into borosilicate screw cap tubes (PTFE-lined caps). Hydrochloric acid (0.1 ml, 1.0 N) and internal standard (5 μg/ml) were added. Analytes were extracted by gentle shaking (15 min) with chloroform/2-propanol (4:1, 2 × 5 ml). Organic layers were evaporated under a gentle stream of nitrogen, and residues were reconstituted into the mobile phase (1 ml). Twenty-microliter portions were injected onto the HPLC column.

Chromatography. Caffeine and its metabolites were resolved by isocratic HPLC with a Waters HPLC system equipped with a 712-WISP autosampler, a C_8 column (Zorbax RX-C8, 25 × 0.46 cm, 3 μm particle size), and a model 486 tunable UV detector operating at 275 nm. Column temperature was

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[1] Abbreviations used are: CYP, cytochrome P450; CYP1A, cytochrome P4501A; CYP1A1, cytochrome P4501A1; CYP1A2, cytochrome P4501A2; $t_{1/2\beta}$, elimination half-life; MRT, mean residence time; CL_S, systemic clearance; V_{ss}, volume at steady state.

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maintained at 30°C. The mobile phase was prepared by diluting a mixture of acetonitrile and phosphoric acid (130 ml + 0.5 ml) with HPLC-grade water (869.5 ml). Solvent flow rate was 1.0 ml/min. Amounts of caffeine in unknown samples were determined by linear regression analysis of unknown and standard sample caffeine/internal standard peak area ratios.

Pharmacokinetics. Plasma concentration-time data from sheep and cattle were fitted to mono- and biexponential equations (PCNONLIN 4.2, SCI Software, Lexington, KY). Goodness-of-fit, as determined from Akaike's Information Criteria (16), supported use of a biexponential equation to predict theoretical plasma concentrations at zero time. Plasma concentration-time data, including predicted concentrations at time 0, were then analyzed according to noncompartmental techniques. Values of pharmacokinetic parameters were calculated according to standard equations (17) and are reported as means ± SD. Differences between sheep and cattle in parameter-calculated values were examined by Welch's t test (GraphPad Instat, version 2.04a, GraphPad Software, San Diego, CA). Statistical significance was assigned at $p < 0.05$.

Identification of Metabolites in HPLC Chromatograms. Chromatographic peaks corresponding to dimethylxanthine metabolites of caffeine were identified in HPLC chromatograms of plasma extracts by comparison of retention times to authentic samples of each metabolite. In addition, samples of plasma (1 ml) collected from caffeine-treated animals were augmented with each dimethylxanthine metabolite and then assayed. Area counts of chromatographic peaks, before and after spiking, were compared to determine specific augmentation.

Results

Recoveries from cattle plasma of caffeine (1 μg/ml), and of the internal standard (5 μg/ml), were 77.3 ± 5.8 and 71.1 ± 3.6% (means ± SD, $N = 6$), respectively. Validation samples prepared in cattle plasma (0.5, 1.0, or 10.0 μg/ml caffeine, $N = 6$) were assayed to contain 0.550 ± 0.018, 1.04 ± 0.09, and 10.15 ± 0.85 μg/ml. Cattle plasma validation samples ($N = 3$) containing 2.0 μg/ml of each metabolite were determined to contain 1.98 ± 0.06 μg/ml theobromine, 1.98 ± 0.06 μg/ml paraxanthine, or 2.02 ± 0.07 μg/ml theophylline. Recovery of the dimethylxanthine metabolites from cattle plasma ranged between 85 and 95%. Standard curves, prepared in either sheep or cattle plasma, were linear over the concentration range from 0.1 to 12.5 μg/ml ($r^2 = 0.995–0.999$). Quality control samples (0.5 or 1.0 μg/ml caffeine) run daily, in parallel to unknown samples, were assayed to contain 0.53 ± 0.07 and 1.00 ± 0.07 μg/ml ($N = 16$) from sheep plasma and 0.53 ± 0.06 and 1.05 ± 0.08 μg/ml ($N = 16$) from cattle plasma.

HPLC chromatograms of extracts of blank and augmented (1 μg/ml) control cattle plasma and of plasma from representative sheep and cattle (300 min posttreatment) are shown in fig. 1. These chromatograms indicate that each of the three possible dimethylxanthine metabolites of caffeine were present in the plasma of sheep and cattle, and that the relative amounts varied between species. Augmentation experiments and retention time comparisons indicated that theophylline was the major metabolite in sheep plasma, whereas in cattle plasma the major metabolite was paraxanthine.

Plots of mean concentrations of caffeine in plasma collected from treated sheep and cattle are shown in fig. 2. Values of pharmacokinetic parameters as determined by noncompartmental techniques are listed in table 1. These data indicate that, whereas the $t_{1/2\beta}$ and MRT of caffeine in plasma did not differ significantly between sheep and cattle, values for CL_s and V_{ss} may be slightly smaller in sheep than in cattle ($p < 0.05$).

Amounts of theophylline in sheep plasma and of paraxanthine in cattle plasma after treatment with caffeine are indicated in fig. 3. Maximum concentrations were ~1 μg/ml, 360–420 min after treatment. Levels of theobromine and paraxanthine in sheep plasma, or of theobromine and theophylline in cattle plasma were generally below 0.2 μg/ml and are not illustrated.

