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COMPARATIVE THEOBROMINE METABOLISM IN FIVE MAMMALIAN SPECIES

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ABSTRACT:

Biotransformation of theobromine (TBR) was compared in rats, mice, hamsters, rabbits, and dogs by assaying urinary metabolites using HPLC after oral administration of a 5 mg/kg dose containing 8-^{14}C-TBR. Recovery of radioactivity ranged from 60–89% of the dose in urine, and from 2–38% of the dose in feces, with most material being excreted during the first 48 hr after dosing. TBR was most extensively metabolized by rabbits and male mice. The primary metabolite excreted by rats and mice was 6-amino-5-[*N*-methylformylamino]-1-methyluracil (6-AMMU); male mice converted TBR to this metabolite more extensively than did female mice. Rabbits and dogs metabolized TBR primarily to 7-methylxanthine (7-MX) and 3-methylxanthine (3-MX), respectively; the major metabolites excreted by hamsters were 6-AMMU and 7-MX. Overall *N*-demethylase activity yielding monomethyl metabolites was greatest in rabbits and lowest in rats. Ring *N*-demethylation at position 3 predominated over 7-*N*-demethylation in all species except the rat and dog. In dogs, TBR was *N*-demethylated primarily at position 7, while *N*-demethylase activity in rats was without apparent positional specificity. Oxidation of methylated xanthines to the corresponding uric acids was a relatively minor metabolic pathway in all species, but had greatest activity in mice. Oxidation of TBR to 3,7-dimethyluric acid was significantly greater in female rats than in male rats. In summary, excretion patterns of TBR and its metabolites were qualitatively similar among species, indicating that TBR is metabolized along similar pathways. Except for the excretion of small quantities of an unidentified but apparently unique metabolite by dogs, only quantitative species- and sex-related differences were observed in the metabolic disposition of TBR.

TBR[1] and the related methylxanthines, caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine), are pharmacologically active alkaloids that occur naturally in a variety of plants. TBR, in concentrations averaging 1.89%, is the predominant methylxanthine found in cocoa; caffeine is the only methylxanthine present in coffee, while tea contains caffeine, TBR, and theophylline in decreasing order of concentration (1).

Since humans ingest methylxanthines in their diet as well as for therapeutic effects, the metabolism of these compounds has been of interest for many years. Cornish and Christman (2) reported the major urinary metabolites of TBR in man to be 7-MX (28–30% of dose), 3-MX (14–21%), and 7-MU (3–4%), with 11–12% of the dose being excreted unchanged. Arnaud and Welsch (3) more recently identified 3,7-DMU and 6-AMMU as two additional TBR metabolites in human urine, and reported the following distribution of metabolites in the urine of TBR-dosed rats: 7-MX (6% of urine radioactivity), 7-MU (4%), 3,7-DMU (3%), 6-AMMU (36%), unchanged TBR (49%), and trace quantities of dimethylallantoin and *N*-methylurea. Shively and Tarka (4) described a similar distribution of metabolites in the urine of female rats treated with TBR, and found that pregnancy had little effect on TBR pharmacokinetics and metabolism.

Few studies have specifically addressed the toxicology of TBR. Friedman *et al.* (5) reported testicular atrophy and impaired spermatogenesis in rats fed high levels of caffeine, TBR, and theophylline. A threshold dose for TBR-induced testicular toxicity was established for the rat by Tarka *et al.* (6) at a dietary level of 0.6% over 28 days (310 mg/kg/day), however mice and hamsters tolerated much higher TBR doses with no apparent adverse testicular effects. Dogs also seem resistant to TBR-induced testicular toxicity as Gans *et al.* (7) found no evidence of adverse testicular effects in dogs administered TBR for 8 months at doses of 100 or 150 mg/kg/day, but did observe a high incidence of fibrotic lesions localized to the right atrium of the heart.

Whether the differential sensitivity of these species to the testicular effects of TBR is related to different patterns of metabolism or to other factors is unclear. Therefore, the present study was undertaken to provide comparative information on TBR metabolism in five mammalian species including the dog, rabbit, hamster, rat, and mouse.

Materials and Methods

Chemicals. Radiolabeled TBR was synthesized by Amersham Corp. (Arlington Heights, IL) with ^{14}C located at position 8 of the xanthine ring. This material had a specific activity of 7.8 mCi/mmol and its radiochemical purity was found to be 98.6% by HPLC as described below. Monomethylxanthines, monomethyluric acids, and 3,7-DMU were obtained from Adams Chemical Co. (Round Lake, IL); TBR, as the free base, was obtained from K & K Laboratories, Inc. (Plainview, NY); 6-AMMU was provided by Dr. Philippossian (Nestle, La Tour-de-Peilz, Switzerland).