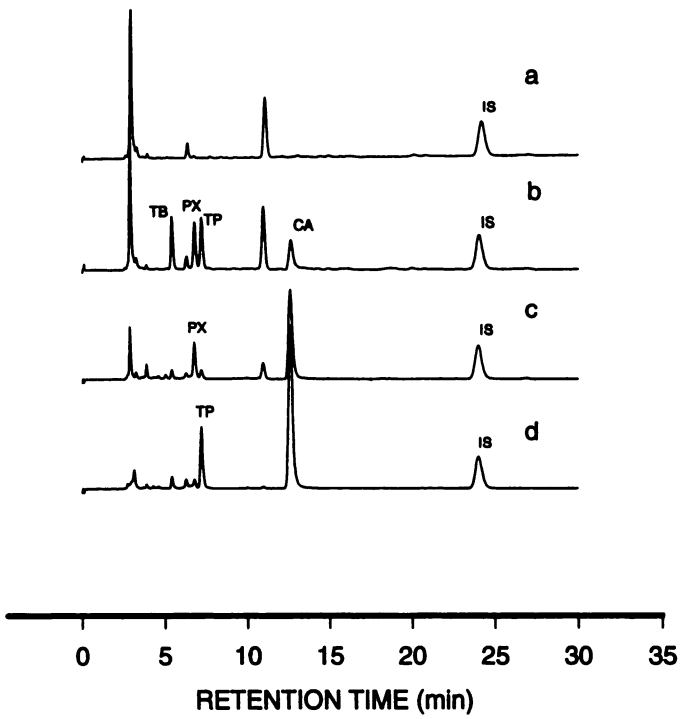

FIG. 1. *HPLC chromatograms of plasma from cattle [(a) blank; (b) theobromine (TB), paraxanthine (PX), theophylline (TP), and caffeine (CA)—each 1 μg/ml; and (c) 300-min posttreatment] and sheep [(d) 300-min posttreatment].*

IS, internal standard, 5 μg/ml.

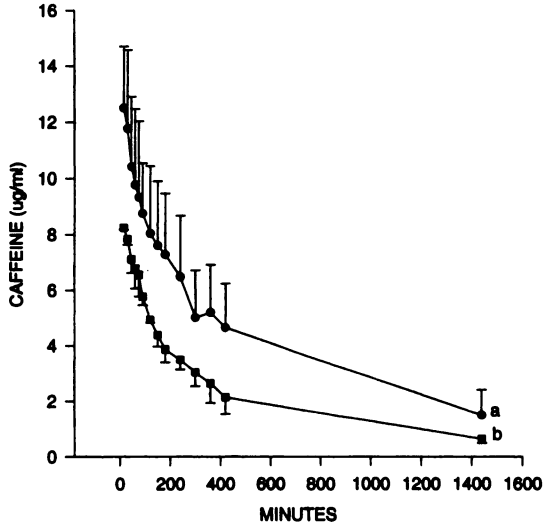

FIG. 2. *Caffeine in plasma from sheep (curve a) and cattle (curve b) after intravenous injection of caffeine (5 mg/kg, N = 3; means ± SD).*

Mean concentrations of caffeine and each dimethylxanthine metabolite in plasma collected 300 min after treatment, plus paraxanthine/caffeine and dimethylxanthine ratios, are indicated in table 2.

Discussion

It has been suggested that hepatocytes from food-producing species may have application in the determination of metabolite patterns of veterinary drugs and crop protection chemicals (18, 19). However, although many food-producing species are ruminants, the bulk of data regarding the metabolism of xenobiotics has been collected in non-

TABLE 1

Pharmacokinetics of caffeine in sheep and cattle plasma after treatment by intravenous injection (5 mg/kg, N = 3, means ± SD)

Parameter	Species	
	Sheep	Cattle
β (min^{-1})	0.0013 ± 0.0003	0.0014 ± 0.0001
$t_{1/2\beta}$	535 min (8.9 hr)	485 min (8.1 hr)
AUC (μg/ml/min)	6330 ± 1950[a]	3690 ± 545
MRT (min)	700 ± 170	590 ± 65
CL_s (ml/min/kg)	0.8 ± 0.2[a]	1.4 ± 0.2
V_{ss} (ml/kg)	560 ± 50[a]	805 ± 90

AUC, area under the zero-moment concentration in plasma/time curve.
[a] Significantly different from values in cattle, $p < 0.05$.

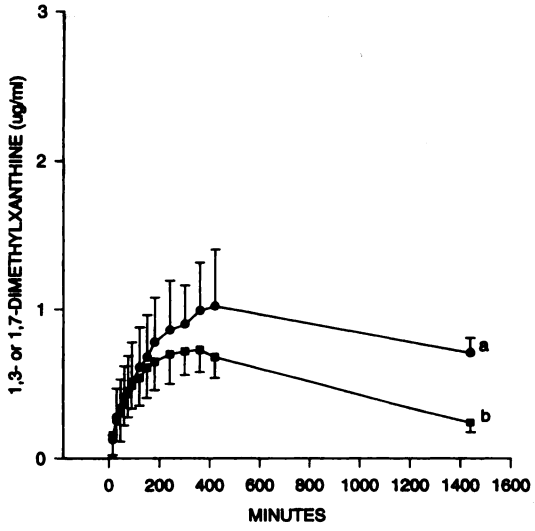

FIG. 3. *Theophylline in plasma from sheep (curve a) and paraxanthine in plasma from cattle (curve b) after intravenous injection of caffeine (5 mg/kg, N = 3; means ± SD).*

ruminant species. Application of these observations to ruminant models may be complicated by *in vitro* observations of differences among ruminant species regarding relative activities of CYP1A enzymes (6). The goal of our experiments was therefore to determine the CL_s and metabolite patterns of an innocuous CYP1A substrate in ruminant species, *in vivo*. Caffeine was chosen as a test compound, because its metabolism by other species is well known, both *in vivo* and *in vitro*, and reports exist regarding the association of CYP1A isoenzymes with its metabolic transformations (8–12).

Our results suggest that CL_s of caffeine in sheep (0.8 ± 0.2 ml/min/kg) and cattle (1.4 ± 0.2 ml/min/kg) occurred at rates similar to those observed in several other nonruminant species, such as dogs (1–2.5 ml/min/kg) (20) and people (1.0 ml/min/kg) (15). The $t_{1/2\beta}$'s of caffeine in sheep (8.9 hr) and cattle (8.1 hr) were also similar to those in dogs (3.2–5.2 hr) (21), people (6.5 hr) (15), or horses (6.6 hr) (22); and the MRT of caffeine in sheep and cattle was similar to that reported in dogs (20). These comparisons of the pharmacokinetics of caffeine among sheep, cattle, and other species suggest that, as in those other species, caffeine behaves as a capacity-limited drug and that its rate of elimination from plasma may be a useful index of hepatic capacity.

TABLE 2

Mean concentrations (μg/ml ± SD) of caffeine and each dimethylxanthine metabolite in the plasma of sheep and cattle (N = 3) 300 min after intravenous injection of caffeine (5 mg/kg)

Analyte	Sheep	Cattle
Caffeine	5.2 ± 1.0	3.1 ± 0.2
Paraxanthine	0.15 ± 0.10	0.70 ± 0.10
Theophylline	0.89 ± 0.29	0.15 ± 0.01
Theobromine	0.19 ± 0.01	0.12 ± 0.01
Paraxanthine Caffeine	0.03 ± 0.01	0.23 ± 0.05
TB/PX/TP ratio[a]	15/12/72	12/72/16

[a] TB, theobromine; PX, paraxanthine; TP, theophylline.

Caffeine was converted metabolically to three dimethylxanthines (theophylline, paraxanthine, and theobromine) by sheep or cattle, although the predominant demthylated metabolite differed from theophylline in sheep to paraxanthine in cattle. Evidence from *in vitro* studies suggests that these primary metabolites are formed from caffeine by CYP enzymes and that the pattern of metabolite formation may indicate expression of CYP1A1/CYP1A2 isoenzymes (8–12). In people, constitutive expression of CYP1A2 exceeds that of CYP1A1, and caffeine is metabolized predominantly to paraxanthine (8, 11, 12). However, in cynomolgus monkeys (*Macaca fasicularis*), expression of either isoenzyme is low and the predominating demethylation of caffeine is to theophylline (8, 23, 24), probably by other CYP enzymes. In that vein, Berthou *et al.* (8) reported that, whereas paraxanthine accounted for 81% in human liver microsomes, theophylline accounted for 89% of total dimethylxanthines produced by cynomolgus monkey liver microsomes. This pattern of *in vitro* demethylation is similar to our current *in vivo* situation, wherein 300 min after treatment, theophylline accounted for 72% of total dimethylxanthine in plasma from sheep and paraxanthine accounted for 76% of the total in cattle.

Fuhr and Rost (11) further suggest that, in people, the ratio of paraxanthine to caffeine in plasma, 5–7 hr after treatment, is a reliable and specific indicator of CYP1A2 activity *in vivo*. Our experiments indicate this ratio to be 0.03 in sheep and 0.23 in cattle (table 2), suggesting that, in comparison with cattle, sheep may less actively express the CYP1A2 isoenzyme. This interpretation is consistent with prior observations of lower 7-ethoxyresorufin-*O*-deethylation by hepatocytes of sheep than of cattle or goats (6). Additional *in vivo* or *in vitro* experiments with other known CYP1A2 substrates, such as phenacetin (25), are required to test this difference more clearly.

Evidence indicates that CYP1A2 enzyme contributes to the metabolism of a wide variety of chemicals and drugs, including phenacetin (25), verapamil (26), propaphenone (27), and clozapine (28), as well as the bioactivation of aflatoxin-B1 (29, 30) and many carcinogenic arylamines (31, 32). If confirmed, decreased CYP1A (or CYP1A2) activity in sheep livers may contribute to an interspecies variation in the metabolism of agricultural and veterinary xenobiotics.

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