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[1] Abbreviations used are: TBR, theobromine (3,7-dimethylxanthine); 6-AMMU, 6-amino-5-(*N*-methylformylamino)-1-methyluracil; 3-MU, 3-methyluric acid; 7-MU, 7-methyluric acid; 7-MX, 7-methylxanthine; 3-MX, 3-methylxanthine; 3,7-DMU, 3,7-dimethyluric acid.

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Animals and Dosing Procedure. Adult animals of the following species were utilized in this study: male and female CD Sprague-Dawley rats (192–221 g), CD-1 outbred mice (24–37 g), and Chinese hamsters (20–30 g); male New Zealand White rabbits (3.6–5.1 kg), and beagle dogs (10.2–14.9 kg) were also used. Rats and mice were obtained from Charles River Breeding Laboratories (Wilmington, MA); hamsters, rabbits, and dogs were obtained from Chick Line (Vineland, NJ), Langshaw Farms (Augusta, MI), and Hodgin's Kennels (Howell, MI), respectively. All animals were housed within stainless steel cages in rooms with controlled temperature and lighting, and were allowed free access to food (Ralston Purina Co.) and tap water prior to dosing.

TBR was administered in aqueous solution at a dose of 5 mg/kg by oral intubation. The dosing solution was prepared immediately prior to administration by mixing aqueous ^{14}C-TBR (16.67 μCi/ml) with a slightly basic solution of unlabeled TBR such that rats, mice, and hamsters received approximately 5 μCi of radioactivity in a volume of 0.2–1.0 ml, while dogs and rabbits received approximately 50 and 20 μCi, respectively, in a volume of 2–9 ml. Immediately after dosing, animals were placed in stainless steel metabolism cages (2 individuals/cage for mice, 1 individual/cage for all other species), and were allowed food and tap water *ad libitum*.

Analytical Procedures. Urine and fecal samples were collected at various time intervals after dosing as shown in tables 1 and 2. The volume of each urine sample was measured, and then duplicate aliquots (25–100 μl) were added to minivials and mixed with 5 ml of ACS scintillation cocktail (Amersham Corp.). The remaining urine was stored at −20°C until analyzed by HPLC.

Fecal samples were dried for 24 hr under partial vacuum in an oven maintained at 75°C, weighed, and then finely pulverized. Analysis of fecal radioactivity was performed in duplicate by oxidation to $^{14}CO_2$ in a ceramic combustion tube packed with copper shavings and platinum-covered ceramic saddles. The combustion tube was maintained at 700°C by two Hoskins electric furnaces (Hoskins Mfg. Co.), and oxygen flow was regulated at 300 ml/min. Under these conditions, a 100-mg fecal sample was completely oxidized in 2 min, and $^{14}CO_2$ was trapped with an efficiency of 95 ± 2% using 15 ml of phenethylamine/methanol, 1:2 (v/v). The radioactivity in 6 ml of trapping agent was counted in 10 ml of Permablend I (Packard Instrument Co.). A Packard TriCarb liquid scintillation spectrometer was used for analysis of radioactivity, and all determinations were corrected for quenching by external standardization.

Labeled compounds in urine were separated by HPLC with a Partisil PXS 10/25 ODS-3 reverse-phase analytical column (Whatman Co.) and a mobile phase of methanol/potassium phosphate buffer (60 mM, pH 2.4); a guard column packed with CO:PELL ODS (Whatman Co.) preceded the analytical column. Mobile phase programming was provided by a microprocessor (model 420, Altex) interfaced with dual metering pumps (model 825, Instrument Specialties Co.). Optimal separation of reference standards with rapid column re-equilibration between sample injections was achieved at a flow rate of 1.5 ml/min with the following program: from time zero to 17 min, the mobile phase consisted of 4% methanol; at 17 min, the methanol composition was increased to 15% in 1 min, and 4 min later was again returned to 4% in 1 min. Urine samples were prepared for HPLC by centrifugation to sediment particulate material. Each sample injection typically contained 3,000–20,000 dpm, and minivials were used to collect column eluate in 300–750-μl fractions for 30 min after which time another injection could be made. Each fraction of eluate was mixed with 5 ml of ACS scintillation cocktail and assayed for radioactivity; recovery of radioactivity averaged 94 ± 1%. Retention times for reference standards were determined at the beginning and end of each day's work by monitoring UV absorption of eluate at 254 nm (UV detector model 153, Altex Co.); these values served as the basis for identification of radiolabeled compounds.

Statistical Methods. Within the same species, sex-related differences in the per cent distribution of urinary metabolites and unchanged TBR were evaluated by Student's *t* test; the level of significance was chosen as $p < 0.05$. When sex differences were not statistically significant, male and female data were combined into a single group for subsequent evaluations of species-related differences. These tests were carried out by the Scheffé method of multiple comparisons subsequent to one-way analysis of variance (8); $p < 0.05$ was chosen as the level of significance.

Results

Percentages of administered radioactivity recovered in the urine and feces of each species are shown in tables 1 and 2. For those species where urine samples were obtained 4 to 5 hr after dosing, approximately 25% of the administered radioactivity had already been excreted. This indicates that TBR is rapidly absorbed from the gut and, along with its metabolites, is readily cleared from blood to urine. Total recovery of radioactivity in urine ranged from a low of 60% for the female mouse, to a high of 89% for the dog.

The amount of radioactivity recovered in feces was quite variable, ranging from 2 to 38% of the dose for the rabbit and male rat, respectively. Erratic absorption of TBR may be partially responsible for this variability in fecal elimination of radioactivity, especially since TBR was administered to animals that had not been fasted, and recent reports indicate that dietary factors may influence the bioavailability of orally administered methylated xanthines (9).

Fig. 1 illustrates the separation of reference standards by HPLC as monitored by UV absorption. The resolution of radiolabeled material in dog urine into several discrete peaks, each eluting with a retention time coinciding with that of reference standard, is also shown. One exception, also noted in urine samples from the other species, was a small fraction of radioactivity that eluted prior to 6-AMMU. Most of the radiochemical impurity present in the administered ^{14}C-TBR had a similar retention time and may largely account for this chemically undefined material. Another unidentified radioactive peak eluting between 3,7-DMU

TABLE 1

Cumulative recovery of radioactivity in urine after oral administration of ^{14}C-theobromine to different species

Data are expressed as percentage of administered radioactivity (mean ± SE); ns, no sample.

Species (*N*)	Sex	Time after Dose						
		4–5 hr	6–8 hr	12 hr	24 hr	36 hr	48 hr	60–96 hr
					%			
Rat (6)	M	25.4 ± 1.7	41.8 ± 2.1	52.5 ± 2.1	63.8 ± 2.1	65.6 ± 2.2	66.2 ± 2.1	ns
Rat (6)	F	26.6 ± 4.3	43.2 ± 4.3	54.6 ± 3.7	66.9 ± 3.5	70.2 ± 3.6	72.2 ± 3.5	ns
Mouse (6)	M	25.1 ± 3.9	ns	60.8 ± 1.5	68.7 ± 2.2	70.2 ± 1.9	72.0 ± 2.0	ns
Mouse (6)	F	27.0 ± 6.0	ns	46.6 ± 8.7	53.2 ± 7.7	56.0 ± 7.4	59.5 ± 7.2	ns
Hamster (3)	M	ns	34.7 ± 5.1	49.8 ± 4.2	58.5 ± 3.5	60.9 ± 4.3	62.9 ± 5.0	68.5 ± 5.6
Hamster (5)	F	ns	31.9 ± 5.7	45.7 ± 5.7	53.6 ± 5.1	57.1 ± 5.1	59.9 ± 4.5	61.5 ± 4.7
Rabbit (4)	M	23.9 ± 9.0	ns	ns	55.6 ± 11.8	75.1 ± 5.6	75.1 ± 5.6	85.3 ± 2.3
Dog (3)	M	ns	14.1 ± 5.7	ns	60.2 ± 1.7	79.3 ± 1.5	79.3 ± 2.8	88.9 ± 1.4

TABLE 2

Cumulative recovery of radioactivity in feces after oral administration of ^{14}C-theobromine to different species

Data are expressed as percentage of administered radioactivity (mean ± SE).

Species (*N*)	Sex	Time after Dose 24–36 hr	Time after Dose 48–72 hr	Total Recovery Urine Plus Feces
		%		%
Rat (6)	M	34.4 ± 0.4	38.2 ± 0.8	104.4 ± 2.4
Rat (6)	F	11.5 ± 1.5	16.2 ± 1.3	88.4 ± 3.1
Mouse (6)	M	7.2 ± 0.5	8.8 ± 1.1	80.8 ± 2.8
Mouse (6)	F	9.2 ± 2.2	11.5 ± 1.8	71.0 ± 7.8
Hamster (3)	M	13.8 ± 5.9	15.0 ± 6.0	83.5 ± 4.0
Hamster (5)	F	13.2 ± 3.2	14.3 ± 3.3	75.8 ± 2.2
Rabbit (4)	M	0.8 ± 0.1	1.6 ± 0.2	86.8 ± 2.2
Dog (3)	M	3.1 ± 0.3	4.5 ± 0.1	93.3 ± 1.4

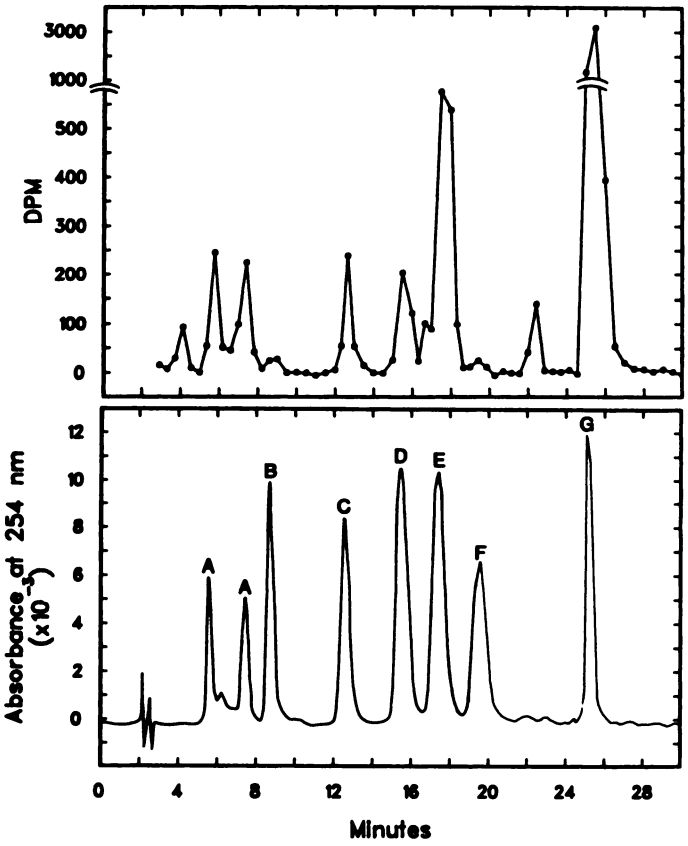

FIG. 1. *HPLC separations of reference standards and radiolabeled compounds excreted in dog urine.*

A 26-μl aliquot of dog urine (321 dpm/μl) excreted 6 hr after ^{14}C-theobromine dosing, and 26 μl of an aqueous mixture of reference standards (each component at an approximate concentration of 20 μg/ml) were each chromatographed as described in *Materials and Methods.* Peak assignments for reference standards are as follows: *A*, 6-AMMU; *B*, 3-MU; *C*, 7-MU; *D*, 7-MX; *E*, 3-MX; *F*, 3,7-DMU; *G*, TBR.

and TBR was evident only in dog urine and accounted for 1.2% of the administered radioactivity. This material was not chemically identified, but its long retention time suggests it is relatively nonpolar. It should also be noted that 6-AMMU eluted from the column as two peaks. One of these may be attributed to 3,7-dimethyldihydrouric acid since Rao *et al.* (10) showed by NMR analysis that the analogous metabolite of caffeine (1,3,7-trimethyldihydrouric acid) exists in equilibrium with its uracilic analog (6-amino-5-(*N*-methylformylamino)-1,3-dimethyluracil) when in solution. For these studies, excretion of 6-AMMU was quantitated by combining the radioactivity under both peaks.

Table 3 shows the cumulative urinary excretion of TBR and its metabolites by each of the species studied. HPLC analysis of radioactivity was performed only on urine samples collected during the indicated time intervals since subsequent samples contained only a few per cent of the radioactive dose (see table 1). Excretion of unchanged TBR was substantial, ranging from 14 to 37% of the administered dose. The rat and mouse converted TBR primarily to 6-AMMU. In contrast, 7-MX and 3-MX were the primary metabolites excreted by the rabbit and dog, respectively. Metabolism of TBR by the hamster was unique in that excretion of a single major metabolite was not clearly observed. Instead, roughly equivalent percentages of the TBR dose were converted to two primary metabolites, 7-MX and 6-AMMU.

For all species, excretion of 7-MU exceeded that of 3-MU, and combined excretion of all methylated uric acids never exceeded 8.2% of the administered dose. Thus, oxidation of the methylated xanthine ring is a relatively minor metabolic pathway.

Approximately 8% of the TBR administered to rats was recovered in urine as monomethyl metabolites. For both male and female animals, excretion of 3-MX exceeded that of 7-MX, ostensibly indicating preferential *N*-demethylation of TBR at position 7. However, this specificity is no longer apparent if the disproportionate excretion of 3-MU and 7-MU is considered in conjunction with monomethylxanthine excretion. This appears to be the most appropriate way to assess the positional specificity of *N*-demethylase activity since excretion of monomethylxanthines and monomethyluric acids reflects differences not only in the rates of *N*-demethylation at positions 3 and 7, but also potential differences in the rates at which 3-MX and 7-MX are subsequently oxidized to methylated uric acids.

Approximately 13 to 17% of the TBR dose was recovered as monomethyl derivatives in both mouse and hamster urine. In these species, ring *N*-demethylation at position 3 clearly predominated over *N*-demethylation at position 7. *N*-Demethylation of TBR was a prominent metabolic pathway in both the rabbit and dog since the respective excretion of monomethyl metabolites by these species accounted for 46.1 and 28.6% of the TBR dose. Similar to that in the mouse and hamster, *N*-demethylase activity in the rabbit strongly favored removal of the 3-methyl group. This was in contrast to overall *N*-demethylase activity in the dog which preferentially removed the methyl group at position 7.

Since urinary data from table 3 reflect group variation in the recovery of radioactive dose, differences in TBR metabolism due solely to species and sex are not appropriately represented. Therefore, these data were normalized to eliminate recovery variability by expressing the excretion of TBR and its metabolites as percentages of recovered urinary radioactivity; these results are presented in fig. 2. *Panel A* shows that approximately 20% of the urinary radioactivity recovered from the male mouse and rabbit was comprised of unchanged TBR. In contrast, TBR represented about 50% of the radioactivity in rat and dog urine. This suggests TBR is most extensively metabolized by the rabbit and male mouse. Furthermore, the TBR composition of urinary radioactivity for male mice was 41% lower than the corresponding value for female mice, implying that male mice metabolize TBR more extensively than do females.

TABLE 3

Urinary excretion of theobromine and its metabolites by different species given a single oral dose of ^{14}C-theobromine

Radioactivity in urine samples collected over the indicated time intervals was separated by HPLC and quantitated by liquid scintillation counting. Excretion of each compound is expressed as a percentage of administered radioactivity (mean ± SE).

Species (*N*)	Sex	Time Interval[a]	Recovery[b]	Metabolites and Parent Compound						
				Uracil Derivative	Uric Acids			Xanthines		
					3-Methyl	7-Methyl	3,7-Dimethyl	3-Methyl	7-Methyl	Theobromine
		hr	%				%			
Rat (6)	M	24	63.8 ± 2.1	16.5 ± 0.9	<0.1	1.3 ± 0.1	2.1 ± 0.2	3.9 ± 0.3	2.5 ± 0.1	32.4 ± 2.2
Rat (6)	F	24	66.9 ± 3.5	18.2 ± 1.2	<0.1	1.4 ± 0.1	3.4 ± 0.3	4.1 ± 0.2	2.7 ± 0.1	32.3 ± 2.5
Mouse (6)	M	36	70.2 ± 1.9	27.8 ± 2.5	0.4 ± 0.1	5.3 ± 0.2	2.5 ± 0.3	3.2 ± 0.2	8.2 ± 0.4	16.4 ± 1.6
Mouse (6)	F	36	56.0 ± 7.4	13.9 ± 2.1	0.3 ± 0.1	4.7 ± 0.6	1.6 ± 0.2	3.0 ± 0.4	5.2 ± 0.9	22.1 ± 3.6
Hamster (3)	M	48	62.9 ± 5.0	15.5 ± 2.3	0.3 ± 0.1	3.2 ± 0.9	2.3 ± 0.6	2.2 ± 0.3	11.6 ± 1.6	20.3 ± 4.9
Hamster (5)	F	48	59.9 ± 4.5	14.6 ± 1.6	0.2 ± 0.03	2.9 ± 0.4	2.0 ± 0.3	2.3 ± 0.2	11.4 ± 1.0	20.0 ± 2.8
Rabbit (4)	M	48	75.1 ± 5.6	10.0 ± 1.1	0.6 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	8.4 ± 0.5	35.5 ± 3.2	13.9 ± 2.7
Dog (3)	M	48	79.3 ± 2.8	7.5 ± 3.1	0.9 ± 0.3	4.4 ± 2.2	0.4 ± 0.1	19.9 ± 2.7	3.4 ± 0.9	36.8 ± 5.9

[a] Time required to collect 88–97% of the total radioactivity recovered in urine.

[b] Percentage of administered radioactivity recovered in urine during the indicated time intervals.

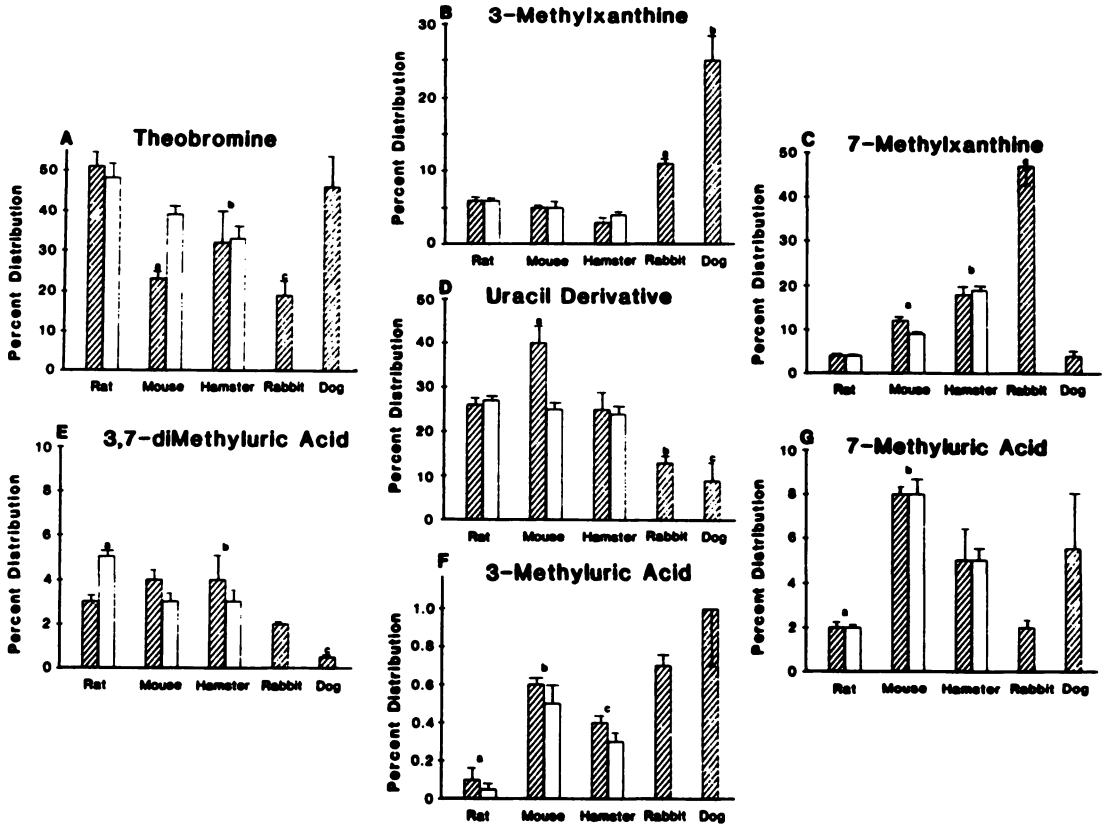

FIG. 2. *Per cent distribution of urinary radioactivity among individual metabolites and unchanged theobromine after oral administration of ^{14}C-theobromine to different species.*

Values are expressed as percentage of total radioactivity recovered in urine over time intervals as indicated in table 3 (mean ± SE). Male and female animals are represented by *cross-hatched* and *open bars*, respectively. *Panel A: a*, male mouse significantly different from female mouse, rat, and dog; *b*, hamster significantly different from rat; *c*, rabbit significantly different from rat, female mouse, and dog. *Panel B: a*, rabbit significantly different from hamster; *b*, dog significantly different from all other species. *Panel C: a*, mouse significantly different from rat and dog; *b*, hamster significantly different from rat, mouse, and dog; *c*, rabbit significantly different from all other species. *Panel D: a*, male mouse significantly different from female mouse and all other species; *b*, rabbit significantly different from rat and female mouse; *c*, dog significantly different from rat, female mouse, and hamster. *Panel E: a*, female rat significantly different from male rat and all other species; *b*, hamster significantly different from rabbit; *c*, dog significantly different from male rat, mouse, and hamster. *Panel F: a*, rat significantly different from all other species; *b*, mouse significantly different from dog; *c*, hamster significantly different from rabbit and dog. *Panel G: a*, rat significantly different from hamster and dog; *b*, mouse significantly different from rat, hamster, and rabbit.

Panels B and *C* show the monomethylxanthine composition of urinary radioactivity for each species. 3-MX comprised the greatest fraction of radioactivity in dog urine at a level which was from 2 to 7 times greater than those found for other species. Great disparity was also noted when comparing percentages of urinary radioactivity comprised of 7-MX. This metabolite accounted for as much as 47% of the radioactivity in rabbit urine, and as little as 4% of the radioactivity in rat and dog urine.

Panel D shows that metabolism of TBR to 6-AMMU was somewhat less extensive in the rabbit and dog than in other species. In addition, 6-AMMU comprised a significantly greater fraction of radioactivity in the urine of male mice as compared to female mice, indicating that an increased conversion of TBR to 6-AMMU may at least partially account for the more extensive metabolism of TBR that was apparently observed in male mice.

A relatively small proportion of urinary radioactivity was comprised of methylated uric acids as illustrated in *panels E* through *G*. For the rat, a sex-related difference was noted in the 3,7-DMU composition of urinary radioactivity. The greater proportion of this metabolite in female rat urine may be attributed to two effects: relatively more 3,7-DMU was formed by ring oxidation of TBR, or relatively less 3,7-DMU was demethylated to form monomethyluric acids.

The combined per cent distribution of all methylated uric acids was highest for mice at approximately 12%, and lowest for the rabbit and rat at 5 and 6%, respectively; intermediate values between 8 and 9% were obtained for the dog and hamster. These data suggest mice have a somewhat greater capacity to ring-oxidize methylated xanthines than do other species; however, this effect is statistically significant only when mice are compared to rabbits and rats.

Total *N*-demethylase activity, as assessed by the monomethyl metabolite composition of urinary radioactivity, was highest for the rabbit and dog at 60 and 36%, respectively. Monomethyl metabolites accounted for only 12% of rat urine radioactivity, whereas intermediate values of 24 and 28% were obtained for mouse and hamster urine, respectively. Statistical analyses of these data indicate both the rat and rabbit to be significantly different from all other species; in addition, the dog is significantly different from the mouse.

For the rabbit, hamster, and mouse, 75–86% of monomethyl radioactivity was comprised of 7-methyl derivatives, indicating that despite significant differences in overall *N*-demethylase activity between these species, there is a predominance of *N*-demethylation at position 3 that appears to be maintained at a fairly consistent level over 7-*N*-demethylation. In contrast, 7-methyl metabolites accounted for only 27% of monomethyl radioactivity in dog urine. This implies that *N*-demethylase activity in the dog is at a comparable level of specificity, but proceeds by preferential removal of the 7-methyl group. *N*-Demethylase activity in the rat was unique in that no apparent positional specificity was detected. Thus, monomethyl radioactivity in rat urine was proportioned equally among 3-methyl and 7-methyl isomers.

Discussion

This study had demonstrated that the rat, mouse, hamster, rabbit, and dog convert TBR to the same metabolites by pathways which primarily entail ring oxidation, ring scission, and *N*-demethylation. These metabolites, along with unchanged TBR are rapidly excreted in urine, but quantitative differences in their patterns of excretion indicate considerable species-related variability in the reltive activities of individual metabolic pathways. The only evidence of qualitative differences in TBR biotransformation was observed in dogs where a small fraction of TBR was apparently converted to a unique metabolite which was not found in the urine of other species; studies are in progress to isolate this material and determine its chemical structure.

Much of the species variation in TBR biotransformation can be attributed to substantial differences in *N*-demethylase activity as evidenced by the wide range of values describing total monomethyl metabolite composition of urinary radioactivity (12–60%). As was reported for methylxanthine metabolism in man (2), no evidence was obtained in the present study to suggest that TBR *N*-demethylation proceeded beyond the monomethyl stage. The positional specificity of *N*-demethylation in most species also paralleled that which was reported for TBR metabolism in man: namely, a predominance of 3-*N*-demethylation over 7-*N*-demethylation (2). However, exceptions to this were noted in the dog and rat where *N*-demethylase activity preferentially removed the 7-methyl group, and was without apparent positional specificity, respectively.

The effect of *N*-demethylation on the biological activity of TBR is likely to depend on which methyl groups are removed from the xanthine ring. This is because 3-MX, unlike other monomethyl isomers, has been shown to have biological activity (11). Thus, relatively less attenuation of biological activity may be associated with TBR *N*-demethylation in the dog than in those species where TBR is preferentially *N*-demethylated at position 3 to form 7-MX.

All species oxidized TBR to both monomethyl- and dimethyluric acids. Collectively, these metabolites comprised from 5 to 12% of urinary radioactivity indicating that ring oxidation is a minor metabolic pathway with relatively little species variation in activity when compared to TBR *N*-demethylation. In man, TBR also undergoes ring oxidation to a much lesser extent than it does *N*-demethylation as Cornish and Christman (2) found only small quantities of 7-MU in the urine of human subjects administered TBR, but substantially greater quantities of 3-MX and 7-MX.

Interestingly, if the methyl group at position 7 of TBR is moved to position 1 to form theophylline, then biotransformation has been shown to proceed primarily by ring oxidation to 1,3-DMU (2, 12, 13). Thus, the quantitative relationship between metabolism by *N*-demethylation and by ring oxidation is markedly influenced by the placement of methyl groups on the xanthine ring. Although it is not precisely known what determines the predominance of one pathway over the other, Cornish and Christman (2) have postulated that a methyl substituent at position 7 may prevent uric acid formation by hindering C-8 oxidation.

Each species metabolized TBR by ring scission to a variable extent as indicated by differences in the 6-AMMU composition of urinary radioactivity ranging from 9 to 40%. Fink *et al.* (14) were first to propose that exogenous methylxanthines such as caffeine might be metabolized by imidazole ring scission *in vivo*, but this was not verified until Khanna *et al.* (15) isolated 1,3,7-trimethyldihydrouric acid from the urine of caffeine-dosed rats and, in a subsequent study, showed that this compound existed in equilibrium with its open ring, *N*-formyl analog (10). These findings were subsequently extended by Arnaud and Welsch (3) who demonstrated that TBR is also metabolized by ring scission in both the rat and man.

Conversion of TBR to 6-AMMU is apparently brought about

by a reaction sequence analogous to that proposed for the ring scission of caffeine; this involves ring hydroxylation at C-8, and rupture of the carbon-nitrogen bond between positions 8 and 9 on the imidazole portion of the xanthine ring (10). Other studies using purine nucleosides have shown that the imidazole ring readily undergoes chemical cleavage between C-8 and N-9 provided a methyl substituent is present at N-7 to provide ring destabilization (16, 17). Methylated xanthines appear to behave similarly in biological systems as Arnaud and Welsch (13) reported that, unlike TBR and caffeine, theophylline which does not possess a methyl substituent at N-7 is not metabolized by ring scission, but primarily by ring oxidation. Thus, methylated xanthines, subsequent to hydroxylation at C-8, appear to undergo either ring scission or further C-8 oxidation to methylated uric acids. Ring destabilization derived from a methyl group at position 7 is apparently one factor that predisposes methylxanthine biotransformation to proceed by ring scission.

The enzymes reponsible for TBR biotransformation have not been clearly identified; however, a variety of studies have implicated the microsomal mixed function oxidase system in both caffeine *N*-demethylation (18, 19), and theophylline oxidation to 1,3-DMU (20). In addition, quantitative changes in the relative excretion of caffeine metabolites by phenobarbital- and β-naphthoflavone-induced dogs have led to the proposal that different forms of P-450 mediate caffeine *N*-demethylation at different positions on the xanthine ring with some degree of specificity (21). Therefore, preferential *N*-demethylation of TBR at positions 3 or 7 by a particular animal species is probably determined by species-related differences in P-450 isozyme profiles. Whether P-450 also mediates ring scission of caffeine and TBR is unknown, but the C-8 ring hydroxylation associated with this reaction seems to argue in favor of P-450 involvement.

Biotransformation of TBR has been previously described in rats for male animals (3), and for pregnant and nonpregnant female animals (4). In both studies, the ratio of urinary to fecal elimination of administered dose was greater than that obtained for rats in the present study; however, urinary excretion patterns for TBR and its metabolites as described by all three studies are in close agreement. The more extensive fecal elimination of radioactivity observed in the present study is probably related to differences in methodology since, unlike the previous studies, TBR was adminstered to animals that had not been fasted, and dietary factors are reported to decrease the bioavailability of orally administered methylated xanthines (9).

The relationship of TBR biotransformation to toxicity has not been well characterized although at least one metabolite, 3-MX, is known to have biological activity and may therefore play a role in TBR-induced toxicities. For example, subacute and chronic administration of TBR to dogs have been associated with right atrial cardiomyopathy; however, lesion severity was not well correlated with plasma concentrations of TBR (7). Since dogs metabolize TBR primarily to 3-MX, this metabolite may also play a role in the development of cardiomyopathy, and lesion severity might be more highly correlated to plasma levels of TBR and 3-MX combined.

Whether the occurrence of testicular damage in TBR-treated laboratory animals is influenced by species differences in TBR biotransformation is difficult to ascertain on the basis of the present data. Rats are reported to be considerably more susceptible than mice and hamsters to the testicular effects of TBR (6), yet the present study provides little indication of substantial metabolic differences between these species. It must be remembered, however, that the distribution of TBR and its metabolites as observed with the present dosing regimen may be altered after repetitive administration of TBR at the much higher doses required to produce testicular damage. Given TBR's wide spectrum of pharmacological activity (22), the factors governing species susceptibility to TBR-induced testicular damage are undoubtedly complex and varied. Although species differences in TBR biotransformation cannot be ruled out, serious consideration must also be given to potential differences in TBR pharmacokinetic and pharmacodynamic parameters.

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